The γ-aminobutyric acid type A (GABA_A) receptor M2–M3 loop structure and its role in gating were investigated using the substituted cysteine accessibility method. Residues from α1Arg-273 to α1Ile-289 were mutated to cysteine, one at a time. MTSET* or MTSES reacted with all mutants from α1R273C to α1Y281C, except α1P277C, in the absence and presence of GABA. The MTSET* closed-state reaction rate was >1000 liters/mol-s at α1N274C, α1S275C, α1K278C, and α1Y281C and was <300 liters/mol-s at α1R273C, α1L276C, α1V279C, α1A280C, and α1A284C. These two groups of residues lie on opposite sides of an α-helix. The fast reacting group lies on a continuation of the M2 segment channel-lining helix face. This suggests that the M2 segment α-helix extends about two helical turns beyond α1N274 (20°), aligned with the extracellular ring of charge. At α1S275C, α1V279C, α1A280C, and α1A284C the reaction rate was faster in the presence of GABA. The reagents had no functional effect on the mutants from α1A282C to α1I289C, except α1A284C. Access may be sterically hindered possibly by close interaction with the extracellular domain. We suggest that the M2 segment α-helix extends beyond the predicted extracellular end of the M2 segment and that gating induces a conformational change in and/or around the N-terminal half of the M2–M3 loop. Implications for coupling ligand-evoked conformational changes in the extracellular domain to channel gating in the membrane-spanning domain are discussed.

The GABA_A receptors mediate inhibitory neurotransmission in the central nervous system (1). They are members of a gene superfamily that includes glycine, serotonin type 3, and nicotinic acetylcholine (ACh) receptors (2, 3). The GABA_A receptors are a major target for drugs used for the induction and maintenance of general anesthesia and for the treatment of anxiety and epilepsy (1, 4).

The GABA_A receptors are composed of five subunits arranged pseudo-symmetrically around the central channel axis. Each subunit has an extracellular N-terminal domain and a C-terminal domain with four transmembrane segments (M1–M4) that are largely α helical (5, 6). The putative ends of the membrane-spanning segments have been defined on the basis of hydrophobicity and sequence analysis (7, 8). There is, however, little experimental evidence to define precisely the ends of the helical membrane-spanning segments.

The subunits of this gene superfamily are built on a modular basis. The extracellular domain forms the agonist binding sites and determines the order of subunit assembly around the channel axis (6, 9–12). The C-terminal domain forms the ion channel that is largely lined by residues from the M2 membrane-spanning segments of each of the five subunits (13, 14). Support for the concept of modular design comes from the ability to generate functional chimeras between the GABA_A receptor extracellular domain and the glycine receptor membrane-spanning domain, or between serotonin Type 3 and ACh α7 receptors (15, 16). In addition, in invertebrates, a number of “chimeras” have evolved that combine extracellular domains that bind ACh, serotonin, or glutamate with a GABA_A-like, anion-selective, channel-forming, membrane-spanning domain (17, 18). The conformational changes by which ligand binding is coupled to movement of the channel gate are unknown.

Two regions of the membrane-spanning domain that might be involved in coupling to the extracellular domain are the pre-M1 region and the M2–M3 loop, the extracellular region that flanks the channel-lining M2 segment (Fig. 1). Depending on how the C-terminal end of M2 and the N-terminal end of M3 are defined, the M2–M3 loop is between 9 and 15 residues long. Only 3 to 5 residues would be necessary to form a β-turn between α helical M2 and M3 membrane-spanning segments. Several lines of evidence have implicated the M2–M3 loop in the signal transduction process. Mutations of three residues in the GABA_A receptor γ2 subunit, two in the M2 segment and one in the M2–M3 loop aligned with α1Ala280, uncouples benzodiazepine binding in the extracellular domain from benzodiazepine potentiation of GABA-evoked currents (19). Mutations in the M2–M3 loop alter agonist efficacy in GABA, ACh, and glycine receptors (20–24). Naturally occurring mutations in the M2–M3 loop of glycine receptors cause startle disease (hypercblepsia) (25) and of the muscle acetylcholine receptors cause slow channel congenital myasthenic syndrome (22). The effect of many of these mutations is to uncouple or to reduce the coupling between ligand binding and channel gating (22–24). In α7/α3 chimeric nicotinic ACh receptors, mutation at Asp-266 in the M2–M3 loop of the α7 subunit exhibited poor response to different nicotinic agonists. Similar results were observed when the aligned residue in the ACh β4 subunit were mutated in α3/β4 ACh receptor (26). All these studies suggest that the M2–M3 loop may be involved in coupling conformational change between the two domains. There is, however, a paucity.
of data on the structure of the M2–M3 loop and little information on its structural dynamics during gating. Consistent with conformational change in this region, in the homo-pentameric glycine receptor, the water accessibility of residues in the N-terminal end of the M2–M3 loop increases in the presence of glycine (27).

To probe the structure and conformational changes that occur in the M2–M3 loop during channel gating, we used the substituted cysteine accessibility method (SCAM) (28, 29). This method assays the water-surface accessibility of engineered cysteine residues by their reactivity with water-soluble, charged sulfhydryl-specific reagents (29). The reagents used in this study were permanently charged derivatives of methanethiosulfonate (MTS), a positively charged derivative, MTS-ethanesulfonate (MTSES) as the 20 residue, 1Leu-261, ACh α1 subunit, the mouse muscle ACh α1 subunit, and the mouse 5-HT₃A subunit. The light gray bars underneath indicate the M2 and M3 segments defined on the basis of hydropathy analysis. The dark gray regions indicate the uncertainty in the ends of the M2 and M3 segments based on sequence analysis. The residues in this region have a large “X” over them in the top line. The underlined regions indicate positions studied by the substituted cysteine accessibility method in this or in other studies: data for GABA receptor α₁ subunit (13, 33), glycine receptor (27), ACh receptor (38, 53), and 5-HT₃A (39). Cysteines substituted for the residues in boldface italic letters are reactive with sulfhydryl reagents. Numbers at the right of each row indicate the amino acid number of the last residue shown. The first residue is the conserved leucine, the “position” in the M2 segment indexing system (35): GABA, α₁Leu-263, β₃Leu-259, γ₂Leu-274, glycine α₁Leu-261, ACh α₁Leu-261, and 5-HT₃A Leu-287.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—The cDNAs encoding the rat GABAₐ receptor α₁, β₁, and γ₂ subunits in the pGEMHE vector were used (30). To ensure that the sulfhydryl reagents only reacted with the engineered Cys all of the endogenous Cys in membrane-spanning segments were mutated to other amino acids. Thus, “wild type” subunits had the following mutations: α₁, C239S and C292S; β₁, C288S; and γ₂, C244S, C303A, and C413A. Hereafter, these “Cys-minus” constructs will be referred to as wild type. The α₁, Cys-minus construct was used as the background for the M2–M3 loop mutations from α₁S275C to 1289C. Mutations in the mouse ACh α₁ receptors were generated by PCR and confirmed by DNA sequencing (31). The α₁ wild type and all α₁ Cys mutants except α₁R273C, α₁N274C, and α₁S275C contained the FLAG epitope tag sequence inserted between residues 4 and 5 in the mature protein sequence. The β₁ subunit was tagged with the myc epitope sequence at the same site as described previously (14). Insertion of these epitope tags had no functional effect (32). α₁R273C and α₁N274C were in the wild type, endogenous-Cys-containing background. The M2 segment residues from α₁E249 to α₁N274 and the M3 segment residues from α₁A290 to α₁V306 were studied previously (13, 33). The 5’-capped cRNA were made using standard procedures (14). Oocytes were injected with 50 nl of RNA (200 pg/nl) mixed in an equal ratio of α₁:β₃:γ₂. After injection, oocytes were incubated at 16–17 °C for 2–3 days in OR3 solution before use in electrophysiological experiments (14).

Residues in the M2 segment are referred to by an indexing number system that numbers a conserved positively charged amino acid, aligned with GABA receptor α₁Arg-254, at the cytoplasmic end of M2 as the ‘position’ and the residues (α₁Asn-274) aligned with the ACh receptor extracellular ring of charge (34) at the extracellular end of M2 as the ‘20 position’ (35).

**Electrophysiology**—Two electrode voltage clamp experiments were performed at room temperature as described previously (13). Holding potential was −60 mV. The oocyte was continuously perfused at 6 ml/min with calcium-free frog Ringer (CFFR), containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl₂, and 10 mM HEPES (pH 7.5). To determine the GABA EC₅₀ concentration for wild type and the Cys mutants, concentration-response curves were generated by recording the currents elicited by increasing concentrations of GABA (Fig. 2). The GABA-induced currents for each GABA concentration were normalized relative to the maximal currents (Iₘax) and then plotted as a function of GABA concentration. The curves were fitted to the Hill equation. Iₘax = [I]EC₅₀ [1 + X], where EC₅₀ is the maximum GABA-induced current, [c] is the GABA concentration, EC₅₀ is the GABA concentration that gives half-maximal current, and n is the Hill coefficient, using Sigma Plot 2000 (SPSS Inc., Chicago, IL).

**Sulfhydryl Reagents**—We used the MTS derivatives MTSET™ and MTSES™ (Biotium, Inc., Hayward, CA). They add −SCH₂CH₂X onto the Cys where X is N(CH₃)₂ for MTSET™ and SO₃ for MTSES™ (28).

**Sulfhydryl Reagent Effects**—Reaction of the MTS reagents with engineered Cys residues was assayed functionally by effects on GABA-induced currents. The effects of MTSET™ and MTSES™ were studied using the following protocol: two 10- to 20-s pulses of submaximal GABA concentration (EC₅₀ – EC₉₀), designated as test pulses, were applied. Next, 1 ms MTSET™ or 10 ms MTSES™ near-saturating GABA concentration (5 times EC₅₀) was applied for 1 min. This was followed by two submaximal GABA test pulses. All applications were separated by 3- to 5-min washes with CFFR to allow complete recovery from desensitization. GABA-induced currents were stable before and after application of the sulfhydryl reagents. Experiments in which the peak currents of the two GABA test responses were not within 5% were discarded. The percentage effects of the sulfhydryl-specific reagents were calculated using the following equation: ([I(test)/I(max)] – 1) × 100. Iₘax designates the average peak current of the two test pulses after MTS reagent application, and I(test) is the average peak current of the two initial GABA test pulses. To avoid MTS reagent hydrolysis, 100 mM CFFR immediately before use (29).

Data are presented as mean ± S.E. of at least three observations. All experiments were performed at least two separate batches of oocytes from different frogs. Statistical significance of effects was determined by one-way analysis of variance (ANOVA) using the Dunnett post hoc test with wild type as control. p < 0.05 was considered significant.

It is important to recognize that MTS-reactive residues were identified based on functional effects of modification. Functional effects were determined by the statistical significance of the effect on a mutant.
relative to the effect on wild type. For some mutants the average effect following MTSET reagent application was small. Whether such an effect was statistically significant depended, in part, on the stringency of the one-way ANOVA post hoc test used. For example, in our previous work on the M3 segment, using the Student-Newman-Kuels post hoc test to determine significance, the effect of pCMBS applied in the presence of GABA was significant at six residues: α1A290C, α1Y293C, α1F297C, α1A299C, α1L300C, and α1E302C. With the less stringent Duncan post hoc test, an additional residue, α1F295C was judged to be reactive with pCMBS applied in the presence of GABA (33). The choice of post hoc test is, unfortunately, somewhat arbitrary. Thus, for Cys mutants where the effects of complete reaction are small, it may be difficult to determine whether reaction has occurred.

For screening experiments 1 mM MTSET$^-$ was applied for 1 min. This combination of time and concentration limits our ability to detect reactive residues. For a given mutant, based on the variability of responses, application of a reagent must cause a net change in current greater than ~50% to be statistically significantly different than wild type by a one-way ANOVA (for n between 3 and 6). Thus, if complete reaction caused 100% inhibition of the GABA-induced current, with a detection threshold of 30% effect and the MTSET$^-$ reaction conditions of 1 mM applied for 1 min, the slowest reaction rate that we can detect must have a second order reaction rate constant of ~7 × 10^5 liters/mol·s. To place this in context, the second order rate constant for MTSET$^-$ reaction with 2-mercaptoethanol in solution at pH 7.0 is ~2.1 × 10^5 liters/mol·s (39).

**Measurement of Reaction Rates**—The reaction rate of MTS reagents with engineered Cys were determined by the method described previously (36). A test pulse of GABA was applied to monitor the GABA-induced current. MTS reagents (1 µM to 1 mM) were applied with or without GABA for 10–60 s. The MTS reagent concentration was constant during an experiment and in vast molar excess to the number of Cys on the oocyte surface. After washing for 3–5 min, a second test pulse of GABA was applied and the peak current was measured. The brief pulses of reagent alternating with GABA test pulses continued until the reaction saturated, as observed by the plateau of the GABA test-pulse current magnitude. The magnitude of GABA-induced, test-pulse currents was normalized, relative to the current induced by GABA test pulses before exposure to the MTS reagents. The normalized currents were plotted as a function of cumulative exposure time to the MTS reagents. Using Prism 3 software (GraphPad Software, Inc., San Diego, CA) the curves were fitted with single- or double-exponential functions. The F-test was used to determine whether the double-exponential fit was significantly better than the single-exponential fit. In all cases, except α1A290C in the presence of GABA, the best fit was obtained with a single-exponential function. The pseudofirst-order rate constant, obtained from the exponential fit, was divided by the molar concentration of MTS reagents to give the second order reaction rate constant. At most positions the pseudo-first order reaction rates were determined using at least two different concentrations of MTS reagent. Similar second order rates were obtained in all cases consistent with the reaction being a simple bimolecular reaction.

**RESULTS**

**Characterization of Cysteine Substitution Mutants**—Oocytes expressing each of the Cys substitution mutants displayed GABA-evoked currents. This indicated that the mutations were tolerated and yielded functional channels. The GABA EC⁵₀ values for the wild type and the mutants were determined (Table I and Fig. 2). Five mutants, α1L276C, α1F277C, α1K278C, α1D286C, and α1P288C, had statistically significant increases in GABA EC⁵₀ (Table I). The Hill coefficient for wild type was 1.5, whereas for the mutants it ranged from 0.9 to 1.7. For wild type the average maximal current (I_max) was ~405 ± 359 pA (n = 28) at a holding potential of ~60 mV. The I_max for the mutants ranged from ~206 ± 41 nA (α1D286C) to ~2496 ± 236 nA (α1L289C) (Table I). For three mutants, α1A282C, α1D286C, and α1P288C, I_max was only 5–8% of the wild type I_max. The currents, however, were sufficiently large for our experiments.

**Effect of MTSET$^-$**—We studied the sensitivity to MTSET$^-$ of wild type and Cys substitution mutants both in the absence and in the presence of GABA. In the absence of GABA, the channels are in the closed state most of the time. Application of 1 mM MTSET$^-$ for 1 min irreversibly potentiated the GABA test pulse current amplitude for the mutants α1R273C, α1N274C, α1S275C, α1L276C, α1V279C, and α1A284C (Figs. 3 and 4A). The GABA-induced currents of the mutant α1Y281C were significantly reduced after exposure to MTSET$^-$ (Fig. 4A). MTSET$^-$ application to wild type receptors and the other Cys mutants caused no functional effects (Fig. 4A). At α1L276C the GABA-induced currents following MTSET$^-$ treatment in the absence of GABA were potentiated by 24%, however, this was not statistically significant.

In the presence of GABA, the channels undergo transitions between the open, desensitized, and closed states. Application of 1 mM MTSET$^-$ in the presence of GABA irreversibly potentiated the subsequent GABA test currents for the mutants α1R273C, α1N274C, α1S275C, α1K278C, α1V279C, and α1A284C (Fig. 4B). The subsequent GABA test currents were irreversibly inhibited for the mutant α1Y281C (Fig. 4B). The mutant α1K278C showed 56% potentiation of subsequent GABA-induced currents when modified in a closed state (Fig.

### Table I

| Mutant   | n | EC⁵₀ (µM) | Hill coefficient | I_max (nA) |
|----------|---|-----------|------------------|------------|
| WT       | 5 | 10 ± 2 ²  | 1.5 ± 0.2 ²      | 4034 ± 359 |
| R273C    | 3 | 12 ± 1   | 0.9 ± 0.1        | 2621 ± 302 |
| N274C    | 4 | 5 ± 0.2  | 1.0 ± 0.1        | 3397 ± 423 |
| S275C    | 3 | 3 ± 0.1  | 1.1 ± 0.1        | 2639 ± 146 |
| L276C    | 4 | 138 ± 12 | 1.7 ± 0.2        | 1190 ± 428 |
| P277C    | 4 | 143 ± 17 | 1.3 ± 0.5        | 1036 ± 112 |
| R273C    | 6 | 51 ± 13  | 1.2 ± 0.1        | 2745 ± 134 |
| V279C    | 3 | 20 ± 5   | 1.0 ± 0.1        | 2633 ± 149 |
| A280C    | 4 | 8 ± 2    | 1.4 ± 0.2        | 2832 ± 509 |
| Y281C    | 3 | 9 ± 2    | 1.0 ± 0.2        | 2891 ± 216 |
| A284C    | 4 | 42 ± 3   | 1.5 ± 0.6        | 292 ± 43  |
| T283C    | 5 | 24 ± 4   | 1.2 ± 0.1        | 2470 ± 227 |
| A284C    | 5 | 12 ± 1   | 1.6 ± 0.3        | 2403 ± 417 |
| M285C    | 3 | 13 ± 1.5 | 1.3 ± 0.1        | 2597 ± 241 |
| D286C    | 4 | 77 ± 17  | 1.0 ± 0.1        | 206 ± 41  |
| W287C    | 8 | 3 ± 0.5  | 1.2 ± 0.1        | 2699 ± 139 |
| F288C    | 4 | 65 ± 3b  | 1.0 ± 0.1        | 257 ± 21  |
| I289C    | 3 | 37 ± 8   | 1.4 ± 0.1        | 3496 ± 239 |

² Mean ± S.E.
² Statistically significantly different from WT by one-way ANOVA with Dunnett’s post hoc test.
MTSET acted with \( \alpha \)-V279C in the absence of GABA irreversibly potentiating subsequent GABA-induced currents. However, when MTSET was applied in the absence of GABA, MTSET reacts silently. An inhibitory effect of pretreatment with MTSET + GABA was reversed by application of 10 mM dithiothreitol between the MTSET + GABA applications (Fig. 3E). This implies that, when applied in the presence of GABA, MTSET covalently modified \( \alpha \)-K278C in a functionally silent manner. However, when applied in the absence of GABA, MTSET modification altered subsequent channel function. The potentiation of GABA-induced currents by MTSET treatment in the closed state was not reversed by subsequent co-application of GABA + MTSET (data not shown). These experiments imply that the cysteines in both \( \alpha \) subunits were reacting in both conditions.

A similar phenomenon was observed with the mutant \( \alpha_1 \)L276C. Modification by 1 mM MTSET alone is not shown. A similar phenomenon was also observed with the mutant \( \alpha_1 \)L276C. Modification by 1 mM MTSET when it was first applied with GABA thereby blocking the possibility of reaction with the engineered Cys when MTSET was applied a second time in the absence of GABA. In D the current during application of MTSET + GABA alone is not shown. E, application of 10 mM dithiothreitol between the MTSET + GABA applications reverses the ability of MTSET + GABA to block the reaction during the second MTSET + GABA application in the absence of GABA. Thus, in contrast to the absence of an effect by the second MTSET + GABA application in D, now the second application causes potentiation of the subsequent currents. All traces are separated by 3- to 5-min washes with CFFR buffer to allow complete recovery from desensitization. The stability of the GABA-induced currents is indicated by the similar magnitudes of the pairs of GABA test currents before and after MTSET + GABA application. Duration of application of GABA and 1 mM MTSET + GABA is indicated by bars above the traces. Near-saturating GABA co-applied with MTSET + GABA was 100 \( \mu \)M.
GABA potentiated the subsequent GABA test pulses by 62% (Fig. 4). Application of 1 mM MTSET$^{+}$ in the absence of GABA did not have a statistically significant effect on the subsequent GABA-induced currents (Fig. 4A). Nevertheless, MTSET$^{+}$ reacted in the absence of GABA with the engineered Cys in \(\alpha_1\)L276C, because it inhibited the ability of a subsequent application of MTSET$^{+}$ in the presence of GABA to potentiate GABA-induced currents (data not shown).

In our original SCAM analysis we reported that application of the MTS reagents had no functional effect on \(\alpha_1\)R273C (13). In those experiments we used only saturating concentrations of GABA for the test pulses. Here we have used submaximal GABA concentrations for the test pulses. The functional effects of covalent modification by MTS reagents can alter single channel conductance or channel gating. If MTS modification only alters gating and not conductance, this may cause a shift in the GABA \(EC_{50}\) but should not affect maximal current elicited by near-saturating GABA on wild type and the M2–M3 loop Cys mutants. Gray bars indicate effects that are significantly different than the effect on wild type by a one-way ANOVA. Negative effect indicates inhibition, and positive effect indicates potentiation of subsequent EC$_{50}$ GABA-induced currents. There are two notable differences between the effects follow application of MTSES$^-$ and MTSET$^{+}$. Note that at \(\alpha_1\)A280C subsequent currents were inhibited following MTSES$^-$ application, but application of MTSET$^{+}$ had no effect on the subsequent currents in this mutant. Also, application of MTSES$^-$ had no effect on subsequent current at \(\alpha_1\)L276C, but MTSET$^{+}$ had a significant effect.

**Effect of MTSES$^-$**—The reaction rate of sulphydryls, such as 2-mercaptoethanol, with MTSES$^-$ (1.7 \(\times\) 10$^4$ liters/mol-s) is about 10-fold lower than the rate with MTSET$^{+}$ (29). Therefore, for MTSES$^-$ screening experiments we used a 10-fold higher concentration of MTSES$^-$ than MTSET$^{+}$. A 1-min application of 10 mM MTSES$^-$, both in the absence and in the presence of GABA, significantly potentiated the subsequent GABA-induced currents of the mutants \(\alpha_1\)R273C, \(\alpha_1\)N274C, \(\alpha_1\)S275C, \(\alpha_1\)V279C, and \(\alpha_1\)A284C and significantly inhibited the subsequent GABA-induced currents of the mutants \(\alpha_1\)A280C and \(\alpha_1\)Y281C (Figs. 5 and 6). MTSES$^-$ treatment of \(\alpha_1\)L276C and \(\alpha_1\)K278C did not have any functional effect on subsequent GABA-induced currents, but it blocked the effect of a subsequent application of MTSET$^{+}$ (data not shown). We inferred that these mutants reacted with MTSES$^-$, but the modification failed to exert any functional changes. Similarly, a 1-min application of MTSET$^{+}$ to \(\alpha_1\)A280C had no functional effects other than blocking the inhibition induced by application of MTSES$^-$ (Fig. 6). Thus, MTSET$^{+}$ reacted silently with \(\alpha_1\)A280C. These findings indicate that the lack of functional effects does not necessarily signify that a residue has not reacted and is not on the water-accessible surface.

**MTSET$^{+}$ Reaction Rates with Cys Substitution Mutants**—We measured the reaction rates of MTSET$^{+}$ with the accessible mutants in different receptor states, i.e. the closed state and in the presence of near-saturating GABA. Because the modification of \(\alpha_1\)A280C by MTSET$^{+}$ had no functional effect, we determined the reaction rates with MTSET$^{+}$ (Fig. 7). The results are summarized in Table II. For the mutants \(\alpha_1\)N274C, \(\alpha_1\)S275C, \(\alpha_1\)K278C, and \(\alpha_1\)Y281C, the closed state MTSET$^{+}$ reaction rates were of similar magnitude, >1100 liters/mol-s. In contrast, for the mutants \(\alpha_1\)R273C, \(\alpha_1\)V279C, \(\alpha_1\)A280C,\(^2\) and \(\alpha_1\)A284C the closed-state MTSET$^{+}$ reaction rates were <300 liters/mol-s.

In the presence of GABA, the MTSET$^{+}$ reaction rates with the residues \(\alpha_1\)N274C, \(\alpha_1\)S275C, \(\alpha_1\)V279C, and \(\alpha_1\)A284C were 3, 8, 7, and 2 times faster, respectively, than in the absence of GABA. The increase in reaction rate could result from changes in multiple factors. These include: 1) conformational changes either in this region or in a region contacting the M2–M3 loop that alters accessibility, 2) increased fractional time spent on the water-accessible surface, and/or 3) changes in local electrostatic potential that either alter the extent of ionization of the Cys or the local concentration of the MTS reagent. We cannot distinguish which of the above factors alters reactivity in the presence of GABA. The MTSET$^{+}$ reaction rate with the residue \(\alpha_1\)Y281C was the same in the absence and presence of GABA, implying that the environment surrounding this residue did not change in the presence of agonist.

Only reaction with MTSES$^-$ had an effect on subsequent currents at \(\alpha_1\)A280C. Applied in the closed state, the MTSES$^-$ reaction rate was 28 ± 5 liters/mol-s. When applied in the presence of GABA the plot of MTSES$^-$ cumulative exposure

---

\(^2\)For \(\alpha_1\)A280C only reaction with MTSES$^-$ had a functional effect, although MTSET$^{+}$ reacted. Thus, we measured the reaction rate with MTSES$^-$.
time versus percent change in current (Fig. 7) was best fit by a double-exponential equation (as judged by F-test, Prism). This gave two reaction rates (121 ± 38 and 695 ± 33 liters/mol-s, n = 3) with MTSES− in the presence of GABA. A possible explanation is that, in the presence of near-saturating GABA (5 times EC50) there are two main populations of channels, i.e. desensitized and open. The two rate constants may reflect different MTSES− reaction rates with open and desensitized channels. Alternatively, there are two α subunits and thus two engineered Cys in each functional channel. If the accessibility of the two Cys was different, we might observe two reaction rates if reaction at each site contributed to the total effect.

**DISCUSSION**

We have shown that Cys substituted for all residues in the N-terminal portion of the M2–M3 loop between α1Arg-273 and α1Tyr-281, except α1Pro-277, reacted with MTS reagents in the absence and in the presence of GABA. Based on the closed state MTSET+ reaction rates these Cys mutants can be divided into two groups (Table II). The reaction rate of one group, which included α1N274C, α1S275C, α1K278C, and α1Y281C, was greater than 1000 liters/mol-s. The reaction rate of the other group, which included α1R273C, α1L276C, α1V279C, and α1A280C, was less than 300 liters/mol-s. On an α helical wheel plot the two groups of residues fall on opposite sides of the helix (Fig. 8A). The helical face containing the fast reacting residues is a direct continuation of the channel-lining face of the M2 segment (Fig. 8B) (13). The GABA_A receptor M2 segment is largely α helical, based on SCAM experiments (13). Likewise, in ACh and 5-HT3 receptors a variety of experiments imply that the M2 segment is largely α helical (6, 37–40). The M2 segment extracellular end is predicted, by amino acid sequence analysis, to lie near the 20′ position (α1Asn-274), aligned with the extracellular ring of charge in the acetylcholine receptor (7, 8, 34, 41). Sequence analysis predicts that the residues beyond the 20′ position, in the M2–M3 loop, are in an extended structure (8). Our present experimental results suggest that the M2 segment α-helix extends beyond the predicted end of the M2 segment to include an additional two helical turns. The residues on the fast reacting face presumably line the wide, extracellular, channel vestibule. At present we do not know the position of these residues relative to the extracellular surface of the membrane.

We infer that MTS reagent access to the slow reacting side of the helix is restricted relative to the fast reacting side. The residues on the slow reacting face may interact with the lipid headgroup region of the bilayer or may interact with other parts of the protein. The identity of protein domains that might interact with the slow reacting face residues might include residues from other membrane-spanning segments or residues from the extracellular domain. Based on the high resolution crystal structure of the homologous acetylcholine binding protein, we can infer which extracellular domain residues may interact with the slow reacting face. In the crystal structure the pre-M1 region, the Cys loop, or the L1 loop are close to the membrane surface and, thus, may interact with the M2–M3 loop residues (12). The interaction with these other protein regions cannot be very tight. The MTS-reactive M2–M3 loop residues must, at least transiently, reach the water-accessible surface during the closed state to react with MTSET+, because the MTS reagents react with ionized thiolate groups 109 times faster than with un-ionized thiols (42), and only water-accessible cysteines will ionize to any significant extent. Thus, MTS-reactive residues are, at least transiently, on the water-accessible protein surface. Several lines of evidence are consistent with the assumption that sulfhydryl reactivity is a measure of water-surface accessibility. In the aspartate chemotaxis receptor, a protein of known crystal structure, reactivity correlated with surface accessibility (43). More recently, the results of SCAM experiments on the dopamine D2 receptor correlated with surface accessibility of the aligned residues in the crystal structure of rhodopsin, a homologous G-protein-coupled receptor (44). Thus, even positions on the slow reacting helix face are, at least transiently, on the water-accessible protein surface to allow reaction with the MTS reagents. SCAM experiments in the homopentameric glycine receptor also reported that Cys substitution mutants in the N-terminal half of the M2–M3 loop were MTS-reactive (27). Similar differences in reaction rates, however, were not observed. In the case of the glycine receptor, each receptor has five engineered cysteines, one in each subunit, that may complicate the results because of the conformation of each subunit may not be identical.

Alanine scanning mutagenesis of the N-terminal half of the M2–M3 loop showed effects on GABA EC50 and efficacy only at α1Arg-273 and α1Leu-276, both on the slow reacting face (23). Another mutagenesis study showed that mutations of slow reacting face residues, α1L276A, α1P277A, and α1Val279A, caused a right shift in the GABA EC50 (24). Thus, it appears that mutation of slow reacting face residues has a greater effect.
ribenzenesulfonate (pCMBS/H11002 MTS reagents (Figs. 4 and 5). We also tested for effects of other no evidence that the Cys substituted for it reacted with the slow reacting, non-channel-lining face of the helix. There was reduced the maximal currents. Mutation to alanine, however, reacting face, aligned with GABA-A helical wheel (A) and an α helical net (B). A, residues with MTSET+ reaction rates > 1000 liters/mol-s are indicated by black squares. Residues with reaction rates < 300 liters/mol-s are indicated by black triangles. The dashed line separates the fast and slow reacting faces of the helix. Note that the fast reacting residues lie on one face of the helix. B, a helical net representation. The extracellular end is at the top. Residues that align vertically are on the same face of the helix. Channel-lining, sulfhydryl-reactive M2 segment residues for which reaction rates are not known are indicated by black circles. The channel-lining face residues are shown in boldface type. Note that the fast reacting residues lie on the same α helical face as the channel-lining residues. The vertical dashed line separates fast and slow reacting faces as in panel A.

on protein function than mutation of fast reacting face residues. An α helical secondary structure in this region of the M2–M3 loop, with one side facing the channel lumen and the other side interacting with protein or lipid, may help to explain the observation by Auerbach and colleagues (22). They found that hydrophobic mutations at adjacent positions in the ACh receptor α subunit M2–M3 loop had opposite effects on channel opening (22). Hydrophobic mutations at the position aligned with α1 Tyr-281, on the fast reacting face, increased the channel opening rate, whereas at the adjacent positions on the slow reacting face, aligned with GABA-A, α1 Val-279 and α1 Ala-280, hydrophobic mutations decreased the channel opening rate (22).

The highly conserved proline at the 23' position lies on the slow reacting, non-channel-lining face of the helix. There was no evidence that the Cys substituted for it reacted with the MTS reagents (Figs. 4 and 5). We also tested for effects of other sulfhydryl reagents at this position, including p-chloromercuribenzenesulfonate (pCMBS-) and MTS-tetramethylrhodamine, but neither had any effect (data not shown). Mutation of α1 Pro-277 to Cys caused a 14-fold increase in GABA EC50 and reduced the maximal currents. Mutation to alanine, however, was reported to decrease the GABA EC50 in one study using the α2 subunit (23) and increase it in another that used the α1 subunit (24). The mutated α1P277C channels were still functional, suggesting that the proline is not essential for channel function. The marked effect of mutating the proline to Cys, and the lack of effect of application of the MTS reagents to this mutant, suggests that these reagents do not react with this engineered Cys, but we cannot rule out silent reaction. The proline may induce a bend in the helix in this region. The extent of a proline-induced bend is difficult to predict (45–48). This proline, or the structure of this region induced by the proline, may have a role in signal transduction from the extracellular ligand binding domain to the gate in the membrane-spanning domain. Perhaps α1 Pro-277 is in tight contact with another part of the protein, or the bend at this position may make the Cys inaccessible to the MTS reagents. We cannot distinguish these possibilities at present.

The MTSET+ reaction rates could be measured both in the absence and presence of GABA at several of the Cys mutants. Reaction rates increased in the presence of GABA at positions on both sides of the helix. It should be remembered that in the presence of GABA the channel undergoes transitions between the open, desensitized, and closed states on a rapid time scale relative to our experiments. We do not know in which state(s) reaction is occurring in the presence of GABA. The GABA-induced rate increases indicate that this region undergoes a conformational change during gating. Increased reaction rates could result from changes in several factors, including: 1) the fractional time on the water-accessible surface could increase due to increased conformational mobility/flexibility, 2) local steric obstructions from neighboring residues or other parts of the protein could be reduced, and 3) changes in the local electrostatic potential could increase the ionization of the Cys or change the local concentration of MTSET+. At present we cannot distinguish between these possibilities, although an electrostatic change affecting local MTS reagent concentration is the least likely, because MTSES− also reacted at these positions. At two positions, α1R273C and α1Y281C, GABA did not induce a significant increase in the MTSET+ reaction rate.

Covalent modification of an engineered Cys can alter macroscopic GABA-induced currents by affecting the single channel conductance or channel gating kinetics. At most reactive positions modification by the positively and negatively charged reagents had similar functional effects, i.e. in most cases potentiation of subsequent submaximal GABA-evoked currents. Furthermore, MTS reagent modification of M2–M3 loop Cys mutants had little effect on currents evoked by saturating GABA concentrations.3 Thus, we infer that the effects of modification on GABA-induced currents were not due to electro-

3 A. K. Bera, M. Chatav, and M. H. Akabas, unpublished observations.

### Table II

| Mutant | Reagent | Closed state | n | > GABA |
|--------|---------|--------------|---|--------|
| R273C  | MTSET+  | 237 ± 13     | 3 | 371 ± 98 |
| N274C  | MTSET+  | 1878 ± 436   | 4 | 4981 ± 789 |
| S275C  | MTSET+  | 1160 ± 231   | 7 | 9743 ± 1281 |
| L276C  | MTSET+  | 61 ± 7       | 4 |        |
| K278C  | MTSET+  | 1163 ± 93    | 3 |        |
| V279C  | MTSET+  | 185 ± 62     | 4 | 1353 ± 91 |
| A280C  | MTSES−  | 28 ± 5       | 4 | 121 ± 38− |
| Y281C  | MTSET+  | 1067 ± 89    | 3 | 1023 ± 31 |
| A284C  | MTSET+  | 39 ± 5       | 3 | 79 ± 7   |

* Units are liters/mol-s, mean ± S.E.
* n = number of oocytes.
* Rate in the presence of GABA is significantly different from the rate in the absence of GABA.
* Unlike other mutants, the data for this mutant in the presence of GABA were better fit by a double-exponential function.

---

A different representation of the helical wheel, highlighting the slow reacting, non-channel-lining face of the helix.
static interactions of the charge on the modified Cys with permeating ions. This implies that the potentiation due to
covalent modification of the engineered Cys residues presumably
results from a left shift in the GABA EC_{50} as we have
observed previously in the M3 segment (33). In the absence of
detailed single channel kinetic analysis of the effects of covalent
modification at each position we do not know whether
modification is affecting channel opening rates, closing rates,
or desensitization rates. Furthermore, in the absence of a high
resolution structure of the channel, it is not possible to provide
a structural explanation for why covalent modification causes poten-
tiation as opposed to inhibition. The problem is similar to
the problem of interpreting the functional effects of mutagen-
esis experiments. Mutagenesis of some positions in this region
alter GABA EC_{50} (23, 24, 49). One does not know whether
these changes occur, because specific interactions between the mu-
tated residue and other residues were altered or because the
mutation caused a long range alteration in protein structure
that altered function. Long range structural effects of mutagen-
esis have been reported. For example, in a crystallographic
study of dihydrofolate reductase, a mutation 15 Å from
the catalytic site caused less than a 1-Å deviation in the backbone
structure resulting in a change in rotamer conformation of a
residue in the catalytic site (50). There is no high resolution
structural information on the extracellular end of the GABAA
receptor channel. From the 9-Å resolution, cryo-electron micro-
scopic images of the homologous ACh receptor the channel
diameter in this region is about 20 Å (37). Charge change
mutations in the ACh receptor at the M2 segment extracellular
ring of charge, aligned with GABAA receptor α_{1}Asn-274, could
not be explained by simple electrostatic effects on conductance
(34, 51). In the Drosophila GABA receptor, the effects of mu-
tations at the adjacent position, aligned with α_{1}Arg-273, were
complicated suggesting that the effects arose from structural
perturbations of the M2 segment (52). Thus, the conforma-
tion of this region may be sensitive to mutation. From disulfide
cross-linking studies we inferred that there was considerable
thermal motion in the extracellular ends of the M2 segments
and this may extend into the M2–M3 loop residues (14). Coval-
ent modification of residues in this region may alter the ther-
mal motion and the conformational changes that this region
undergoes during GABA-induced gating.

At two positions on the slow reacting face, α_{1}L276C and
α_{1}A280C, both MTSET^{+} and MTSES^{-} reacted, but only one
reagent caused a functional effect on subsequent GABA-in-
duced currents, MTSET^{+} at α_{1}L276C and MTSES^{-} at
α_{1}A280C (Figs. 4 and 5). Because these two MTS reagents are
of comparable size and could fit in a right cylinder 10 Å long
with a 6-Å diameter, it is likely that the functional effects of
modification at these two positions are due to electrostatic
interactions rather than steric effects. Although electrostatic
forces seem to be important for the effects of modification at
these two positions, they do not follow simple charge rela-
tionships and thus we do not believe that they result from inter-
actions with permeating ions. If this region has an α helical
secondary structure, the Cα carbons of these two residues
would lie ~4.5 Å and ~10.5 Å above the Cα carbon of α_{1}Arg-
273. Thus, the electrostatic interaction might be with this
positively charged arginine or with another charged residue
from elsewhere in the protein.

At two positions, α_{1}L276C and α_{1}K278C, MTSET^{+} reacted
in both the absence and presence of GABA but reaction only had
a functional effect on the subsequent GABA-induced currents if
it occurred in the presence of GABA at α_{1}L276C and in the
absence of GABA at α_{1}K278C (Figs. 3 and 4). If MTSET^{+}
was applied sequentially in the absence and presence of GABA,
then whichever condition MTSET^{+} was applied first had the
controlling effect on subsequent currents (Fig. 3). For example,
if MTSET^{+} was applied to α_{1}K278C in the presence of GABA,
it had no functional effect. A subsequent application of
MTSET^{+} in the absence of GABA did not change the currents
(Fig. 3D). However, if MTSET^{+} were first applied in the pres-
ence of GABA, the subsequent currents were potentiated and
a subsequent application of MTSET^{+} in the absence of GABA
had no effect on the potentiated currents. This implies that the
engineered cysteines in both α subunits in each functional
receptor complex were modified during the first application of
MTSET^{+}, whether it was in the absence or in the presence of
GABA. This prevented the subsequent application from having
an effect. This implies that the conformations of the engineered
Cys at positions 276 and 278 must be different in the closed and
in the GABA-activated states. Once modified, the conformation
is trapped, depending on whether the MTS reagent reacted in
the absence or in the presence of GABA. The structural basis of
this phenomenon is unclear, however, a similar effect was
observed in the ACh receptor M2 segment at αs252C (38).

In the C-terminal half of the M2–M3 loop, from α_{1}Ala-282 to
α_{1}Ile-289, there was only one MTS-reactive residue, α_{1}A284C.
The reaction rate was less than 100 liters/mol-s (Table II). At
the other positions there was no functional effect of MTS rea-
gent application in either the absence or in the presence of
GABA. We must interpret with caution results at positions
where MTS reagent application had no effect, because there are
two alternative explanations. One, the reagents did not react at
a measurable rate either because the Cys is buried or local
steric or electrostatic factors prevented access of the reagent
and, two, the reagent did react but had no functional effect on
GABA-induced currents (29). Our electrophysiological assay
cannot distinguish between these possibilities. If these resi-
dues are buried they may be tightly interacting with other parts
of the protein, such as the extracellular domain. Of note, the Cys mutants at positions α_{1}Asp-286 and α_{1}Phe-288
causd significant increases in GABA EC_{50} and reductions in
the maximal GABA-induced currents compared with wild
type (Table I). At α_{1}A282C only the maximal current was
significantly affected by the mutation. As discussed above, in
the absence of a high resolution structure it is difficult to
discern the structural basis for these functional consequences
of mutations.

In summary, our results suggest that the M2 segment α-helix
extends two helical turns beyond the predicted end of the
M2 segment. The channel-lining face of this region may help to
line the wide extracellular channel vestibule. This region of
the protein undergoes a conformational change during GABA-
duced gating that increases the reactivity of Cys-substituted
mutants. There were no functional consequences of MTS rea-
gent application to Cys substituted for residues in the C-
terminal end of the M2–M3 loop. This region may be tightly
packed in the protein structure or covalent modification may be
functionally silent. Further experiments will help to elucidate
the role of this region in the transduction of GABA binding into
opening of the transmembrane ion channel.

Acknowledgments—We thank Dr. Moez Bali, Dr. Jeff Horenstein,
and Paul Riegelhaupt for helpful discussions and comments on this
manuscript.

REFERENCES
1. Rabow, L. E., Russek, S. J., and Farb, D. H. (1995) Synapse 21, 189–274
2. Ortelis, M. O., and Lunt, G. G. (1995) Trends Neurosci. 18, 121–127
3. Le Novere, N., and Changeux, J. P. (1999) Nucleic Acids Res. 27, 340–342
4. Krasowski, M. D., and Harrison, N. L. (1999) Cell Mol. Life Sci. 55, 1278–1303
5. Macdonald, R. L., and Olesen, R. W. (1994) Annu. Rev. Neurosci. 17, 569–602
6. Karlin, A., and Akabas, M. H. (1995) Neuron 15, 1231–1244
7. Schofield, P. R., Darlison, M. G., Fujita, N., Burt, D. R., Stephenson, F. A.,
Rodriguez, H., Rheer, L. M., Ramachandran, J., Reale, V., Glenorse, T. A.,

N-terminal End of GABA\textsubscript{A} Receptor M2–M3 Loop Is a Helical
GABA<sub>A</sub> Receptor M2–M3 Loop Secondary Structure and Changes in Accessibility during Channel Gating
Amal K. Bera, Maya Chatav and Myles H. Akabas

*J. Biol. Chem.* 2002, 277:43002-43010.
doi: 10.1074/jbc.M206321200 originally published online September 10, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206321200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 53 references, 17 of which can be accessed free at http://www.jbc.org/content/277/45/43002.full.html#ref-list-1