Published in final edited form as:

Protein Expr Purif. 2009 October ; 67(2): 76–81. doi:10.1016/j.pep.2009.05.001.

Effect of homologous serotonin receptor loop substitutions on the heterologous expression in *Pichia* of a chimeric acetylcholine-binding protein with α -bungarotoxin-binding activity

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Abstract

The molluscan acetylcholine-binding protein (AChBP) is a soluble homopentameric homolog of the extracellular domain of various ligand-gated ion channels. Previous studies have reported that AChBP, when fused to the ion pore domain of the serotonin receptor (5HT_{3A}R), can form a functional ligand-gated chimeric channel only if the AChBP loop regions between β -strands β 1 and β 2 (β 1– β 2), β 6 and β 7 (β 6– β 7), and β 8 and β 9 (β 8– β 9) are replaced with those of the 5HT_{3A}R. To investigate further the potential interactions among these three important loop regions in a membrane- and detergent-free system, we designed AChBP constructs in which loops β 1– β 2, β 6– β 7, and β 8– β 9 of the AChBP were individually and combinatorially substituted in all permutations with the analogous loops of the 5HT_{3A}R. These chimeras were expressed as secreted proteins using the *Pichia pastoris* yeast expression system. [¹²⁵I]- α -Bungarotoxin-binding was detected in the culture media obtained from homologous recombinant clones expressing the wild-type AChBP, the β 1– β 2 loop-only chimera, and the chimera containing all three 5HT_{3A}R loop substitutions. The remaining chimeras failed to show [¹²⁵I]- α -bungarotoxin binding, and further analysis of cellular extracts allowed us to determine that these binding-negative chimeric constructs accumulated intracellularly and were not secreted into the culture medium. Our results demonstrate that coordinated interactions among loops β 1– β 2, β 6– β 7, and β 8– β 9 are essential for formation of a functional ligand-binding site, as evidenced by [¹²⁵I]- α -bungarotoxin-binding, and for efficient protein secretion. In addition, the constructs described here demonstrate the feasibility of utilizing soluble scaffolds to explore functionally important interactions within the extracellular domain of membrane-bound proteins.

INTRODUCTION

The acetylcholine-binding protein (AChBP) is a naturally-occurring soluble homolog of the extracellular ligand-binding domain (LBD) of the Cys-loop, ligand-gated ion channel (LGIC) superfamily, which includes nicotinic acetylcholine receptors (nAChRs) and the 5-hydroxytryptamine (serotonin) type 3A receptor (5HT_{3A}R) [1]. In accordance with structural predictions for these transmembrane receptors, each monomer of the homopentameric AChBP

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is composed of a set of ten β -strands in which the corresponding inter- β -strand loops are arranged in a novel immunoglobulin fold (Figure 1) [2]. The AChBP, originally discovered in the central nervous system of the freshwater mollusk, *Lymnaea stagnalis*, as a modulator of cholinergic response, also recognizes many ligands which are commonly used to study vertebrate nAChRs, such as acetylcholine, nicotine, epibatidine, α -bungarotoxin, α -cobratoxin, and tubocurarine [2]. As the AChBP is a secreted protein, it can facilitate the investigation of the ligand-binding properties of these receptors in a membrane- and detergent-free system.

α -Bungarotoxin (α -bgtx), a neurotoxin found in the venom of the Taiwanese banded krait, *Bungarus multicinctus*, has been used extensively to identify, localize, and purify specific nAChRs [3,4]. In addition to binding the muscle-type and homopentameric $\alpha 7$ subunit-containing nAChRs, this neurotoxin is a known ligand of the AChBP from *L. stagnalis* [5]. α -Bgtx binds at the interface of two subunits in a pocket of hydrophobic and aromatic residues that is approximately 40Å away from the tips of the interstrand loops, $\beta 1$ – $\beta 2$, $\beta 6$ – $\beta 7$, and $\beta 8$ – $\beta 9$ of the AChBP. The corresponding loops of the 5HT_{3A}R, are oriented towards the membrane interface [2,6].

Upon ligand-binding, conformational changes in the LBD of LGICs propagate to the ion pore domain (IPD). Structural evidence from AChBP and muscle-type nAChR studies suggest that interactions among certain inter- β -strand loops, particularly loops $\beta 1$ – $\beta 2$, $\beta 6$ – $\beta 7$, and $\beta 8$ – $\beta 9$, function in coupling ligand-binding to channel-gating [2,7–10]. The significance of these interactions has been emphasized further by a study investigating a functional chimeric acetylcholine-activated cation channel which was produced by the fusion of the $\alpha 7$ nAChR LBD and the 5HT_{3A}R IPD [11]. More recently, the soluble AChBP was linked to the 5HT_{3A}R IPD, but unlike the $\alpha 7$ -5HT_{3A}R chimera, the AChBP-5HT_{3A}R chimera, as it is, does not form a functional ion channel [12]. Electrophysiological responses to acetylcholine were attained in this fusion construct only following the replacement of three loops ($\beta 1$ – $\beta 2$, $\beta 6$ – $\beta 7$, and $\beta 8$ – $\beta 9$) of the AChBP with the corresponding loop sequences of the 5HT_{3A}R, suggesting that interactions of these loops with membrane embedded sequences of the 5HT_{3A}R IPD were necessary for the formation of a functional receptor.

To investigate further the mechanistic basis underlying the observations made with the transmembrane chimeric AChBP-5HT_{3A}R, we have constructed soluble hexahistidine-tagged 5HT_{3A}R loop-substituted AChBP chimeras for expression in *Pichia pastoris* with every permutation of $\beta 1$ – $\beta 2$, $\beta 6$ – $\beta 7$, and $\beta 8$ – $\beta 9$ loop replacements. In addition, we noted that in the published AChBP-5HT_{3A}R fusion construct, the C-terminus of the AChBP had been truncated and replaced with the 5HT_{3A}R-derived amino acid residues connecting the tenth β -strand with the first transmembrane helix ($\beta 10$ -M1 linker or preM1). As this region is suspected also to be in close proximity to and potentially interact with the three aforementioned loops, we designed additional sets of chimeras to test whether the preM1 region of the 5HT_{3A}R had any effect on proper protein expression and α -bgtx binding when compared to the native C-terminus of the AChBP [1,13].

We expressed all chimeric constructs as secreted proteins using the methylotrophic yeast, *Pichia pastoris*, protein expression system, which provides for robust expression and secretion of the native AChBP into *Pichia* culture medium [14]. We investigated whether the media collected from cultures of the various AChBP-5HT_{3A}R loop-substituted chimeric constructs showed [¹²⁵I]- α -bgtx binding activity, and we then determined for each chimeric AChBP construct whether the chimeric AChBP was secreted or retained intracellularly. Our studies demonstrate that although the short hairpin loop, DEKNQV, of $\beta 1$ – $\beta 2$ derived from the 5HT_{3A}R, could be well tolerated, incorporation of either of the two other loops, $\beta 6$ – $\beta 7$ or $\beta 8$ – $\beta 9$, alone or in combination with each other, did not lead to successful secretion. Constructs containing loop substitution $\beta 6$ – $\beta 7$ or $\beta 8$ – $\beta 9$ were only successfully secreted when there was

full three-fold complementarity of the 5HT_{3A}R loops β 1– β 2, β 6– β 7, and β 8– β 9. These findings suggest that interactions among these three loop regions are required for proper folding and trafficking of the chimeric AChBP, and by extension, are critical for receptor biogenesis. Our results provide further evidence that, even in the absence of an ion pore domain or membrane bilayer, coordinated interactions among these three 5HT_{3A}R loops are likely to be important for the overall structural and functional integrity of receptors in the Cys-loop superfamily.

EXPERIMENTAL PROCEDURES

Materials

[¹²⁵I]- α -Bungarotoxin (NEX-126) was purchased from Perkin Elmer (Shelton, CT). Yeast nitrogenous base (YNB; 239210), peptone (294312), and yeast extract (212720) were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). Talon cobalt-affinity resin (635501) was obtained from Clontech Laboratories (Mountain View, CA). The *Pichia pastoris* EasyComp transformation kit (K173001) was from Invitrogen (Carlsbad, CA). Mouse anti-hexahistidine tag antibody (70796) was from EMD Biosciences (San Diego, CA). Tween-20 (X251-07) was from Mallinckrodt Baker (Phillipsburg, NJ). SuperSignal West Pico chemiluminescence substrate (34080) was from Pierce (Rockford, IL). Horse-radish peroxidase-conjugated goat anti-mouse antibody (A8924), colloidal Coomassie blue (B2025) and the remaining chemicals were from Sigma-Aldrich (St. Louis, MO).

AChBP expression constructs

cDNA encoding the C-terminal hexahistidine-tagged AChBP protein sequence in the pPIC9 vector was a kind gift from A.B. Smit. This vector also encodes an upstream *Saccharomyces cerevisiae* α -mating factor signal sequence, which directs secretion of the recombinant AChBP into the culture media. Amino acid substitutions were introduced via the QuikChange Mutagenesis XL Kit (Stratagene, La Jolla, CA) following the manufacturer's specifications. Table 1 lists the primers used for the substitutions of the loops (β 1– β 2, β 6– β 7, and β 8– β 9) and the preM1 (pM1) segment. All loop substitutions reported here were verified by dideoxy sequencing (Genewiz, South Plainfield, NJ).

Pichia growth and AChBP expression

AChBP was overexpressed in *Pichia pastoris* strain GS115 following Invitrogen's specifications. Briefly, AChBP constructs were linearized using BglII, transformed into competent GS115 cells using the Invitrogen *Pichia* EasyComp kit, and successful recombination was verified via colony PCR. Ten milliliters of BMGY media (1.34% yeast nitrogenous base, 2% peptone, 100 mM potassium phosphate pH 6.0, 4×10^{-5} % biotin, 1% glycerol) were inoculated with a single *Pichia* colony and the culture was grown overnight at 30°C on an orbital shaker at 300 rpm. After 16 hours of growth, the cells were resuspended in 10 ml of BMMY expression media (1.34% yeast nitrogenous base, 2% peptone, 100 mM potassium phosphate, pH 6.0, 4×10^{-5} % biotin, 1% methanol) and supplemented daily with additional 1% methanol. After 48 hours of expression, the cleared media was used immediately for binding studies, or for immobilized metal-affinity bead sedimentation followed by SDS-PAGE. Untransformed GS115 *Pichia* cultures were processed in parallel as negative controls for our [¹²⁵I]- α -bgtx-binding assays, Talon bead sedimentations, and Western blotting experiments. For large-scale cultures, chimeric AChBPs can be purified using immobilized cobalt-affinity chromatography following the manufacturer's instructions (GE Healthcare, Piscataway, NJ) and buffer-exchanged with PT8 buffer (150 mM NaCl, 2 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 20 mM Tris base, 0.1% Tween-20, pH 8.0).

[¹²⁵I]-α-Bgtx-binding assay

We screened ten recombinant colonies (identified as recombinant via colony PCR) of each AChBP chimera construct for [¹²⁵I]-α-bgtx binding. Two 100 μl aliquots from each cleared *Pichia* culture supernatant (representing a technical repeat), were each incubated with 5 nM of [¹²⁵I]-α-bgtx (greater than 10-fold molar excess over AChBP) in a total volume of 500 μl in PT8 buffer with gentle rotation at 4°C. Following a 1 hour incubation, a Talon bead slurry (50 μl) was added, and the sample was gently rotated for an additional hour at 4°C. After removal of unbound [¹²⁵I]-α-bgtx, the radioactivity in the lower portion of the microcentrifuge tube retaining the bead/AChBP/[¹²⁵I]-α-bgtx complexes was determined using a Wallac 1275 gamma counter (Perkin Elmer, Shelton, CT). On the basis of [¹²⁵I]-α-bgtx-binding, a single representative colony for each chimera construct was analyzed via Talon bead sedimentation followed by SDS-PAGE and Western blotting, as described in the following sections.

Talon bead sedimentation and SDS-PAGE

A 5 ml aliquot of cleared culture supernatant media was incubated with 50 μl of Talon beads for 1 hour at 4°C. The binding capacity of this resin is 5 to 20 mg of protein per ml of resin, which is well in excess of the typical amount of wild-type AChBP in a 5 mL culture. After removal of unbound protein, total protein was solubilized from the beads by the addition of 20 μl of Laemmli buffer (100 mM Tris-HCl, pH 8, 2% SDS, 100 mM DTT, 20% glycerol, 0.002% bromophenol blue) and incubation for 30 minutes at 55°C. Protein samples were analyzed on 15% SDS-PAGE gels which were stained with colloidal Coomassie blue.

Western blotting

The cell pellets from 5 ml *Pichia* cultures expressing the AChBP chimeras were washed twice with Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4). The pellets were resuspended and homogenized with 35 strokes of a Potter-Elvehjem homogenizer in 500 μl of lysis buffer (TBS with 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS). Ten microliters of cleared lysate was added to an equal volume of 2X Laemmli buffer and incubated for 30 minutes at 55°C. Following separation on a 15% SDS-PAGE gel, proteins were electroblotted onto a nitrocellulose membrane for 1.5 hours at 100 V. After the membrane was blocked in a 5% milk solution in TBS-T (TBS plus 0.1% Tween-20) for 30 minutes, mouse anti-hexahistidine tag primary antibody was added to a final concentration of 1 μg/ml in TBS buffer and incubated for 2 hours at room temperature. The membrane was again washed with TBS-T, followed by TBS, and was incubated for 1 hour with a final concentration of 50 ng/ml of horseradish peroxidase-conjugated anti-mouse antibody. The membrane was washed with TBS-T, followed by TBS, and treated with SuperSignal West Pico chemiluminescence substrate following the manufacturer's protocol. After a 30 second exposure to the membrane, the X-ray film was developed.

RESULTS

Loop substitutions

The primers used to replace the specified regions of the AChBP (loops β1–β2, β6–β7, and β8–β9, and the pre-M1 linker region) with those of the 5HT_{3A}R are listed in Table 1. The sequences chosen for the preparation of these chimeras were based on the prior work of Bouzat, *et al.* [12]. All constructs described here were prepared in our laboratory, as when this work began, the constructs used in Bouzat, *et al.* were not being made available. Because of the larger number of substituted residues in the β8–β9 loop, two successive mutagenesis reactions were necessary to replace this loop. The amino acid substitutions are illustrated in Figure 2. With the exception of the triple loop chimeras (AChBP[3L] and AChBP[3L]-pM1), the chimeric

AChBPs are referenced by the substituted regions, for example, the $\beta 1\beta 2$ - $\beta 6\beta 7$ -pM1 AChBP chimera contains loops $\beta 1$ - $\beta 2$ and $\beta 6$ - $\beta 7$, as well as the preM1 linker from the 5HT_{3A}R.

[¹²⁵I]- α -Bgtx binding

The ability of the various AChBP chimeras to bind α -bgtx was assessed by performing [¹²⁵I]- α -bgtx-binding assays on culture media. In these assays, cleared culture medium samples are incubated with [¹²⁵I]- α -bgtx. We determined the relative radioactivity content due to [¹²⁵I]- α -bgtx binding to chimeric hexahistidine-tagged AChBPs following sedimentation with immobilized cobalt-affinity chromatography beads. Relative [¹²⁵I]- α -bgtx binding was evaluated with respect to the average radioactivity (counts per minute) obtained from an equivalent volume of the AChBPwt sample. For each set of AChBP chimeric constructs, recombinant colonies that were identified via yeast colony PCR for incorporation of AChBP sequences, showed expression levels that were within the standard deviation of technical replicates of each sample. The data for the [¹²⁵I]- α -bgtx-binding assay in Figure 3 (top) were generated from cultures inoculated with single representative colonies secreting AChBP as determined in preliminary [¹²⁵I]- α -bgtx-binding assays. The cultures from these selected colonies were subsequently analyzed using Talon bead sedimentation followed by SDS-PAGE (Figure 3 middle) and Western blotting (Figure 3 bottom).

Wild-type AChBP (AChBPwt), the $\beta 1\beta 2$ chimera, along with the pM1 and $\beta 1\beta 2$ -pM1 chimeras (with the preM1 region of the 5HT_{3A}R substituted into the AChBPwt and $\beta 1\beta 2$ chimera, respectively) showed the highest levels of [¹²⁵I]- α -bgtx binding. No significant [¹²⁵I]- α -bgtx binding was detected in the cultures prepared from the remaining single- or double-loop AChBP chimeric constructs, similar to those of the negative control (cleared untransformed GS115 *Pichia* culture media). However, in cultures prepared with the chimeras containing all three loop substitutions (AChBP[3L] and AChBP[3L]-pM1), α -bgtx binding activity was restored to approximately 50% of the AChBPwt level. In addition, we noted that the replacement of residues at the C-terminus of the AChBP with those of the 5HT_{3A}R pre-M1 segment (pM1) resulted in a consistent 10–15% decrease in α -bgtx binding activity. The error bars represent the standard deviation of two technical repeats of cleared *Pichia* culture media derived from a single colony.

AChBP expression and secretion

The absence of detectable [¹²⁵I]- α -bgtx binding activity in the culture media of certain constructs may be a result of disrupted protein expression, secretion of misfolded or inactive protein, or intracellular retention. The efficiency of AChBP chimera secretion was investigated by SDS-PAGE analysis of immobilized cobalt-affinity bead sedimentations prepared from *Pichia* expression cultures secreting the hexahistidine-tagged AChBP chimeric constructs (Figure 3 middle). In agreement with results from the binding assays, AChBPwt, $\beta 1\beta 2$, pM1, and $\beta 1\beta 2$ -pM1 showed the highest levels of secreted protein. The amount of the triple-loop chimeras, AChBP[3L] and AChBP[3L]-pM1, that was secreted into the medium was approximately 50% that of the AChBPwt construct, which is consistent with the binding assays. The remaining chimeric AChBPs containing either a $\beta 6$ - $\beta 7$ or $\beta 8$ - $\beta 9$ loop substitution, alone or in combination, were not detected in the culture media by affinity precipitation and SDS-PAGE analysis. Similarly, no protein was sedimented from cleared untransformed GS115 *Pichia* culture media.

Intracellular retention of expressed AChBPs was assessed by Western blotting analysis of *Pichia* cell lysates (Figure 3 bottom). These results revealed that high levels of intracellular chimeric AChBP were present in those samples in which no secreted AChBP was detected (Figure 3 middle). Furthermore, binding assays performed on these yeast cell lysates showed no significant [¹²⁵I]- α -bgtx-binding activity (data not shown), demonstrating that these

chimeras are not only retained intracellularly, but they also are incapable of binding α -bgtx. In samples from both triple loop chimeric constructs, AChBP[3L] and AChBP[3L]-pM1, a small fraction of the chimeric protein was retained intracellularly, which agrees with the observed decrease in [125 I]- α -bgtx binding activity in the *Pichia* culture media (Figure 3 top) and the compromised secretion (Figure 3 middle) of the triple loop chimeras relative to AChBPwt. Also, as expected, no protein was detected from untransformed GS115 *Pichia* lysates via Western blotting. The combined results, summarized in Table 2, shows that intracellular retention of certain AChBP chimeras correlates inversely with the [125 I]- α -bgtx binding activity observed in the culture media (Figure 3 top).

DISCUSSION

Our experiments demonstrate that the AChBP can tolerate the substitution of loop β 1– β 2 and the pM1 linker region of the 5HT_{3A}R, but that single- or double-loop substitutions having either loop β 6– β 7 or β 8– β 9 are detrimental to proper protein expression. The absence of [125 I]- α -bgtx-binding activity in those cases was a result of failed protein secretion, rather than impaired translation. In addition, the inability to demonstrate α -bgtx binding to the intracellularly accumulated material is consistent with protein misfolding of those constructs. In contrast, AChBP chimeras containing all three loop substitutions are secreted at levels of ~50% of AChBPwt and are capable of binding α -bgtx. The data illustrate that compatibility among the three 5HT_{3A}R-derived loops is necessary for proper expression and secretion of α -bgtx-binding AChBP chimeras, and suggest that the three loop regions interact with each other either within or across subunit monomers.

Our expression analysis of AChBP chimeras was consistent with previous studies of the AChBP-5HT_{3A}R chimeric transmembrane receptor expressed in mammalian cells [12,15]. These studies revealed that surface expression of this AChBP-5HT_{3A}R fusion protein, as determined by [125 I]- α -bgtx [12] or [3 H]-epibatidine [15] binding, tolerated the analogous substitution of loop β 1– β 2, but surface expression was greatly reduced, or undetectable, following single or double substitutions involving loops β 6– β 7 or β 8– β 9 originating from the 5HT_{3A}R. As in our study, functional expression was rescued in chimeras possessing all three loop substitutions (β 1– β 2, β 6– β 7, and β 8– β 9). In agreement with the conclusions of Bouzat, *et al.* [12] and Grutter, *et al.* [15], we present very convincing evidence that these same loop combinations are necessary for the successful expression of soluble, α -bgtx-binding AChBP chimeras. Due to the fact that our AChBP-5HT_{3A}R loop chimeras are not associated with an ion pore domain, our findings suggest that these loop interactions may be sufficient on their own to explain the functional expression of the full-length receptor chimeras. Interactions with the membrane bilayer or transmembrane domain of ligand-gated receptors may be necessary for ion gating, but the folding and biogenesis of the extracellular domain appears to be largely independent of these interactions.

The current model of the muscle-type nAChR derived from cryoelectron microscopy data indicates that the β 1– β 2, β 6– β 7, and β 8– β 9 loops are in close proximity to each other, similar to their arrangement in the AChBP crystal structures [6]. In addition to biochemical data, molecular dynamics calculations modeled using the muscle-type nAChR and AChBP structures have also suggested the interaction among loops β 1– β 2, β 6– β 7, and β 8– β 9 [16–19]. However, in the full-length transmembrane receptor, these loops are also positioned near the cell membrane and the extracellular linker between the second and third transmembrane helix (M2–M3 linker) of the IPD [20]. In addition, several studies in other LGICs have revealed potential interactions between the extracellular loops and the M2–M3 linker. In the GABA_A receptor, for example, there is evidence for electrostatic interactions between loop regions in the LBD and the M2–M3 linker of the transmembrane domain [21]. Furthermore, docking of the AChBP onto the crystal structure of the M2–M3 transmembrane linker of the glycine

receptor has suggested the presence of a salt bridge and potential hydrophobic interactions between loop $\beta 1$ – $\beta 2$ and the M2–M3 linker [22]. Similarly, it has been suggested that a *cis-trans* proline isomerization within the M2–M3 linker may be required for gating of the 5HT_{3A}R channel [23]. Thus, multiple lines of evidence support the importance of the molecular interactions of these membrane-facing loops with the M2–M3 linker. Such interactions, although not essential for the expression of our soluble AChBP-5HT_{3A}R triple loop chimeras, may be a prerequisite for the propagation of the conformational change initiated by ligand-binding and culminating in the opening of the ion pore.

To test for such inter-domain interactions in a soluble environment with our hexahistidine-tagged AChBP[3L]-pM1 chimera, we designed and expressed a series of constructs of varying lengths of the 5HT_{3A}R M2–M3 linker and performed reciprocal affinity precipitations and Western blotting. These constructs included hexahistidine-tagged and GST-fusion M2–M3 linker constructs, in addition to a four- α -helical bundle-scaffolded M2–M3 linker. However, we were unable to detect any binding interaction between the M2–M3 linker-containing protein and either of the triple loop chimeras (AChBP[3L] and AChBP[3L]-pM1) with our assays. We reasoned that under our experimental conditions, we could not effectively mimic the multiple low-affinity cooperative interactions that are thought to be present between the membrane-facing loops and the associated M2–M3 linker of the 5HT_{3A}R in its native environment.

The substitution of the pM1 region did not significantly alter the protein expression, secretion, or α -bgtx-binding ability of our soluble constructs when compared to the $\beta 6$ – $\beta 7$ and $\beta 8$ – $\beta 9$ loop substitutions. In the full-length receptors, however, the pM1 may have a greater structural significance, as studies suggest that this region is important for salt bridge formations that may be essential for the receptor gating mechanism [1,13]. More recently, a similar interaction involving salt bridge formations among loop $\beta 1$ – $\beta 2$, the pM1 linker region, and the M2–M3 linker has been suggested to be important for channel gating in the muscle-type nAChR [19].

We have shown that the presence of the M2–M3 linker is not essential for the proper expression and secretion of soluble α -bgtx-binding chimeric AChBP in which loops $\beta 1$ – $\beta 2$, $\beta 6$ – $\beta 7$, and $\beta 8$ – $\beta 9$ have been replaced with those of the 5HT_{3A}R. If these three loops are required to act together functionally in a critical manner, it is not surprising to expect these loops to stabilize the protein structure during biogenesis. Our data indicates that the short $\beta 1$ – $\beta 2$ loop alone may be altered without negatively affecting AChBP secretion or the ability of the chimera to bind α -bgtx. However, the longer loop replacements ($\beta 6$ – $\beta 7$ and $\beta 8$ – $\beta 9$) appear less structurally compatible with the native AChBP sequences, possibly due to structural perturbations arising from lengthy segments of altered sequences. Such local structural modifications may result in compromised protein folding and subsequent impaired trafficking through the yeast secretory pathway. In the triple loop chimeras, the $\beta 1$ – $\beta 2$ loop substitution may stabilize loops $\beta 6$ – $\beta 7$ and $\beta 8$ – $\beta 9$ through a network of amino acid interactions. Further mutational analysis of the short, six-residue $\beta 1$ – $\beta 2$ loop at the single amino acid level could be used to help define the essential interacting residues given that the 5HT_{3A}R sequence, DEKNQV, is permissive and necessary for functional formation of the chimeric AChBP containing 5HT_{3A}R-derived $\beta 6$ – $\beta 7$ and $\beta 8$ – $\beta 9$ loops, while the corresponding *Lymnea* $\beta 1$ – $\beta 2$ sequence, NEITNE, is non-permissive.

We have demonstrated that complementarity among three inter- β -strand loop regions is necessary for proper folding and efficient protein trafficking of the AChBP-5HT_{3A}R loop chimeras examined in this study. Subsequent structural studies aimed at further elucidating the detailed interactions between or among the interacting 5HT_{3A}R-derived loops, in the presence or absence of α -bgtx, should now be facilitated by the availability of the soluble AChBP chimeras described here. In the absence of crystals of the homologous transmembrane receptors, these soluble hybrid AChBPs should provide useful models for studying those

factors that contribute to protein folding and stability of the extracellular domains of this functionally important receptor family.

Acknowledgments

This work was supported by National Institutes of Health grant, #GM32629. Some of the work presented here was done in partial fulfillment of the requirements for a Ph.D. degree (J.A.P.) from Brown University.

We thank A. B. Smit for the wild-type AChBP cDNA and Dr. P. Caffery for his critical reading of the manuscript.

ABBREVIATIONS

AChBP	acetylcholine-binding protein
α-bgtx	α -bungarotoxin
BMGY	buffered glycerol-complex medium
BMMY	buffered methanol-complex medium
5HT_{3A}R	5-hydroxytryptamine (serotonin) receptor type, 3A
IPD	ion pore domain
LBD	ligand-binding domain
LGIC	ligand-gated ion channel
nAChR	nicotinic acetylcholine receptor

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FIGURE 1. Structure of the AChBP highlighting the substituted loops

A) AChBP monomer. B) Two adjacent monomers, as would be arranged in the native pentamer. Loops $\beta 1-\beta 2$, $\beta 6-\beta 7$, and $\beta 8-\beta 9$, and the pM1 linker are labeled. Structure coordinates were imported from PDB 119B [2], modified with SwissPdb Viewer [24], and rendered in VMD [25].

```
AChBP      -----LDRADILYN---IRQTSRPDVIPTQ-RDRPVAVSLSLKFINILEVNEITNEVDVV 51
5-HT3R     RRSRNTTRPALRLSDYLLTNYRKGVPRVDRWRKPTTVSIDIVIYAILNVDEKNQVLTTY 60
AChBP [3L]  -----LDRADILYN---IRQTSRPDVIPTQ-RDRPVAVSLSLKFINILEVDEKNQVVDVV 51
                                                    □□□□□□

AChBP      FWQQTTWSDRTLAWNSS--HSPDQVSVPISSLWVPDLAAAYNAISKPEVLTPQLARVVSDG 109
5-HT3R     IWYRQYWTFDEFLOWNPEDFDNITKLSIPTDSIWVPDILINEFVDVGKSPNIPYVYIRHQG 120
AChBP [3L] FWQQTTWSDRTLAWNSS--HSPDQVSVPISSLWVPDLAAAYNAISKPEVLTPQLARVVSDG 109

AChBP      EVLYMPSIRQRFS CDVSGVDTESGAT-CRIKIGSWTHHSREISVDPT---TENSDDSEYF 165
5-HT3R     EVQNYKPLQVVTACSLDIYNFPFDVQNCSLTFTSWLHTIQDINISLWRLPEKVKSDRSVF 180
AChBP [3L] EVLYMPSIRQRFSCLDIYNFPFDVQNCRIKIGSWTHHSREISVDPT---TENSDDRVSF 166
                ■■■■■■■■■■■■■■                                ○○○○

AChBP      SQYSRFEILDVTQKKNSVTYSCCPEAYEDVEVSLNFRK---KGRSEIL----- 210
5-HT3R     MNQGEWELLGVLPHYFREFSMESS-NYYAEMKFYVVRRRRPLFYVV----- 224
AChBP [3L] MNQGEWEILDVTQKKNSVTYSCCPEAYEDVEVSLNFRRRPLFKDDDDKHHHHHH 220
○○○○○●●●●●
```

FIGURE 2. Primary sequence alignment of AChBP, presumed extracellular domain of the 5HT_{3A}R, and AChBP[3L]-pM1

The substituted regions are denoted by the following symbols: loop $\beta 1$ - $\beta 2$, $\square$; loop $\beta 6$ - $\beta 7$, $\blacksquare$; loop $\beta 8$ - $\beta 9$, $\circ$; and pM1, $\bullet$. The sequence alignment was performed using the ClustalW algorithm [26].

FIGURE 3. [¹²⁵I]-α-bgtx binding and secretion of chimeric AChBPs

Top: An [¹²⁵I]-α-bgtx-binding assay showing which AChBP chimeras were secreted into the *Pichia* culture media and bind α-bgtx. Middle: SDS-PAGE analysis of metal-affinity isolated proteins indicates which AChBP chimeras were secreted irrespective of their ability to bind α-bgtx. Bottom: The corresponding Western blot was probed with anti-hexahistidine tag antibody identifying intracellularly accumulated AChBP chimeras.

Table 1

Primers used for loop replacements.

Loop	Primer sequences for loop replacement
$\beta 1$ – $\beta 2$	5'-TCAACATCTTGGAAGTGGATGAAAAAACCAGTTGTGGACGTGGTCTTTTGGC-3' 5'-GCCAAAAGACCACGTCCACAACCTGGTTTTTTCATCCACTTCCAAGATGTTGA-3'
$\beta 6$ – $\beta 7$	5'-CGGTTCTCCTGCTCTCTAGATATTTACAACCTCCCATTCGACGTTCAAACTGTCGGATCAAA-3' 5'-TTTGATCCGACAGTTTGAACGTCGAATGGGAAGTTGTAAATATCTAGAGAGCAGGAGAACCG-3'
$\beta 8$ – $\beta 9$ 1 st set	5'-GTGATGATTCTGAATACTTCATGAACCAAGGTGAATGGGAAATCTTGGACGTCACACA-3' 5'-TGTGTGACGTCCAAGATTTCCCATTCACCTTGGTTCATGAAGTATTCAGAATCATCAC-3'
$\beta 8$ – $\beta 9$ 2 nd set	5'-GAAAATAGTGATGATCGTTCAGTCTTCATGAACCAAGGTGAATGGGAAATCTTGGACGTC-3' 5'-GACGTCCAAGATTTCCCATTCACCTTGGTTCATGAAGACTGAACGATCATCACTATTTTC-3'
preM1	5'-GAAGTGAGTCTCAATTTCCGGAGAAGACCACTCTTCAAGGACGACGACGACAAGCATC-3' 5'-GATGCTTGTGTCGTCGTCCTTGAAGAGTGGTCTTCTCCGAAATTGAGACTCACTTC-3'

Table 2

Summary of chimeric AChBP results.

Chimera	able to bind α - bgtx	extracellular secretion	intracellular accumulation
AChBPwt $\pm$ pM1	+	+	–
Single loop chimeras:			
β 1 β 2 $\pm$ pM1	+	+	–
β 6 β 7 $\pm$ pM1	–	–	+
β 8 β 9 $\pm$ pM1	–	–	+
Double loop chimeras:			
β 1 β 2- β 6 β 7 $\pm$ pM1	–	–	+
β 1 β 2- β 8 β 9 $\pm$ pM1	–	–	+
β 6 β 7- β 8 β 9 $\pm$ pM1	–	–	+
Triple loop chimeras:			
AChBP[3L] $\pm$ pM1	+	+	+/-