Roles for Loop 2 Residues of α1 Glycine Receptors in Agonist Activation*

The present study tested the hypothesis that several residues in Loop 2 of α1 glycine receptors (GlyRs) play important roles in mediating the transduction of agonist activation to channel gating. This was accomplished by investigating the effect of cysteine point mutations at positions 50–60 on glycine responses in α1GlyRs using two-electrode voltage clamp of Xenopus oocytes. Cysteine substitutions produced position-specific changes in glycine sensitivity that were consistent with a β-turn structure of Loop 2, with odd-numbered residues in the β-turn interacting with other agonist-activation elements at the interface between extracellular and transmembrane domains. We also tested the hypothesis that the charge at position 53 is important for agonist activation by measuring the glycine response of wild type (WT) and E53C GlyRs exposed to methanethiosulfonate reagents. As earlier, E53C GlyRs have a significantly higher EC50 than WT GlyRs. Exposing E53C GlyRs to the negatively charged 2-sulfonatoethyl methanethiosulfonate, but not neutral 2-hydroxyethyl methanethiosulfonate, positively charged 2-aminooxyethyl methanethiosulfonate, or 2-trimethylammonioethyl methanethiosulfonate, decreased the glycine EC50 to resemble WT GlyR responses. Exposure to these reagents did not significantly alter the glycine EC50 for WT GlyRs. The latter findings suggest that the negative charge at position 53 is important for activation of GlyRs through its interaction with positive charge(s) in other neighboring agonist activation elements. Collectively, the findings provide the basis for a refined molecular model of α1GlyRs based on the recent x-ray structure of a prokaryotic pentameric ligand-gated ion channel and offer insight into the structure-function relationships in GlyRs and possibly other ligand-gated ion channels.

Glycine is a major inhibitory neurotransmitter in the adult mammalian central nervous system (1, 2). It reduces central nervous system excitability via activation of a ligand-gated receptor linked to an integral chloride channel, the strychnine-sensitive glycine receptor (GlyR).2 GlyRs are members of a superfamily of ligand-gated ion channels (LGICs) known as Cys-loop receptors (3, 4), whose members also include γ-aminobutyric acid type A (GABA_A), nicotinic acetylcholine (nACh), and 5-hydroxytryptaminergic (5-HT), all of which assemble into ion channels with a pentameric structure. Cys-loop receptor subunits share significant sequence homology and consist of four transmembrane (TM) α-helical segments, an intracellular component for cytosolic interactions, and a large, extracellular ligand-binding domain (5–8).

Considerable evidence indicates that Loop 2 in the extracellular domain of Cys-loop receptors (loop terminology as defined by Sixma and co-workers (6)) is important for coupling agonist binding to channel gating in the TM domain (4, 9–12). The importance of the α1GlyR Loop 2 region in agonist activation was first noted when the phenotype of the spastic mouse was traced to a naturally occurring alanine-to-serine exchange at position 52 that results in a significant reduction in glycine sensitivity without affecting agonist binding characteristics (13, 14). These findings with the A52S mutation of the α1GlyR were supported by subsequent experiments, which expressed recombinant wild type (WT) and mutant GlyRs in Xenopus oocytes (15). Moreover, a splice variant of the α2GlyR revealed that replacing residues at positions 58 and 59 in α2GlyRs with the residues from homologous sites in α1GlyRs (Ile42 and Ala47) increased α2GlyR glycine sensitivity to resemble that of the α1GlyR (16). Additional studies found that mutating the charged residues in Loop 2 of the α1GlyR (positions 53 and 57) also altered GlyR glycine sensitivity (10). Taken together, these findings in GlyRs suggest that Loop 2 residues are not involved in agonist binding but that several of these residues play a significant role in transducing agonist activation.

2 The abbreviations used are: GlyR, glycine receptor; AChBP, acetylcholine-binding protein; GABA_A, γ-aminobutyric acid type A; GABA_B, γ-aminobutyric acid type B receptor; LGIC, ligand-gated ion channel; MTSEA, 2-aminooxyethyl methanethiosulfonate; MTSET, 2-hydroxyethyl methanethiosulfonate; MTSES, 2-sulfonatoethyl methanethiosulfonate; MTSET, 2-trimethylammonioethyl methanethiosulfonate; nAChR, nicotinic acetylcholine receptor; WT, wild type; TM, transmembrane; MTS, methanethiosulfonate.

* This work was supported, in whole or in part, by National Institutes of Health, NIAAA, Grants AA03972 (to R. L. A.), AA013890 (to D. L. D.), AA013922 (to D. L. D.), and AA013378 (to J. R. T.) and the University of Southern California School of Pharmacy. This work was conducted as partial fulfillment of the requirements for the Ph.D. degree in Neuroscience, University of Southern California (D. K. C.). Portions of these findings were presented at the Annual Meeting of the Society for Neuroscience in Atlanta, GA (Program 233.7/D32, 2006 Neuroscience Meeting Planner, Atlanta, GA, Society for Neuroscience). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Molecular modeling of GlyRs and GABA<sub>A</sub>Rs suggested that the extracellular region of these channels contains a large extraneous vestibule and a small oval chamber, with the latter being bounded, in part, by residues from Loop 2 (17, 18). The location of Loop 2 in the extracellular oval chamber suggests that Loop 2 may influence chloride ion movement prior to entering the TM segment of the pore. This suggestion is supported by Brownian dynamics simulations, which revealed that the charged residues in and near Loop 2 create an energy barrier to chloride ion movement (18). Based on these findings, the authors suggested that these acidic residues must move away from the pore or be partially neutralized during agonist activation in order to reduce the effective electrostatic barrier and permit chloride ion movement from the extracellular chamber deeper into the TM channel.

In contrast, site-directed mutagenesis and molecular modeling of residues in Loop 2, and in particular residue Glu<sub>53</sub>, suggest that this conserved residue can form a salt bridge with positive residues in the pre-TM1 region (GlyR Arg<sup>218</sup>) (4, 9, 19, 20). In addition, Glu<sub>53</sub> was suggested to form a salt bridge with residues in the TM2–3 linker (GlyR Lys<sup>276</sup>) (9, 21). It seems likely that Glu<sub>53</sub> participates in a distributed electrostatic interaction between mostly negatively charged residues in the ligand-binding domain and positively charged residues in the TM domain (4). Collectively, these studies suggest that Loop 2 plays a role in activation of GlyRs and GABA<sub>A</sub>Rs.

This convergence of evidence led us to hypothesize that individual residues in Loop 2 play important but different roles in mediating the transduction of agonist activation to channel gating. The current investigation tested the hypothesis by systematically studying the role that each residue in Loop 2 plays in glycine activation. To accomplish this, we tested the effect of cysteine point mutations in Loop 2 (positions 50–60) on α1GlyR agonist responses. We further tested the hypothesis by investigating the effect of charge in Loop 2 by binding methanethiosulfonate (MTS) reagents with different charges to cysteine substitutions at position 53 in Loop 2 (Glu<sup>53</sup>).

**EXPERIMENTAL PROCEDURES**

**Materials**—Adult female *Xenopus laevis* frogs were obtained from Nasco (Fort Atkinson, WI). Glycine was purchased from Sigma. 2-Aminoethyl methanethiosulfonate (MTSEA), 2-hydroxyethyl methanethiosulfonate (MTSEH), 2-aminoethyl thiosulfonate (MTSET), and 2-triethylammoniomethyl methanethiosulfonate (MTESET) were purchased from Toronto Research Chemicals, Inc. (North York, Toronto, Canada).

**Loop 2 Cysteine Mutagenesis and Expression of α1GlyR Subunit CDNA**—To investigate the role of Loop 2 residues in α1GlyR agonist activation, we performed cysteine mutagenesis on residues in and adjacent to Loop 2. Loop 2 is defined as positions 51–57 per alignment with the acetylcholine-binding protein (AChBP), as suggested by Sixma and co-workers (6) and recently confirmed in nAChRs (22). The residues tested in the present study extend beyond this defined range to positions 50–60 to test if positions near but outside of Loop 2 play a role in agonist activation. Site-directed mutagenesis in the human GlyR α1 subunit was performed on cDNA subcloned into the pBK-CMV N/B 200 vector using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Point mutations were verified by partial sequencing (DNA Core Facility, University of Southern California). *X. laevis* oocytes were isolated and injected with 1 ng of WT or mutant α1GlyR cDNA using procedures previously described (23–25).

**Electrophysiology**—Electrophysiological measurements were made 2–10 days after oocyte injection, as previously described (23–25). Briefly, oocytes expressing WT and mutant α1GlyRs were perfused in a 100-μl volume oocyte bath with modified Barth’s saline with or without drugs at 4.0 ml/min using a peristaltic pump (Rainin Instruments, Oakland, CA). MBS contains 83 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM MgSO<sub>4</sub>, 2.4 mM NaHCO<sub>3</sub>, 0.91 mM CaCl<sub>2</sub>, and 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub> adjusted to pH 7.5. Oocytes were impaled with two electrodes (1.2-mm inner diameter thick-walled filamented glass capillaries) back-filled with 3 mM KCl with resistances of 0.5–3 megaohms and voltage-clamped (~70 mV) using a Warner Instruments model OC-725C (Hamden, CT) oocyte clamp. Currents were continuously recorded with a strip chart recorder (Barnstead/Thermolyne, Dubuque, IA).

**Glycine Concentration Responses**—Oocytes expressing WT or mutant α1GlyRs were exposed to glycine for 30 s, using 5–15-min washouts between applications to ensure complete resensitization (15, 24–26). Pilot experiments found that WT and cysteine mutant GlyR agonist responses using a 1-min glycine application reached a steady state equilibrium with results (EC<sub>50</sub> = 98 ± 9 μM, n = 4) that did not differ appreciably from results using 30-s applications (EC<sub>50</sub> = 94 ± 17 μM, n = 4; not significant by Student’s t test). Therefore, we used the shorter application time to increase efficiency and to minimize desensitization at the higher glycine concentrations. Responses were normalized to the maximal glycine response. Concentration response curves were analyzed using nonlinear regression.

**MTS Reagent Protocol**—The present experiments focused on assessing the functional effect of binding different charged MTS reagents to position 53. Preliminary studies, using previously described techniques (25), found that each of the residues in Loop 2 in α1GlyRs were accessible to modification by propyl methanethiosulfonate (data not shown). Oocytes expressing WT or E53C GlyRs were exposed to MTSEA (1 mM), MTSEH (10 mM), MTESET (10 mM), or MTSET (1 mM) for 2 min in order to fully saturate the substituted cysteine residues. Following the 2-min saturation exposure, oocytes were transferred to the recording chamber and tested as described above for the glycine concentration response study. The concentrations tested were based on prior studies (27–29). MTS solutions were prepared immediately before testing. This saturation protocol yielded MTSERs results (EC<sub>50</sub> = 189 ± 31 μM, n = 4) that did not differ appreciably from results using the perfusion protocol (EC<sub>50</sub> = 109 ± 2 μM, n = 2) described under “Electrophysiology.”

**Cell Surface Biotinylation and Immunoblotting**—Biotinylation of surface-expressed proteins was performed as previously described (30). Four days after cDNA injections, oocytes (15 oocytes/group) were incubated with 1.5 mg/ml membrane-impermeable sulfouscumimidyl 2-(biotinamido)-ethyl-1, 3-dithiopropionate (Sulfo-NHS-SS-biotin) (Pierce) for 30 min at
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room temperature. After washing once with 25 mM Tris (pH 8.0) and twice with phosphate-buffered saline, oocytes were homogenized in 500 μl of lysis buffer (40 mM Tris (pH 7.5), 110 mM NaCl, 4 mM EDTA, 0.08% Triton X-100, 1% protease inhibitor mixture (Vector Laboratories, Burlingame, CA)). The yolk and cellular debris were removed by centrifugation at 3600 × g for 10 min. Aliquots of the supernatant were mixed with 5× SDS loading buffer and stored at −20 °C to assess total receptor fraction. The remaining supernatant was incubated with streptavidin beads (Pierce) overnight at 4 °C. Beads were washed three times with lysis buffer, and the biotinylated proteins were eluted using SDS loading buffer. The surface and total proteins were separated using SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated overnight with rabbit anti-GlyR antibody (1:500 dilution; Chemicon International, Temecula, CA), followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized using enhanced chemiluminescence (Pierce). The blots were then scanned and analyzed using Scion Image software (Scion Corp., Frederick, MD).

Molecular Modeling—To help visualize Loop 2 characteristics that incorporated the current findings, we built a model of the α1GlyR by threading the human α1GlyR subunit primary sequence onto the backbone coordinates of a template, essentially as previously described (25, 31, 32). In the present case, the template was the recently released 3.3 Å resolution x-ray structure of prokaryotic pentameric LGIC from the prokaryotic Erwinia chrysanthemi (ELIC; Protein Data Bank code 2VLO) (33) rather than the 4 Å resolution cryoelectron micrograph of the nAChR (Protein Data Bank code 2BG9) (8). The sequence of the human GlyR α1 was obtained from the National Center for Biotechnology Information. The amino acid sequence and three-dimensional coordinates of ELIC were obtained from the Research Collaboratory for Structural Biology. An initial BLAST sequence search was performed at the National Center for Biotechnology Information using the ELIC sequence. Among the highest scored homologous human sequences (chance of random alignment ~10−18) were those of the GlyRα1. BLAST was also used to construct a relative phylogeny tree for several LGIC sequences and ELIC. The BLAST-derived score and phylogeny tree suggest a closer homology between the LGICs and ELIC than any previously derived crystal structure (31, 34, 35). In particular, among the human LGICs sequences examined, the GlyRα1 was a closer relation to ELIC than nAChRs. Subsequent ClustalW alignment of GlyR with ELIC demonstrated ~23% sequence identity and 46% sequence similarity.

The coordinates of ELIC and the sequence of GlyRα1 were imported into Discovery Studio 2.0 (DS2.0 Accelrys, San Diego, CA). The amino acid sequence of each of five chains in ELIC was aligned with the sequence of GlyRα1 using the DS 2.0 Align123 algorithm (a derivative of ClustalW) to build five subunits of the GlyRα1 homomer. The DS 2.0 Modeler module was used for assignment of coordinates for aligned amino acids, the construction of loops for unaligned amino acids, and the initial refinement of amino acid side chains. Each GlyRα1 subunit was then merged into one construct to form the final homopentamer. Subsequent refinement of the entire construct was performed with CHARMM-based molecular mechanics optimization to an energy gradient of 0.1 kcal/mol/Å with the amino acid backbone fixed. The integrity and validity of the protein structure was checked using the DS 2.0 Protein Health module.

Data Analysis—Data for each experiment were obtained from oocytes from at least two different frogs. n refers to the number of oocytes tested. Results are expressed as mean ± S.E. Where no error bars are shown, they are smaller than the symbols. We used Prism (GraphPAD Software, San Diego, CA) to perform curve fitting and statistical analyses. Concentration response data were analyzed using nonlinear regression analysis, \[ I = I_{\text{max}}[A]^{n_H}/([A]^{n_H} + EC_{50}), \] where I is the peak current recorded following application of a range of agonist concentrations, \([A]; I_{\text{max}}\) is the estimated maximum current; \(EC_{50}\) is the glycine concentration required for a half-maximal response, and \(n_H\) is the Hill slope. Data were subjected to one-way analysis of variance with Dunnett’s multiple comparison post-test when warranted. Statistical significance was defined as \(p < 0.05\).

RESULTS

Glycine Concentration Responses—We first tested the effect of cysteine point mutations at positions 50–60 on the glycine sensitivity of α1GlyRs (Fig. 1). Inward Cl− currents were evoked in a concentration-dependent manner by glycine in WT and all mutant GlyRs. The responses were analyzed using nonlinear regression. The glycine \(EC_{50}\) and Hill slope for WT α1GlyRs agree with previous studies (Table 1) (13–15, 24, 25). One-way analysis of variance revealed significant differences between WT and several cysteine mutant GlyRs in \(EC_{50}\) Hill slope (\(n_H\)), and/or maximal current amplitude \((I_{\text{max}})\) (Table 1). Five mutations (I51C, E53C, T55C, M56C, and D57C) significantly right-shifted and one mutation (T54C) significantly left-shifted the glycine concentration-response curve as compared...
GlyR and GABAAR subunits. Similar impairment of receptor function plays an important role in agonist activation.

The methionine residue at this position is conserved across many of the residues in the Loop 2 region and is critical for agonist activation (37). Here, we determined the effect of changing the charge at position Glu53 on the glycine concentration response, resulting in a left-shifted response back toward WT GlyR glycine sensitivity.

MTS Experiments—Previous results using site-directed mutagenesis and molecular modeling of residues in Loop 2, and in particular residue Glu53, suggested that this conserved residue plays an important role in agonist activation (37). Here, we tested the effect of changing the charge at position Glu53 on the response to glycine by substituting a cysteine at position 53 and then covalently binding positive (MTSEA and MTSET), neutral (MTSEH), or negatively (MTSES) charged MTS reagents to the substituted cysteine residue.

WT GlyRs—The glycine responses of WT and mutant GlyRs exposed to MTS reagents are shown in Fig. 3 and Table 2. The glycine EC_{50} and Hill slope for WT GlyRs exposed to MTSEA, MTSEH, MTSES, and MTSET did not significantly differ from WT GlyRs that were not exposed to an MTS reagent. This absence of a change in base-line function or agonist activation in WT GlyRs indicates that either 1) the MTS reagents do not bind to cysteine residues in WT GlyRs or 2) any MTS binding that occurs in WT GlyRs does not appreciably alter receptor function.

E53C GlyRs—Replacing the negatively charged glutamic acid at position 53 in WT GlyRs with the neutral cysteine (E53C) right-shifted the glycine concentration response, resulting in a

**TABLE 1**

Summary of nonlinear regression analysis results for the glycine concentration responses in WT and mutant α1GlyRs.

| GlyR         | EC_{50} (μM) | Hill slope (n) | I_{max} (nA) |
|--------------|--------------|----------------|--------------|
| Wild type    | 95.6 ± 17    | 1.1 ± 0.2      | 11313 ± 2176 |
| S50C         | 95.3 ± 17    | 1.1 ± 0.2      | 11313 ± 2176 |
| I51C         | 2856.5 ± 637 | 1.4 ± 0.1      | 5525 ± 1495  |
| A52C         | 1580.0 ± 13  | 1.1 ± 0.1      | 13372 ± 1375 |
| T54C         | 18.1 ± 1     | 2.9 ± 0.4      | 8938 ± 739   |
| T55C         | 726.8 ± 176  | 1.2 ± 0.1      | 4900 ± 1268  |
| M56C         | 366.4 ± 97   | 1.0 ± 0.1      | 5663 ± 1541  |
| S50C         | 57.4 ± 14    | 1.1 ± 0.1      | 12375 ± 3145 |
| R59C         | 143.8 ± 12   | 1.9 ± 0.3      | 13063 ± 724  |
| V66C         | 155.5 ± 15   | 1.8 ± 0.1      | 16375 ± 3642 |

*P < 0.01.

#P < 0.05.
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A significant increase in the glycine EC$_{50}$ (Fig. 3 and Table 2). Exposure to negatively charged MTSes significantly left-shifted the glycine concentration response curve of the E53C GlyR back to an EC$_{50}$ that did not significantly differ from WT GlyRs. In contrast, adding a positive charge at position 53 by exposing E53C GlyRs to MTSEA or MTSET did not significantly change glycine sensitivity. Similarly, exposing E53C GlyRs to neutral MTSEH did not significantly change the glycine sensitivity of E53C GlyRs. Together, these MTS findings provide evidence that a negative charge at position 53 increases response to the agonist, whereas neutral and positive substitutions do not.

It should be noted that the lack of change in glycine sensitivity of E53C GlyRs to neutral and positive MTS reagents could reflect a lack of accessibility due to their charge or lack thereof. To assess this possibility, we tested the effects of MTSES on E53C GlyRs that had already been exposed to MTSEA, MTSET, or MTSEH. We reasoned that MTSES would bind to position 53 and change glycine responses only if the first exposure to MTS agents did not bind to the sites. Therefore, if the follow-up exposure to MTSES changed glycine response, it would indicate that the lack of response in the initial test reflected inaccessibility. However, pilot studies did not support this possibility in that an initial exposure to MTSEA, MTSET, or MTSEH blocked the effect of subsequent exposure to MTSES. These results indicate that position 53 is accessible to MTS reagents regardless of charge. Therefore, the lack of response to MTSEA, MTSET, and MTSEH cannot be explained by their lack of accessibility to position 53. Furthermore, this lack of effect by MTSEA, MTSET, or MTSEH cannot be explained by the physical-chemical characteristics of the MTS reagents, since each has a polarity and molecular volume similar to that of MTSES. Therefore, the results suggest that the negative charge at position 53 is important for agonist activation of GlyRs.

**Molecular Modeling**—The molecular model of the interface between the extracellular and TM domains of GlyR shown in Fig. 4 is based on the 3.3 Å resolution structure of a prokaryotic pentameric LGIC, ELIC (33). The ligand-binding domain of this structure was very similar to the crystal structure of the water-soluble ligand-binding domain of the AChBP (6). We noted that the pore of the ion channel in ELIC is in a fully closed state compared with the ion channel in the 4 Å resolution structure of nAChR (Protein Data Bank code 2BG9) that is closed only at the intracellular end of the ion channel. As a result, there was reason to question if the electrostatic interactions at the interface between the ligand-binding and transmembrane domains of ELIC would be applicable to the domain interface in GlyR. However, inspection of those interactions (shown in Fig. 4C) revealed that they are consistent with those predicted by mutagenesis and homology modeling (4, 9, 20, 31).

The molecular model presented here illustrates the proximity of residues in Loop 2 (Glu$^{53}$) to Loop 7 (Asp$^{148}$), the pre-TM1 segment (Arg$^{218}$), and the TM2-TM3 linker. For example, in this model, Glu$^{53}$ in Loop 2 and Arg$^{218}$ in the Pre-TM1 segment are in ideal positions to form a salt bridge between them. There is *in vitro* (20, 38), molecular modeling (4, 20), and clinical (19) evidence for the importance of this salt bridge.

### Table 2

| Receptor | No MTS | MTSES | MTSET | MTSEA |
|----------|--------|-------|-------|-------|
| WT       | 91 ± 7 | 21 ± 2 | 16813 | 1515  |
| E53C     | 21 ± 2 | 16813 | 1515  | 1515  |

**Summary of nonlinear regression analysis results for the glycine concentration responses in MTS-exposed WT and E53C GlyRs**

| Receptor | EC$_{50}$ (mM) | nI$_{H}$ | Hill slope (n) | I$_{max}$ (pA) |
|----------|----------------|---------|----------------|---------------|
| WT       | 1897 ± 105     | 1.6     | 0.1            | 1861 ± 15     |
| E53C     | 1897 ± 105     | 1.6     | 0.1            | 1861 ± 15     |

MTSES reagent exposure significantly left-shifted the glycine concentration response curve of the E53C GlyR back to an EC$_{50}$ that did not significantly differ from WT GlyRs. In contrast, adding a positive charge at position 53 by exposing E53C GlyRs to MTSEA or MTSET did not significantly change glycine sensitivity. Similarly, exposing E53C GlyRs to neutral MTSEH did not significantly change the glycine sensitivity of E53C GlyRs. Together, these MTS findings provide evidence that a negative charge at position 53 increases response to the agonist, whereas neutral and positive substitutions do not.

**GlyR.** However, inspection of those interactions (shown in Fig. 4C) revealed that they are consistent with those predicted by mutagenesis and homology modeling (4, 9, 20, 31).
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In Fig. 4, the carboxylate group of Glu\textsuperscript{53} shares a salt bridge with Asp\textsuperscript{148} on Loop 7. The latter salt bridge is also known to be important, since the mutation D148A increases the glycine EC\textsubscript{50} from 27 to 932 μM (39). The Gly\textsuperscript{53} residue in Loop 2 is also well positioned to participate in the electrostatic interactions with the TM2-TM3 linker (9, 40, 41). This overlapping set of electrostatic interactions is consistent with a redundant potential prokaryotic ancestor to the superfamily of Cys-loop receptors (34). Although the prokaryotic channels actually lack the two cysteine residues that distinguish the Cys-loop superfamily, they share a high structural (33) and sequence (34) homology to the family. As described under “Experimental Procedures,” we found that the GlyR studied here had the highest sequence homology to the prokaryotic ion channels. The structure of ELIC, the prokaryotic ion channel used as a template for the model in Fig. 4, was refined by 10-fold symmetry averaging to yield a resolution of 3.3 Å. As a result, it was possible to locate individual amino acid residues at the interface between the ligand-binding and TM domains with greater accuracy than was possible in the cryoelectron micrographs of nAChR (8). In addition, a recent high resolution crystal structure (<2.0 Å resolution) of the ligand-binding domain of nAChR demonstrated a remarkable conservation of structure in the Loop regions in that domain (22) that is essentially identical to the high resolution structure of the AChBP (6). As a result, although the ion pore of the ELIC ion channel appears to be collapsed, we have increased confidence that the interac-

![Molecular model of an α1GlyR](image)

**Figure 4.** Molecular model of an α1GlyR. A, the structure of a pentameric GlyR based on the template of a prokaryotic ligand-gated ion channel (ELIC). The ligand-binding domain is mostly β sheet, and the TM domain is mostly α-helical. The intracellular vestibule is omitted, because it is not a part of the prokaryotic channels and is not well visualized in the cryoelectron micrographs of nAChR. The amino acid backbone of the each subunit is shown as a colored ribbon. The horizontal lines show the approximate boundaries of the phospholipid bilayer, B, the structure of a single subunit viewed from the plane of the membrane. Interactions between Loop 2 and the TM domain are more clearly seen. C, zoom view of the domain interface. To visualize residues tested in the current study, the region surrounding Loop 2 was expanded and rotated to focus on the interface between the extracellular and TM domains. A salt bridge is formed between Glu\textsuperscript{53} in Loop 2, Arg\textsuperscript{218} in the pre-TM1 segment, and Asp\textsuperscript{148} in Loop 7 (Cys-loop). The atoms of those residues are rendered as ball and stick and are colored red, black, white, and blue for oxygen, carbon, hydrogen, and nitrogen, respectively. Isoleucine 51 is rendered as ball and stick and colored magenta to illustrate its position at the transition from a β-strand to a β-turn. D, detailed view of residues in Loop 2; odd-numbered (pink, rendered as ball and stick) and even-numbered (colored as atoms and rendered as space-filling CPK). All odd-numbered (pink) residues face toward Loop 7 and the pre-TM1 segment in C. Residue Ala\textsuperscript{52} faces toward the ion channel, Glu\textsuperscript{53} faces toward Arg\textsuperscript{218}, and Thr\textsuperscript{277} is at the tip of Loop 2.

numbered residues 51, 53, 55, and 57 (Table 1). This periodicity is graphed in Fig. 5. Although Loop 2 is a slightly twisted β-turn in all available structures (AChBP (6), nAChR (8, 22), and the prokaryotic pentameric LGIC (33)) that we used as a template for the model in Fig. 4, the odd-numbered residues are clearly on the side of Loop 2 that faces inward toward the center of the subunit and the putative alcohol-binding pocket that we described previously (25).

Another interesting feature of Loop 2 is residue Ile\textsuperscript{51}. The mutation I51C caused the greatest increase in glycine EC\textsubscript{50} measured in this study (Table 1; 2865 for I51C GlyRs versus 98 μM for WT GlyRs). This residue is conserved as a small hydrophobic residue (Ile or Val) throughout the Cys-loop (6) and prokaryotic (33, 34) LGIC superfamily. As shown in Fig. 4, Ile\textsuperscript{51} is located at the transition between β-strand 1 and the β-turn. This position is adjacent to residue Ala\textsuperscript{52}, which has been shown to be important for potentiation of the receptor by alcohol (15, 25) and is on the same odd-numbered side of Loop 2 as the important residue Glu\textsuperscript{53} (Table 1).

The LGIC from Gloeobacter violaceus was proposed previously as a potential prokaryotic ancestor to the superfamily of Cys-loop receptors (34). Although the prokaryotic channels actually lack the two cysteine residues that distinguish the...
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FIGURE 5. Comparison of the changes in agonist EC50 produced by cysteine substitutions for α1GlyRs and α7 nAChRs. The data provided for α1GlyRs were from findings presented by McLaughlin et al. (42). Residues 50–58 of the α1GlyR were aligned with residues 41–48 of the α7 nAChR, as suggested by Sixma and co-workers (6). The pattern of changes in EC50 is consistent with a β-turn structure for Loop 2 for both α1GlyRs (black) and α7 nAChRs (gray). Note that the lack of expression of GlyR M56C resulted in its omission from the graph of glycine EC50. Statistical significance from WT α1GlyRs or α7 nAChRs was assessed using one-way analysis of variance with Dunnett’s post-test. *, $p < 0.05$ for WT α1GlyRs; †, $p < 0.05$ for WT α7 nAChRs.

DISCUSSION

The current study systematically investigated the role that Loop 2 residues play in agonist activation of α1GlyRs. The findings build upon previous work (10, 13–16) and add new evidence that multiple residues within Loop 2 are important for GlyR agonist activation. Further, the current results provide insight into the relationship between the structure of Loop 2 and the function of α1GlyRs.

The pattern of changes in glycine sensitivity induced by point mutations in Loop 2 of α1GlyRs provides the first evidence for the secondary structure of Loop 2 in the extracellular domain of GlyRs. Cysteine substitutions at positions 51, 53, 55, and 57 increased the glycine EC50. In contrast, cysteine substitution at position 54 decreased the glycine EC50. These position-specific changes in glycine sensitivity are consistent with a β-turn structure of Loop 2 that extends beyond β-strand definitions previously reported in the AChBP (6). Moreover, these changes suggest that the odd numbered positions in this sequence face toward and interact with other agonist-activation elements at the interface between extracellular and TM domains.

Similar secondary structure was recently observed in a crystal structure of the mouse α1 nAChR (22) and the prokaryotic LGIC ELIC (33). Moreover, recent work in the chick α7 nAChR, which also utilized cysteine-scanning mutagenesis of the Loop 2 region, found that cysteine mutations in the Loop 2 region of nAChRs display a pattern of changes in agonist sensitivity similar to that found in α1GlyRs (42) (i.e. nAChRs exhibit significant rightward shifts in agonist sensitivity at residues that appear to correspond to positions 52, 54, and 56 of the α1GlyR). However, the residue-specific pattern of changes in agonist sensitivity for α7 nAChRs appears to be opposite to those for α1GlyRs in the present study (Fig. 5). This pattern reversal suggests that there are small differences in the structure of Loop 2 between GlyRs and nAChRs. These subtle structural differences might underlie differences in the role of certain Loop 2 residues in agonist activation observed previously in Cys-loop receptors (4, 9, 10, 12, 42–45) and provide further evidence for heterogeneity in the gating mechanism among receptors in this superfamily.

On the other hand, the pattern reversal might reflect a difference in sequence alignment between α1GlyRs and α7 nAChRs in which the alignment is off by a single residue in this region. Analysis of the sequence alignment, which varied the gap penalties and the scoring matrices used in ClustalW, did not support this notion. As noted above, there is a motif that is absolutely conserved throughout the Cys-loop and prokaryotic LGIC superfamily (6, 33, 34): a valine or isoleucine at positions in Loop 2 homologous to residue 51 and an aspartate or glutamate at positions homologous to position 53. This conserved motif suggests that the present alignment of GlyR, nAChR, AChBP, and ELIC is correct. Perhaps the most important difference to explain the altered periodicity of GlyR versus nAChR in Fig. 5 is that the lysine residue in the TM2–3 linker (GlyR Lys276), which is conserved in the GlyR and GABAAR families, is absent in the nAChR family (35, 40). This lysine residue in GlyRs (Lys276) was proposed to interact with Glu53 in Loop 2 (9). In the absence of this positive residue, other distributed interactions must take over (4).

The pattern reversal between GlyRs and nAChRs might also result from changes in hydrogen bonding within the close confines of Loop 2 (i.e. that the residues that correspond to the β-turn in nAChRs may be displaced in the N-terminal direction by one residue in GlyRs, resulting in Loop 2 residues with differing propensities to be in these positions) (46). We tested this possibility in Loop 2 of 1) ELIC (33), the prokaryotic ion channel that we used as our template for the model in Fig. 4; 2) the nAChR structure of Unwin (8); and 3) the AChBP (6). All three structures show some latitude in the way they fit the criteria of a hydrogen bond between positions $i$ and $i + 2$ (where $i =$ position 52 in α1GlyRs) as well as a distance less than 7 Å between the C-α carbons of residues $i$ and $i + 3$ (47). Further research is necessary to investigate each of these scenarios. Regardless, these findings are consistent with Loop 2 having a β-turn structure in GlyRs, nAChRs, and possibly other LGICs.

Dellisanti and co-workers (22) studied the region around Loop 2 in the 1.94 Å resolution crystal structure of the extracellular domain of nAChR. This structure shows nAChR Glu55 (homologous to GlyR Glu53) projecting outward from the middle of a β-turn in Loop 2. Their high resolution allowed them to observe a hydrogen-bonded network of water molecules that extended from Thr52 in Loop 2 and Ser126 outward to reach the highly conserved surface residue Asn94 (homologous to GlyR Val160, Leu136, and Asn102). They suggested that the localized water molecules were part of a “hydration cavity inside the β-sandwich core of the nAChR subunit.” This hydration cavity is especially pertinent to our present study, because we had previously identified a cavity at the interface between the extra-
cellular and TM domains that was accessible to MTS reagents and, therefore, presumably to water molecules (25). It is possible that these two cavities, at the core of the β-sandwich and at the interface between the extracellular and TM domains, could communicate.

Based on the current and earlier findings, we refined our previous model of the α1GlyR to depict Loop 2 with a β-turn structure between adjacent β-strands. This model suggests that residues at either end of Loop 2 (positions 51 and 57 in α1GlyRs) could act like a hinge that provides flexibility to allow movement of Loop 2 with respect to the more stable sheet structure that makes up most of the extracellular domain. The notion of a hinge and the resulting flexibility in relation to other structures at the interface between the extracellular and TM domains provides a mechanism for Loop 2 movement in the transduction of agonist binding to channel gating within the TM region.

The current study also supports the hypothesis that Loop 2 plays a role in influencing the activation of the ion channel through charge interactions with neighboring agonist activation elements. The cytochrome mutation at position 53 eliminated the negative charge associated with a glutamic acid residue in WT GlyRs and resulted in a significant right shift in glycine sensitivity. If the hypothesis that the charge at residue 53 affects activation of GlyR is true, then replacement of the original WT charge at position 53 would restore ion movement in the channel.

As predicted, we found that binding a negatively charged MTS reagent (MTSES) to the substituted cysteine residue at position E53C shifts agonist sensitivity back to resemble that of WT GlyRs. We interpret the restoration of glycine activation by binding of a negative moiety as replacement of a distributed electrostatic interaction between residue 53 and other positively charged residues near the domain interface, such as Arg218 in the pre-TM1 segment or Lys276 in the TM2-TM3 linker (4, 9, 20). Note that this conclusion, regarding the orientation of position 53, contrasts with models based on Brownian dynamics (17, 18). Additional studies are necessary, such as those done in the TM domain (e.g. current-voltage relationship, ion selectivity, relative ion permeability), before conclusions can be drawn. Nonetheless, the present findings add new direct support for the previous molecular models and suggest that the negative charge at position 53 is important for agonist-induced activation of GlyRs.

Interestingly, binding a positively charged MTS reagent (MTSEA or MTSET) to the substituted cysteine residue did not significantly shift glycine sensitivity beyond the level induced by mutation. Therefore, the distance between position 53 and potential salt bridge partner residue(s), such as Arg218 or Lys276, could be outside of a repulsive distance that would produce deleterious effects should a positive charge be substituted into position 53. In addition, given the flexibility of Loop 2 proposed above, Loop 2 could accommodate some degree of repulsion and could maintain a low energy state without significantly affecting overall agonist activation. Experiments are currently under way to investigate these possibilities in order to better understand the molecular level interactions of Loop 2 residues and their effect on agonist activation.

Collectively, the current and previous findings suggest that the extracellular domain plays an important role, both structurally and functionally, in GlyR agonist activation. The findings add evidence that 1) each residue within Loop 2 is important for GlyR agonist activation; 2) the pattern of changes in glycine sensitivity is consistent with a β strand-like structure of the loop; and 3) the negative charge at position 53 is important for activation of GlyRs. The present study also provides a refined molecular model of the α1GlyR that offers insight into the structure-function relationships in GlyRs and possibly other LGICs.

Acknowledgments—We thank Miriam Fine for technical assistance, Dr. Liana Asatryan for performing the biotinylation experiments, and Dr. Peter Schofield for providing the WT α1 GlyR cDNA.

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