Modeling Anesthetic Binding Sites within the Glycine Alpha One Receptor Based on Prokaryotic Ion Channel Templates: The Problem with TM4

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Ligand-gated ion channels (LGICs) significantly modulate anesthetic effects. Their exact molecular structure remains unknown. This has led to ambiguity regarding the proper amino acid alignment within their 3D structure and, in turn, the location of any anesthetic binding sites. Current controversies suggest that such a site could be located in either an intra- or intersubunit locale within the transmembrane domain of the protein. Here, we built a model of the glycine alpha one receptor (GlyRa1) based on the open-state structures of two new high-resolution ion channel templates from the prokaryote, *Gloebacter violaceus* (GLIC). Sequence scoring suggests reasonable homology between GlyRa1 and GLIC. Three of the residues notable for modulating anesthetic action are on transmembrane segments 1−3 (TM1−3): (ILE229, SER 267, and ALA 288). They line an intersubunit interface, in contrast to previous models. However, residues from the fourth transmembrane domain (TM4) that are known to modulate a variety of anesthetic effects are quite distant from this putative anesthetic binding site. While this model can account for a large proportion of the physicochemical data regarding such proteins, it cannot readily account for the alterations on anesthetic effects that are due to mutations within TM4.

INTRODUCTION

The class of proteins known as ligand-gated ion channels (LGICs) is ubiquitous within the nervous systems of organisms throughout nature. These are transmembrane proteins that allow the passage of ionically mediated currents which form the basis of synaptic communications underlying conscious states. As such, they are also likely sites of general anesthetic action. Such proteins seem to be an evolutionarily successful means of ion transduction. Even in more primitive prokaryotic organisms there exist homologous proteins with similar ion-regulating capabilities that may have been the phylogenetic precursors to their subsequent counterparts in eukaryotic organisms. Determining the structure of such ion channel proteins is paramount to any detailed understanding of the molecular mechanisms of neural communications, and in particular, how such are further modulated by general anesthetics. Due to the transmembrane nature of these proteins and the difficulties associated with gaining structural data at a level greater than 4 A,[1] our work has focused on the use of molecular modeling techniques to build atomic level models of the γ-aminobutyric acid (GABA) receptor and the glycine alpha one receptor (GlyRa1) in order to examine their interactions with anesthetics.[2−4] While initial low-resolution models made it possible to postulate mechanisms for channel gating dynamics and how anesthetics might affect this motion, the amino acid localization and alignment within such lower resolution models has fallen into question. In particular, only slight alterations in amino acid alignments change previous suggestions of an intrasubunit binding site for anesthetics to one that may lie between subunits within the transmembrane domain of the protein.

Our original work in this area was performed without the benefits of the newer prokaryotic templates utilized in this manuscript. Those efforts involved the construction of the transmembrane domain (TMD) model by two methods and mating this to a model of the extracellular ligand binding domain (LBD) created by a third method. We had initially predicted the topology of the individual TMD subunit of GlyRa1 through the consensus of 10 transmembrane secondary structure prediction algorithms superimposed on the multiple sequence alignments of 6 LGICs via ClustalW.[5] These calculations predicted that the four transmembrane segments within the transmembrane domain of each subunit were α helices. This secondary structure and sequence information was used in conjunction with the SeqFold algorithm to find a modeling template[6] that was then aligned with the sequence of GlyRa1. Once a four-helix bundle was constructed, a template forcing algorithm was used to align the TM2 α helix of this tetramer onto a pore-lining α helix of a pentameric mechanosensitive ion channel from a *Mycobacterium tuberculosis* (1MSL.pdb) template. This was repeated with five-fold symmetry to produce a pentamer of tetramers totaling 20 alpha helices to form the entire TMD. The sequence of the extracellular LBD subunit of the GlyRa1 had to be built separately from a different template found in

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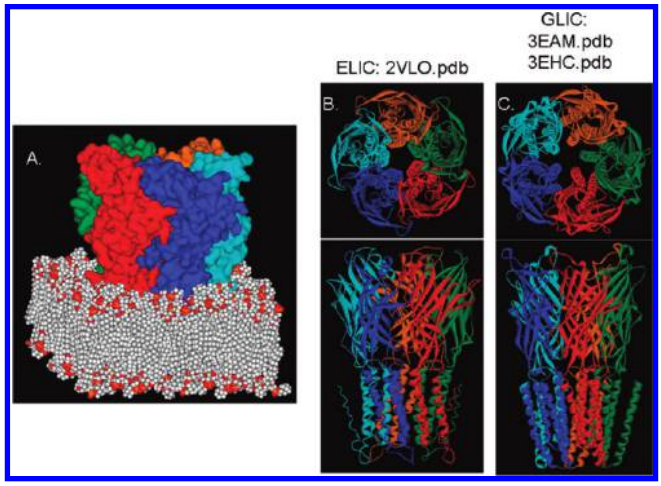

Figure 1. (A) Model of a membrane-spanning pentameric ion channel embedded in a lipid bilayer. (B and C) Side-by-side comparison of the ELIC (closed) vs GLIC (open) templates for LGIC modeling.

the crystal structure of the partially homologous snail acetylcholine binding protein. The five-fold axes of the two pentameric domains (TMD and LBD) were then aligned and mated together. This composite structure showed that specific amino acid residues known to modulate anesthetic potency were in direct proximity to one another.[4] Within each of the four helix bundle TMD subunits, there was a clearly visible cavity that was bounded by residues that, when mutated, were known to alter anesthetic-mediated modulations of native ligand binding effects. These residues were Leu 229 (TM1),[7] Ser 267 (TM2),[8] and Ala 288 (TM3).[9] Additionally, this model of an anesthetic binding site predicted that residues from TM4 should have direct bearing on anesthetic binding and modulation of these channels, a result confirmed by the experiments of several others.[10,11] This model further explained the results from cysteine modification studies showing irreversible anesthetic effects with methanethiosulfonate reagents introduced for TM2 and TM3[12] as well as the disulfide cross-link experiments for TM3−4.[13]

In the current study, we build a model of the GlyRa1 based on two new cation channel templates (PDB ID's 3EAM and 3EHZ)[14,15] from the prokaryote *Gloebacter violaceus* (GLIC, Figure 1) that contrasts greatly with the more contrived constructs of our previous models described above. These template proteins have sequences with reasonable similarity to GlyRa1, which provides for much higher quality alignments and subsequently better structural models[16] than in our previous work. This enables computationally robust models of GlyRa1 to be constructed via two completely independent methods that converge to a common structural solution. Additionally, these two templates are thought to be close to the open state of the ion channel. This feature is important for examining any possible anesthetic effect since anesthetics are thought to bind and stabilize the open state of the GlyRa1. Putative anesthetic binding sites have been identified in these models as well, by the convergence of key residues that, when mutated, alter the effect of anesthetics on channel current flow in this or homologous receptors.[7−11] Of greatest importance is the fact that this new model provides support for an anesthetic binding site composed of residues from transmembrane domains 1−3 that is present between subunits, as opposed to the intrasubunit binding site proposed in previous works. However, all models that purport an intersubunit anesthetic binding site place the fourth transmembrane helix from each subunit (TM4) at great distance from the binding site. As such, these models cannot yet explain how mutations on TM4 directly impinge on any intersubunit anesthetic binding site but can be used to suggest future mutational experiments to further converge on a more refined solution to this issue.

METHODS

Multiple Sequence Alignment of LGIC Homologues. An initial BLAST[17] sequence search was performed at the National Center for Biotechnology Information (NCBI) using the entire sequence from a GlyRa1 target monomer, including both the TMD and the LBD. Both the nicotinic acetylcholine receptor (2BG9) and GLIC were among the top hits. The probability of a random alignment with the template GLIC sequence (p-value) was less than 10^{-12}. The transmembrane as well as ligand binding domains of the target are very well aligned, both to sequences with known structure and those without, with the latter still being useful in the Hidden−Markov model (HMM)-based construction of the consensus sequence alignment presented below.[18] Despite an accurate alignment between GlyRa1 and 2BG9, it is not possible to build a high-resolution homology model since the lower resolution of the 2BG9 template implies that one cannot unambiguously and reliably map the sequence of homologous amino acids involved in GlyRa1 onto its derived structure.

Model Building Based on HHpred. The server for homology detection and structure prediction by the HMM comparisons (HHpred server) was used with default settings to construct a monomer model based on the GLIC X-ray structure template (3EAM). HHpred utilizes HMMs for both the query and the target together with the predicted secondary structure using PSIPRED[19] to produce higher quality alignments even for relatively distant sequences. Modeler[20] was then used to build 3D coordinates from the alignment. To simplify the modeling, the extracellular and membrane domains were first modeled separately for only one of the five subunits. Next, a complete model of one subunit was constructed by superposition of the extracellular and membrane domains onto the subunits in the template followed by rebuilding of the linker region coordinates. Finally, this single subunit homology model was superimposed onto the full pentameric template oligomer to generate a five-fold symmetric complex. As a last step the SCWRL program[21] was used to rebuild all side chains and to achieve good packing between the different subunits. In the process, the extended intracellular loop between helices TM3 and TM4 in GlyRa1 was cut, as it does not have any homologous match in GLIC. Instead, it was replaced with a short glycine loop connecting the two helices.

Model Building with Discovery Studio. To show that the new model is not a result of a particular method but due to good alignments to new higher resolution templates, we repeated the homology modeling with Discovery Studio (DS2.0.1 Accelrys, San Diego, CA) to get a somewhat independent confirmation of 3D coordinate assignments. The coordinates of the known structures in 3EAM, 3EHZ, and 2BG9 (torpedo nicotinic acetylcholine receptor alpha 1 subunit) and the sequence of GlyRa1 were imported into

Discovery Studio 2.0.1. The structures of each of the five chains with known 3D structures were placed into separate files. The three known structures underwent initial structural alignment for optimum coordinate overlap. The amino acid sequence of each chain within the 3D templates was aligned with the sequence of the GlyRa1 using the Align123 algorithm (a derivative of ClustalW)[22] so as to build the five separate subunits of the GlyRa1 homomer. The Modeler[20] module was then used for assignment of coordinates for the aligned amino acids, the construction of possible loops for unaligned amino acids, and the initial refinement of amino acid side chains using the averaged coordinates of 3EAM and 3EHZ. Each GlyRa1 subunit was then merged into one construct to form the final homomeric pentamer. Subsequent refinement of the entire construct was performed with Charmm-based molecular mechanics optimization[23] with the backbone fixed and an energy gradient of 0.1 kcal/mol/A. The integrity and validation of the protein structure was checked using the Protein Health module. The mapping of residues relevant to various experimentally determined effects was examined using the visualization components within DS2.0.1.

Visualizations and Analyses. Molecular visualizations were carried out using a combination of Discovery Studio, VMD,[24] and PyMol software (DeLano Scientific). Root-mean-square deviation (rmsd) analyses were performed using VMD[24] and DeepView[25] software.

RESULTS

The BLAST-derived score and the phylogeny tree suggest a closer homology between the LGICs and GLIC than many previously derived templates in the National Protein Coordinate Database. Subsequent alignment of the GlyRa1 with GLIC demonstrates approximately 21% sequence identity and 41% sequence similarity. Figure 2 shows the multiple sequence alignment with high-scoring structures derived from HHpred, and Supporting Information, Figure 1 displays an alignment with all top sequences used in deriving the HMM for the query sequence. The final model construct after refinement shows a secondary structure closely matching the template both in the TMD (helices) and the LBD (sheets). To further assess the quality of the structure, it was processed with the ProQ model quality assessment predictor (Stockholm Bioinformatics Institute). Figure 3 shows a monomer colored according to the predicted quality (low-quality indicated by red and high- quality by blue). The model of the TMD is predicted to have very high to high quality and the LBD high to medium. As is expected for less well-defined protein regions involving protein loops which are highly mobile, some loops are predicted to be of lower quality.

The model of the GlyRa1 is composed of a homomer with pentameric symmetry about a central ion pore. Unlike other models based on the "closed" ELIC template, the current model based on the "open" GLIC templates shows a continuously open pore from the extracellular to the intracellular region. Most of those residue positions noted by experiment to be labeled by hydrophobic reagents face the lipid bilayer (Figure 4). Most of those residue positions noted by experiment to be labeled by hydrophilic reagents face either the central ion channel or the regions within TMD subunits (Figure 5). Several of the residues notable for salt bridge formation between the LBD loops and the TMD 2−3 loops are well approximated. Three of the residues notable for modulating anesthetic action on the GlyRa1 within TMD's 1−3 (ILE229, SER 267, and ALA 288)[7−9] line the intersubunit interface, in contrast to our previous models. The general location of the latter three residues remains in the outer third of the transmembrane four-helix bundles. However, residues from TM4[10,11] that are known to modulate a variety of anesthetic effects on this or homologous LGICs are present but quite distant from any intersubunit anesthetic binding site (Figure 6).

Using the rmsd of sets of points within the models derived from each of the aforementioned methodologies as a measure of similarity, we derived values using α carbon atoms and all backbone atoms. The rmsd of the two models based on analyses of 1474 α carbon components of the protein backbone was 0.87 A, and even if all backbone atoms are included it only increases to 0.91 A. Analyses based on side chain orientation similarities were not performed due to the large conformer space available to the protein. However, the key residues relevant to anesthetic actions on this channel tended to significantly overlap in 3D space. Thus, despite the two completely separate means of GlyRa1 sequence alignment to the newest high-resolution GLIC templates, from a qualitative and functional point-of-view these models are practically equivalent.

DISCUSSION

Value of the Current Model of the Glyra1. However useful the previous model noted in the Introduction has been for furthering our understanding of the molecular mechanisms of anesthetic action and guiding new experimental directions, one major criticism has been that it was constructed based upon three separate homology modeling templates which bear only modest similarities to the LGIC sequences in question. Furthermore, the initial models of LBD and TMD had to be independently merged into an overall structure in somewhat of an educated but less rigorous manner. This is in stark contrast to the current model which has been built using templates which bear much more reasonable homology to the LGIC sequences having both the LBD and TMD components within one modeling template. This avoids the uncertainties involved in docking several independently constructed protein subunits into one composite model. Remarkably the current model presented in this manuscript actually appears to account for hydrophilic and hydrophobic labeling in homologous positions within the family of LGIC's to a much greater degree than our previous model (Figures 4 and 5).[26−28] Likewise, data from a set of cross-linking studies involving cysteine mutations between TM1 and TM3 as well as TM2 and TM3[29,30] are more reliably accounted for with the model put forth in this manuscript. The current model also maintains a virtually seamless conical channel that forms a continuous pore for the passage of ions spanning the entire length of the protein. In fact, future studies may show how models can be built from both the closed form of the template (ELIC) and the open form of the template (GLIC) so as to observe the changes that may be occurring in the general direction of channel gating. The current model was further validated, though, by virtue of the fact that it could be reproduced by

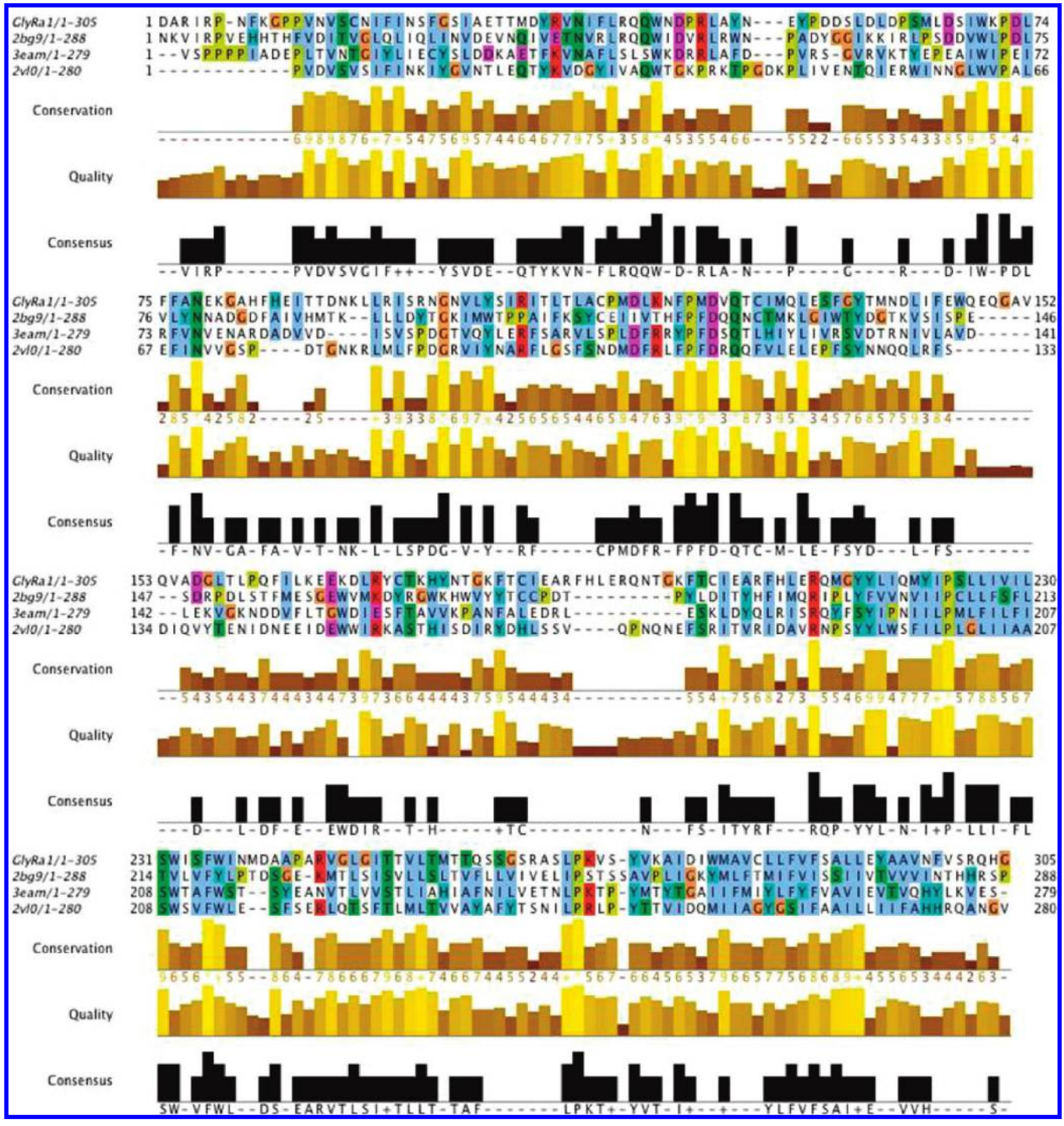

Figure 2. Multiple sequence alignment with high-scoring structures derived from Hhpred.

completely independent efforts and methodologies, with both techniques resulting in virtually the same model based on rmsd and 3D orientation of critical amino acid residues.

Novel Anesthetic Binding Site. However, the real surprise that arises from the current analysis is with regards to the nature of the anesthetic binding site within this new model of the GlyRa1. Those residues noted to alter the ability of anesthetics to modulate ion channel function in the presence of native ligand (Leu 229 from TM1, Ser 267 from TM2, and Ala 288 from TM3) now line the intersubunit interface between transmembrane four-helix bundles instead of lining a common cavity within a single transmembrane four-helix bundle (Figure 6). This, too, satisfies the amphipathic nature of the anesthetic binding site as well as certain size and cysteine modification studies noted above. However, this is in marked contrast to previous models in which the putative anesthetic binding site was located within the intrasubunit portion of each subunit's TMD composed of a four-helix bundle. While this is not the first time that intersubunit binding of anesthetics has been proposed, it is the first noted for this class of LGIC's based upon homology modeling to these very high-resolution templates. There have been others who have suggested the intersubunit location for a putative anesthetic binding site, but the vast majority of these were based upon the somewhat intermediate resolution structure of the nAChRa1 from cryoelectron microscopy.[29−35] Our current model presents an intriguing new means by which an anesthetic may access an intersubunit binding site in that

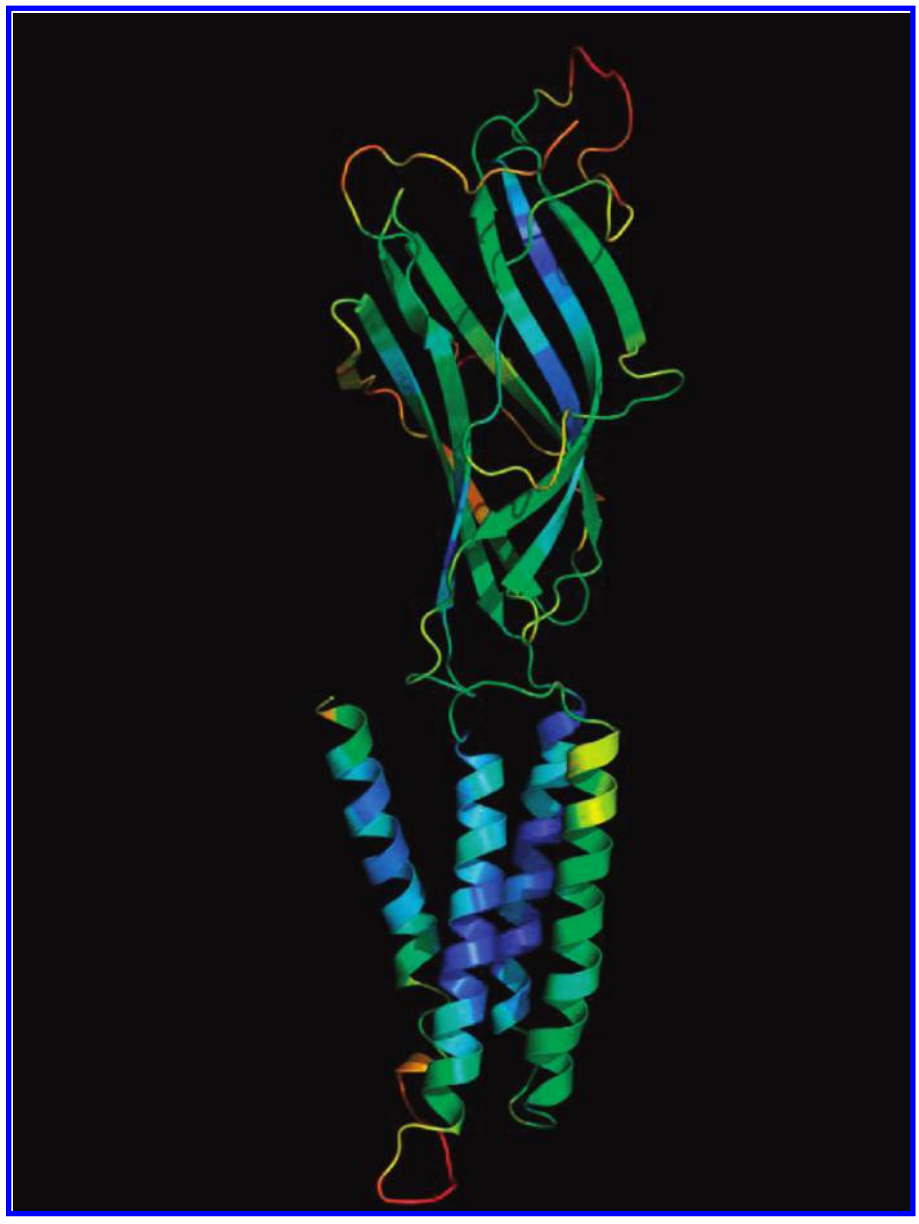

Figure 3. GlyRa1 homology model monomer colored according to predicted quality. A version of the ProQ model program (modified by retraining on membrane proteins) was used to predict local quality for each residue. Blue indicates high model quality, and red indicates low quality. The TMD involved in anesthetic binding has high (surface) to very high (buried residues) quality. Only some loops are predicted to be low quality. It should also be noted that TM3−TM4 loop is much longer and more complex in the real GlyRa1 than in the abbreviated representative here.

pathways could include passage from the pore for small molecules, from the vestibule present between LBD and TMD, as well as directly by diffusing from the lipid bilayer. The latter may make lipid solubility an important characteristic for anesthetics to gain access to what is otherwise a quite amphipathic binding site, a feature which has been experimentally correlated with anesthetic potency since Meyer and Overton's work[36] over a century ago.

The Quandry. While both models account for the proximity of residues from TM1−TM3 that are known to modulate anesthetic effects on LGIC's, there is some difficulty with the current model accounting for the effects of point mutations in TM4[10,11] (Figure 6). An intrasubunit binding site for anesthetics that is formed within the cavity outlined by a single four-helix bundle can be bounded by amino acid contributions from all four transmembrane helices of the bundle. An intersubunit binding site (at the interface between subunits within the transmembrane domain) is bounded by amino acids from TM2 and TM3 of one subunit on one side of the cleft and TM1 from the adjacent subunit on the other. In all such models, TM4 is quite distant from such a site. One could postulate that mutations of TM4 positions could still alter the orientation of adjacent TM1 or TM3 helices in an allosteric manner, but this would imply necessary interactions with additional residues on the latter helices which have not currently been demonstrated by experiment. Furthermore, no channel motion studies to date have suggested the possibilities for large enough TM4 helical movements to account for these results with this model. Just to complicate matters a bit more, there even exists data which demonstrate binding pockets clearly present in both inter- and intrasubunit locations that are large enough for possible cholesterol binding.[37]

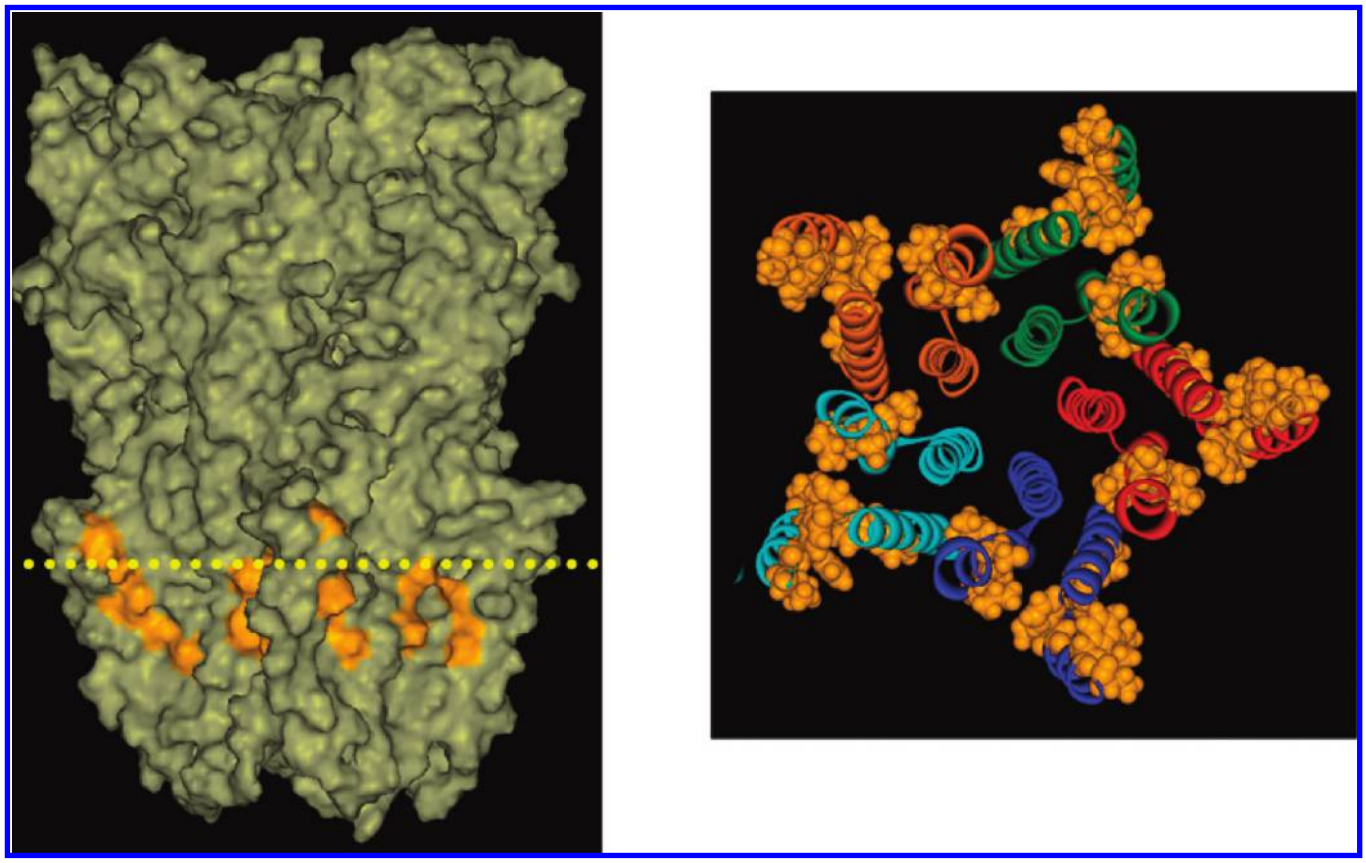

Figure 4. Surface view of entire GlyRa1 model from the side (left) with projections from residues labeled by hydorphobic reagents (orange). Cross-sectional view of the transmembrane domain (right) looking down the pore at the level of the yellow dotted line noted on the surface view. Note the predominantly lipid-facing positions of these hydrophobically labeled residues.

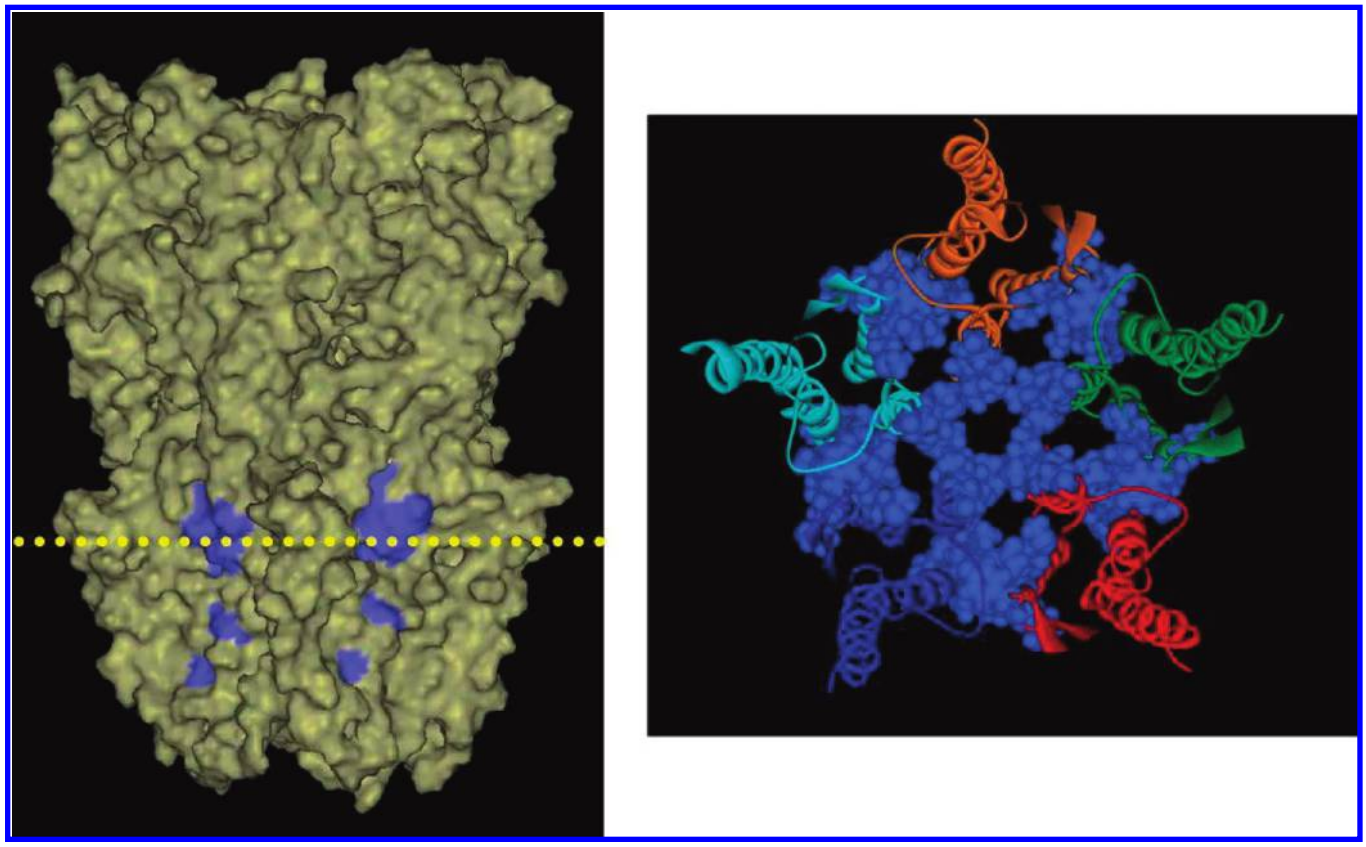

Figure 5. Surface view of entire GlyRa1 model from the side (left) with projections from residues labeled by hydrophilic reagents (blue). Cross-sectional view of the transmembrane domain (right) looking down the pore at the level of the yellow dotted line noted on the surface view. Note the predominantly pore-facing positions of these hydorphilically labeled residues.

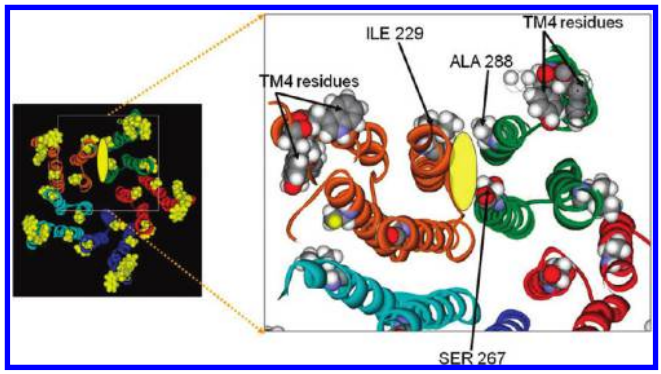

Figure 6. The glycine receptor transmembrane subunit demonstrating amino acid residues relevant for anesthetic effects on the LGIC's. Note the intersubunit binding site for anesthetics in this model. Also note the great distance of TM4 residues from any intersubunit anesthetic binding site. To date, no residues from either TM1 or TM3 that face relevant TM4 residues in this model have been noted to alter anesthetic effects on these ion channels when mutated.

CONCLUSIONS

A reasonable model of the glycine alpha one receptor (GlyRa1) can be constructed using homology modeling based on the high-resolution prokaryotic templates from *Gloebacter violaceus* (GLIC). This model is consistent with a great deal of experimental data regarding the positions of a variety of amino acids from GlyRa1 and homologous proteins within the ligand-gated ion channel (LGIC) family. This model positions an intersubunit site for anesthetic binding that may communicate with the intrasubunit region of each TMD. However, distinct questions remain regarding an explanation for the effects of TM4 mutations on anesthetic modulation of these channels.

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Supporting Information Available: Figure 1 displays an alignment with all top sequences used in deriving the Hidden−Markov model for the query sequence. This information is available free of charge via the Internet at http://pubs.acs.org.

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