

Unique assignment of inter-subunit association in GABA_A $\alpha 1\beta 3\gamma 2$ receptors determined by molecular modeling

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Received 23 April 2002; received in revised form 2 July 2002; accepted 3 July 2002

Abstract

Recent publications defined requirements for inter-subunit contacts in a benzodiazepine-sensitive GABA_A receptor (GABA_A $\alpha 1\beta 3\gamma 2$). There is strong evidence that the heteropentameric receptor contains two $\alpha 1$, two $\beta 3$, and one $\gamma 2$ subunit. However, the available data do not distinguish two possibilities: When viewed clockwise from an extracellular viewpoint the subunits could be arranged in either $\gamma 2\beta 3\alpha 1\beta 3\alpha 1$ or $\gamma 2\alpha 1\beta 3\alpha 1\beta 3$ configurations. Here we use molecular modeling to thread the relevant GABA_AR subunit sequences onto a template of homopentameric subunits in the crystal structure of the acetylcholine binding protein (AChBP). The GABA_A sequences are known to have 15–18% identity with the acetylcholine binding protein and nearly all residues that are conserved within the nAChR family are present in AChBP. The correctly aligned GABA_A sequences were threaded onto the AChBP template in the $\gamma 2\beta 3\alpha 1\beta 3\alpha 1$ or $\gamma 2\alpha 1\beta 3\alpha 1\beta 3$ arrangements. Only the $\gamma 2\alpha 1\beta 3\alpha 1\beta 3$ arrangement satisfied three known criteria: (1) $\alpha 1$ His¹⁰² binds at the $\gamma 2$ subunit interface in proximity to $\gamma 2$ residues Thr¹⁴², Phe⁷⁷, and Met¹³⁰; (2) $\alpha 1$ residues 80–100 bind near $\gamma 2$ residues 91–104; and (3) $\alpha 1$ residues 58–67 bind near the $\beta 3$ subunit interface. In addition to predicting the most likely inter-subunit arrangement, the model predicts which residues form the GABA and benzodiazepine binding sites.

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Keywords: GABA; Acetylcholine binding protein; Threading; Ligand-gated ion channel; Molecular modeling; Comparative modeling

1. Introduction

Members of the superfamily of ligand-gated ion channels (LGICs) include nicotinic acetylcholine (nAChR), GABA_A, glycine, and 5-HT₃ receptors. We have previously built molecular models of homopentameric LGICs, for example the glycine $\alpha 1$ receptor [1–3]. These models were useful for visualizing the effect of site-directed mutations on potentiation of GABA and glycine currents by inhalational anesthetics [2,4] and alcohols [5]. However, there is a need to expand these studies to include putative anesthetic binding sites in heteropentameric GABA_A receptors [5–7]. The heteropentameric receptors are important for mediating the effects of benzodiazepines [8,9] as well as alcohol and inhalational anesthetics [10–13]. The present study investigates the correct assignment of intersubunit associations in

a GABA_A $\alpha 1\beta 3\gamma 2$ receptor in preparation for building a molecular model of this heteropentameric ion channel.

GABA_A receptors are heteropentamers formed from subunits belonging to multiple classes: α , β , γ , δ , ϵ , π , and ρ [14,15]. Although functional receptors can be made from α and β subunits, one γ subunit is required for expression of sensitivity to benzodiazepines [9,16]. The frequently studied GABA_A $\alpha 1\beta 3\gamma 2$ receptor is now known to be assembled from two $\alpha 1$, two $\beta 3$, and one $\gamma 2$ subunit [17]. However, Tretter et al. [17] showed six possible ways to arrange these five subunits into a receptor. The six possible subunit arrangements were reduced to two when Klausberger et al. demonstrated that GABA_A $\alpha 1$ residues (58–67) mediate assembly with GABA_A $\beta 3$ subunits [18] and that GABA_A $\alpha 1$ residues (80–100) make inter-subunit contacts with $\gamma 2$ residues (91–104) but not with $\beta 3$ subunits [19] (Fig. 1).

Similar progress has been made on the structure of the benzodiazepine-binding site in these receptors [15]. Two studies have demonstrated that GABA_A $\alpha 1$ His¹⁰² is involved in binding of the benzodiazepine analog flunitra-

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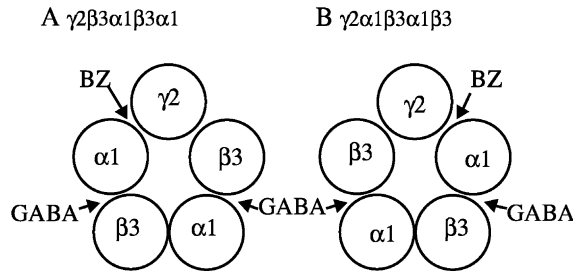


Fig. 1. The two subunit arrangements described by Klausberger et al. [19]. (A) The subunits are arranged in an $\gamma 2\beta 3\alpha 1\beta 3\alpha 1$ sequence (clockwise from extracellular view). (B) The subunits are arranged in an $\gamma 2\alpha 1\beta 3\alpha 1\beta 3$ sequence. The proposed inter-subunit binding sites for GABA and benzodiazepines (BZ) are indicated.

zepam [8,20]. In addition, two tyrosine residues in the $\alpha 1$ subunit, Tyr¹⁶⁰ and Tyr²¹⁰ (numbered Tyr¹⁵⁹ and Tyr²⁰⁹ in Amin et al.), were shown to be crucial for benzodiazepine binding [21]. Expression of a γ subunit is necessary for benzodiazepine binding although photolabeling of this subunit with [³H] flunitrazepam was much less than the α subunit [22]. On the other hand, site-directed mutations in the $\gamma 2$ subunit demonstrated that Thr¹⁴² [23], Phe⁷⁷ [24], and Met¹³⁰ [24] modulate response to application of GABA.

The key to using comparative modeling to assign subunit associations is the recent publication of the crystal structure of the acetylcholine binding protein (AChBP) [25]. The possibility of using this three-dimensional structure as a template for preparing molecular models of other LGICs was predicted in an overview [26]. A composite model of the Torpedo NACHR was made by comparison of the AChBP structure with the electron density in cryoelectron micrographs [27]. Recently, models of three major types of

nAChRs have been built by threading the corresponding primary sequences onto the structure of the AChBP [28]. We have used a similar threading technique to prepare a model of the ligand-binding domain of a homopentameric glycine $\alpha 1$ receptor in order to interpret the effect of a D97R mutation in that receptor {5820}.

Here we use comparative modeling and combine available data to distinguish the remaining two possibilities of GABA_A subunit assembly: The arrangements of $\gamma 2\beta 3\alpha 1\beta 3\alpha 1$ versus $\gamma 2\alpha 1\beta 3\alpha 1\beta 3$ that are shown in Fig. 1. The GABA_A sequences are known to have 15–18% identity with the AChBP and higher homology with conserved residues. The GABA_A sequences were aligned with those of the AChBP template and were then threaded onto the template in the $\gamma 2\beta 3\alpha 1\beta 3\alpha 1$ or $\gamma 2\alpha 1\beta 3\alpha 1\beta 3$ arrangements. Loops were generated in regions of the GABA_A sequences where there were no corresponding residues in the AChBP template. A successful model of the subunit arrangements should satisfy three known criteria described above: (1) $\alpha 1$ His¹⁰² binds at the $\gamma 2$ subunit interface in proximity to $\gamma 2$ residues Thr¹⁴², Phe⁷⁷, and Met¹³⁰; (2) $\alpha 1$ residues 80–100 bind near $\gamma 2$ residues 91–104; and (3) $\alpha 1$ residues 58–67 bind near the $\beta 3$ subunit interface. Only the $\gamma 2\alpha 1\beta 3\alpha 1\beta 3$ arrangement satisfied these criteria.

2. Materials and methods

Coordinates of the AChBP [25] were obtained from the Protein Data Bank (1I9B). This structure contains 205 amino acid residues that were shown, using multiple sequence alignment, to have 24% sequence identity with nAChR alpha 7 and 15–18% identity with GABA_A recep-

| | | |
|-----------------|------|---|
| AChBP | (1) | LDRADILYNIRQTSRPDVIPTQRDRPVAVSLSKFINILEV |
| GABA $\alpha 1$ | (13) | TVFTRILDRLLDGYDNRLRPGLGERVTEVKTDIFVTSFGPV |
| GABA $\beta 3$ | (35) | SFVKETVDKLLKGYDIRLPDFGGPPVCVGMNIDIASIDMV |
| GABA $\gamma 2$ | (25) | GDVTVILNNLLEGYDNKLRPDIGVKPTLIHTDMYVNSIGPV |
| AChBP | | NEITNEVDVFWQQTWSDRTLAWNSSHS.PDQVSVPISSLWVPDLAAYN.AISKPEVLTPQ |
| GABA $\alpha 1$ | | SDHDMYEYIDVFVRQSWKDERLKFKGPMTVLRLNNLMASKIRTPDTFFHNGKKSVAHNMTMP |
| GABA $\beta 3$ | | SEVNMDYTLTMYFQQYWRDKRLAYSIGIPLNLTLDNRVADQLWVPDITYFLNDKKSFFVHGVTVK |
| GABA $\gamma 2$ | | NAINMEYIDIFFAQMWYDRRLKFNSTIKVLRLNSNMVGKIWIPTDFFRNSKKADAHWITTP |
| AChBP | | LARVVS..DGEVLYMPSIRQRFSCDVSGVDTESG.ATCRIKIGSWTHHSREISVDPTTE...NSDD |
| GABA $\alpha 1$ | | NKLLRITEDGTLLYTMRLTVRAECPMHLEDFPMDAHACPLKFGSYAYTRAEVVYEWTPREPARSVVV |
| GABA $\beta 3$ | | NRMIRLHPDGTVLYGLRITTTAACMMDLRRYPLDEQNCTLEIESYGYTTDDIEFYWRGG..DKAVT |
| GABA $\gamma 2$ | | NRMLRIWNDGRVLYSLRLTIDAECQLQLHNFPMDEHSCPLEFSSYGYPREEIVYQWKRS...SVEV |
| AChBP | | SEYFSQYSRFEILDVTQKKNSVTYSCPEAYEDVEVSLNFRKKG |
| GABA $\alpha 1$ | | AEDGSRLNQYDLLGQTVDSGIVQSSTG..EYVMTTHFHLKRKI |
| GABA $\beta 3$ | | GVERIELPQFSIVEHRLVSRNVVFATG..AYPRLSLSFRLKRNI |
| GABA $\gamma 2$ | | GDTRSWRLYQFSFVGLRNTTEVVKTTT.GDYVVMVSYFDLSRRM |

Fig. 2. The alignment of the $\alpha 1$, $\beta 3$, and $\gamma 2$ GABA_A sequences with the AChBP template is based on the alignment of $\alpha 1$ and $\beta 1$ GABA_A by Brejc et al. [25]. The $\beta 3$ and $\gamma 2$ GABA_A sequences were aligned with the ClustalW algorithm [29]. The sequence number of the first residue in the mature sequence (signal sequence removed) is shown in parentheses. Gaps in the sequence alignment are shown as periods.

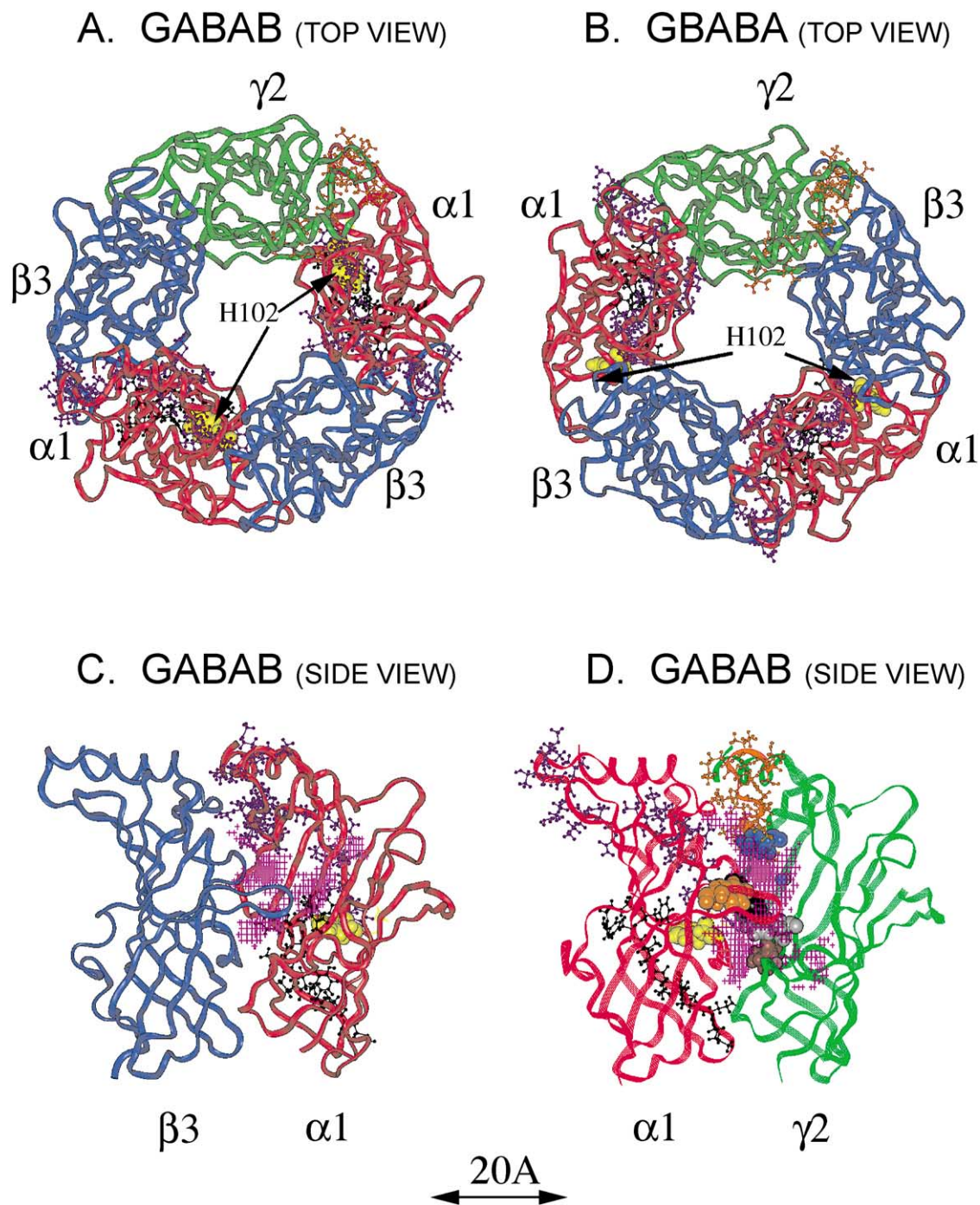


Fig. 3. The amino acid sequences of subunits of GABA_Aα1γ2β3 (human) were aligned to correspond to the sequence in the PDB crystal structure of the AChBP. Then the coordinates of corresponding backbone atoms from one subunit of the crystal structure were assigned to the appropriate GABA_A subunit sequence. In all models the backbone traces of α1, β3, and γ2 subunits are colored red, blue, and green, respectively. In models A and B the subunits are arranged in γ2α1β3α1β3 (GABAB) and γ2β3α1β3α1 (GBABA) sequences, respectively. Residues γ2 (91–104) are colored orange, residues α1 (80–100) are colored purple, residues α1 His¹⁰² are rendered as space-filling surfaces and colored yellow, and residues α1 (58–67) are colored black. Only model A satisfies the known criteria for inter-subunit association. (C) is a side view of the β3/α1 interface that reveals the GABA binding site. (D) is a side view of the α1/γ2 interface that shows the close spatial proximity of γ2 subunit residues (all rendered as space-filling surfaces) in the γ2α1β3α1β3 model in (A): Thr¹⁴⁶ (brown), Phe⁷⁷ (gray), and Met¹³⁰ (blue) with α1 subunit residues H102 (yellow), Tyr¹⁶⁰ (black, partially obscured), and Tyr²¹⁰ (orange). These six residues have been shown to be involved with benzodiazepine binding.

tors [25]. The alignment of the $\alpha 1$, $\beta 3$, and $\gamma 2$ GABA_A sequences with the AChBP template is based on the alignment of $\alpha 1$ and $\beta 1$ GABA_A by Brejc et al. [25] except that the $\beta 3$ and $\gamma 2$ GABA_A sequences were aligned with ClustalW [29] (Fig. 2).

The backbone atoms of the appropriate GABA_A sequences were assigned the corresponding coordinates from the crystal structure of the AChBP using the Homology module of Insight 2000 (Accelrys, San Diego, CA.). Two models were made, one with the arrangement of $\gamma 2\beta 3\alpha 1\beta 3\alpha 1$ and the second with arrangement $\gamma 2\alpha 1\beta 3\alpha 1\beta 3$ (clockwise when viewed from the extracellular side, they are the mirror image models described by Klausberger et al. [19] and Tretter et al. [17]). It was immediately apparent that only the $\gamma 2\alpha 1\beta 3\alpha 1\beta 3$ model fulfilled the three criteria described above. In order to use the latter model to predict important residues near the GABA binding site and the α/γ or α/β subunit interfaces, loops were built to fill in the gaps between the GABA_A sequences and the template sequence (Fig. 2). The default values of the loop/generate command were used with a minimum of three residues in the “flexible” loop-forming region. This model was then refined by an iterative procedure of finding the optimum position of all possible side-chain rotomers in each substituted residue using the auto-rotomer feature in the Biopolymer module of Insight 2000 (Fig. 3). The backbone atoms (C, Ca, N) of each GABA_A residue were tethered to the coordinates of corresponding residues in the AChBP template with a force constant of 5 kcal/Å² and the structure was optimized with the Discover 2000 module of Insight 2000. The optimization was made using the CFF91 force field and Polak Ribiere conjugate gradient algorithm for 5000 steps with a movement limit of 0.2 Å per step to a final derivative of 1 kcal/Å. This optimization made a large improvement in the structure by eliminating unfavorable van der Waals contacts and improving electrostatic interactions ((beginning total potential energy = 950,146,451,676 kcal/mol, final = −35,552 kcal/mol). Then 5000 steps (1 fs) of molecular dynamics were performed at 298 °K with the velocity-scaled NVT ensemble while the backbone atoms were restrained to the original coordinates as above. The 5000 steps improved the optimization by giving the molecule enough thermal energy to get out of local minima. However, they are far too few to make an adequate sample of conformational space and no attempt was made to derive thermodynamic properties from this simulation. The model was then re-optimized using the same force constant for the tether of the backbone atoms. This procedure successfully eliminated adverse van der Waals contacts and improved electrostatic interactions (final total potential energy = −36,975 kcal/mol). Additional optimization cycles would yield only modest improvements in the structure.

The major result was that only the subunit arrangement $\gamma 2\alpha 1\beta 3\alpha 1\beta 3$ satisfied the three criteria described in the Introduction. Therefore, this model was used to define proximity of relevant residues in neighboring subunits. In each case, a residue known to be important, for example

GABA_A $\alpha 1$ His¹⁰², was selected and then all residues within spheres of radius 5 or 10 Å were identified.

Although a study of binding of either GABA or a benzodiazepine is beyond the scope of this study, the program Binding Site Analysis (Accelrys) was applied to the model to see if there were cavities at the α/γ or α/β subunit interfaces of sufficient size to contain these molecules. The default parameters for cutoff of surface invaginations were used. This program constructs a cubic lattice with a grid spacing of 1 Å and tries to find unoccupied lattice points that are contiguous and that define a space with a total volume greater than a specified cutoff; in this case 100 Å³.

3. Results

The models in Fig. 3A and B make it clear that only the subunit arrangement in Fig. 3A ($\gamma 2\alpha 1\beta 3\alpha 1\beta 3$, viewed from the extracellular side in a clockwise direction) satisfied the three criteria described in the Introduction. We will discuss how well the models in Fig. 3 satisfy these criteria.

3.1. $\alpha 1$ His¹⁰² binds at the $\gamma 2$ subunit interface in proximity to $\gamma 2$ residues Thr¹⁴², Phe⁷⁷, and Met¹³⁰

Fig. 3A shows that $\alpha 1$ His¹⁰² (yellow space filling residue) is at the interface with the $\gamma 2$ subunit as predicted. In addition in Fig. 3D, the following residues in $\gamma 2$ are within 5 Å of His¹⁰²: Phe⁷⁷ (gray space filling residue in $\gamma 2$ at the benzodiazepine site that was identified by Wingrove et al. [24]) and Thr¹⁴⁶ (brown space filling residue at the benzodiazepine site identified by Mihic et al. [23]. This residue was numbered Thr¹⁴² in that paper). It is important that $\gamma 2$ Met¹³⁰ (blue space filling residue) is proximate to $\alpha 1$ His¹⁰² because it is the $\alpha 1$ residue involved in binding of flunitrazepam [8,20]. The sequence alignment (Fig. 2) shows that GABA_A $\alpha 1$ His¹⁰² corresponds to AChBP Tyr⁸⁹, an important residue in the center of “loop A” at the binding site for acetylcholine [25]. The following additional residues in $\gamma 2$ are within 10 Å of $\alpha 1$ His¹⁰²: Tyr⁵⁸–Ser⁶¹, Ser¹⁴², Arg¹⁴⁴, Asp¹⁹²–Trp¹⁹⁶, and Leu¹⁹⁸.

In Fig. 3D, all six of the residues that were implicated by Smith and Olsen [8], Amin et al. [21], Wingrove et al. [24], and Mihic et al. [23] in binding benzodiazepines using site-directed mutagenesis were at the $\alpha 1/\gamma 2$ subunit interface. In addition, all were within approximately 5 Å of $\alpha 1$ residue His¹⁰².

3.2. $\alpha 1$ residues 80–100 bind near $\gamma 2$ residues 91–104

This criterion is only satisfied by the model in Fig. 3A. Even though the segment $\alpha 1$ 80–100 (purple) is long and spans the width of the $\alpha 1$ subunit, $\gamma 2$ residues 91–104 (orange) are clustered at a single interface of $\gamma 2$. Since the

long segment 80–100 is too large to use to define a sphere of interest, residues were visually identified to be at the $\alpha 1/\gamma 2$ interface. The following pairs of residues in $\alpha 1$ and $\gamma 2$ are within 5 Å of each other: $\alpha 1\text{Arg}^{95}/\gamma 2\text{Asn}^{101}$, $\alpha 1\text{Thr}^{96}/\gamma 2\text{Met}^{130}$, $\alpha 1\text{Asp}^{98}/\gamma 2\text{Asn}^{99}$, and $\alpha 1\text{Thr}^{125}/\gamma 2\text{Arg}^{144}$.

3.3. $\alpha 1$ residues 58–67 bind near the $\beta 3$ subunit interface

The segment 58–67 (black) spans the GABA_A $\alpha 1$ subunit and has residues near a $\beta 3$ subunit in both models. In Fig. 3A, residues $\alpha 1\text{Asp}^{63}$ and Phe^{65} were identified near the $\alpha 1/\beta 3$ interface. They were within 5 Å of $\beta 3\text{Tyr}^{99}$, Leu^{101} , Lys^{102} and Lys^{103} .

The program Binding Site Analysis (Accelrys) revealed cavities (shown as clusters of pink crosses in Fig. 3C and D) at the α/γ (763 Å³) and α/β (445 Å³) subunit interfaces. These cavities are of sufficient size and are in positions appropriate to contain benzodiazepines and GABA, respectively.

4. Discussion

It is remarkable how well the crystal structure of AChBP served as a template for the three GABA_A subunits. For example, GABA_A $\alpha 1\text{His}^{102}$ A in Fig. 3A is right at the interface with a $\gamma 2$ subunit as predicted [8,20]. Moreover, His^{102} maps exactly to AChBP Tyr^{89} , an important residue in the center of “loop A” at the binding site for acetylcholine [25].

In that the sequence identity of the GABA_A receptors with the AChBP is only 15–18% [25], the appropriateness of the latter structure for the ligand-binding domain of GABA_A receptors must be considered [26]. One point in favor of using the AChBP as a template for the superfamily of LGICs is how well the essential features of nAChRs, for example the loops that define ligand binding, could be located in AChBP [25]. A second point is that the sequence homology is especially high for residues conserved throughout the LGIC superfamily, in particular the two cysteine residues that anchor the “cis-loop” [25]. A third point is that the three models of nAChR prepared by threading onto AChBP reproduced in detail the experimentally determined alignments of residues, including intersubunit alignments [28]. Finally, the crystal structure of AChBP was recently shown to fit well within the electron density of the Torpedo AChR [27].

A study by Baumann et al. [30] addressed the question of inter-subunit arrangement of GABA_A receptors by expressing tandem subunit dimers. We support their conclusion that all GABA_A receptors are pentamers, as opposed to tetramers. However, they suggested a complicated model in which one subunit dimer could rearrange to form a $\gamma 2\beta 3\alpha 1\beta 3\alpha 1$ pentamer, the opposite of the conclusion based on our model. They are presently trying the difficult task of expressing a subunit trimer to resolve this issue [30].

In summary, the model in Fig. 3A provides a unique assignment of the inter-subunit association in GABA_A: clockwise from the extracellular side, $\gamma 2\alpha 1\beta 3\alpha 1\beta 3$. This result resolves the ambiguity posed by Klausberger et al. [19]. In addition, Fig. 3D reveals a close spatial proximity of $\gamma 2$ subunit residues Thr^{142} , Phe^{77} , and Met^{130} with $\alpha 1$ subunit residues H102 , Tyr^{160} , and Tyr^{210} . It is possible that these six residues may define a pharmacophore for benzodiazepine binding.

Acknowledgements

The author thanks Dr. Ed Bertaccini for many helpful discussions. This research was funded by NIGMS grant 1RO1 GM64371 to JRT.

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