Nuclear Magnetic Resonance Solution Conformation of α-Conotoxin AuIB, an α3β4 Subtype-selective Neuronal Nicotinic Acetylcholine Receptor Antagonist*

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The neuronal nicotinic acetylcholine receptors constitute a highly diverse group, with subtypes consisting of pentameric combinations of α and β subunits. α-Conotoxins are a homologous series of small peptides that antagonize these receptors. We present the three-dimensional solution structure of α-conotoxin AuIB, the first 15-residue α-conotoxin known to selectively block the α3β4 nicotinic acetylcholine receptor subtype. The pairwise backbone and heavy-atom root mean square deviation for an ensemble of 20 structures are 0.269 and 0.720 Å, respectively. The overall fold of α-conotoxin AuIB closely resembles that of the α4/7 subfamily α-conotoxins. However, the absence of Tyr15, normally present in other α4/7 members, results in tight bending of the backbone at the C terminus and effectively renders Asp14 to assume the spatial location of Tyr15 present in other α4/7 α-conotoxins. Structural comparison of α-conotoxin AuIB with the α3β2 subtype-specific α-conotoxin MII shows different electrostatic surface charge distributions, which may be important in differential receptor subtype recognition.

The α-conotoxins are small neuropharmacologically active peptides of Conus origin that antagonize the nicotinic acetylcholine receptor (nAChR) (1). The natural diversity of biosynthesized conotoxin peptides has led to the classification of a wide spectrum of disulfide-bridged peptides, which attack various ligand and ion-gated channels and receptors (1). The nicotinic acetylcholine receptors exhibit considerable diversity in their own right because of the different compositions found in the pentameric subunits constituting each nAChR subtype (2). Although the mammalian neuromuscular subtype comprises (α1)2βγδ or (α1)2βδε subunits, the neuronal subtypes are comparatively more diverse with their hetero- or homopentameric combinations of α (α2 - α9) or β (β2 - β7) subunits (3, 4). The general conotoxin strategy of diversification is "combinatorial" (5), in which amino acid residues are varied within a given disulfide framework to specifically and selectively bind various subtypes of the target channel or receptor. For the case of α-conotoxins, target selectivity is essentially defined depending on which subunit interface of the nAChR (e.g. α1/γ, α1/δ, and α3/β2) each individual α-conotoxin preferentially binds to (1). Highly selective α-conotoxins that permit differential blocking of diverse nAChR subtypes have served as effective tools in studying these receptors (1).

Of recent particular interest are the α-conotoxins that act on neuronal nAChRs. For example, α-conotoxin AuIB specifically targets the α3β4 subtype (6), whereas α-conotoxin MII selectively blocks the α3β2 subtype (7). On the other hand, α-conotoxin ImI, the smallest of all α-conotoxins and distinct because of its α4/3 disulfide framework (8), is a specific antagonist of the homomeric α7 subtype (8). In addition, other neuronal α-conotoxins such as PnIA (9), PnIB (9), and EpI (10) that are less selective to a particular nAChR subtype have also been identified (Table I).

As shown in Table I, most neuronal α-conotoxins belong to the α4/7 subfamily, in which the amino acid sequence varies within disulfide bridge-enclosed loops of four and seven residues. Interestingly, α-conotoxin El, unique in its specificity for the α1/δ subunit interface in Torpedo neuromuscular nAChR, belongs to the same α4/7 subfamily (11). The recent increase in the understanding of these conotoxins has been attributable to the advances in the structural characterization of these molecules. High-resolution three-dimensional structures of α4/7 subfamily α-conotoxins PnIA (12), PnIB (13), MII (14, 15), and [Tyr15]EpI (16) show that, when backbone-superimposed, their backbone fold is extremely similar. The recently solved NMR structure of α-conotoxin El also has the same overall molecular fold as the α4/7 neuronal α-conotoxins despite the variation in its sequence both within the disulfide loops and at the N terminus (Table I). These observations reaffirm that conotoxins are based on common three-dimensional scaffolds and that their subtype selectivity is conferred through sequence variation of a selected number of residues (5).

Through structural elucidation of these highly selective α-conotoxins and their analogs (17, 18), we have been using the reverse-mapping approach first to identify receptor subtype specific determinants in the ligands and second to indirectly probe the regions in nAChR responsible for binding agonists

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‡ The abbreviations used are: nAChR, nicotinic acetylcholine receptor; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; COSY, correlation spectroscopy; RMSD, root mean square deviation.

§ The α-conotoxins are additionally grouped according to the number of amino acid residues enclosed within each disulfide loop. Loop sizes of four residues in the first loop and seven in the second are denoted as α4/7. Other subfamilies include α3/5 and α4/3.

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and antagonists. We hereby present the high-resolution solution structure of α-conotoxin AuIB that has a unique αβ4 nAChR subtype selectivity (6). αβββ-like receptors are believed to be important in peripheral ganglionic transmission and centrally mediated norepinephrine release. α-Conotoxin AuIB inherently lacks Tyr15 found in other α/γ/7 members such as α-conotoxins PnIA, PnIB, Epl, and even AuIA and AuIC (Table I). Of the three α-conotoxin AuIs found in Conus aulicus (6), α-conotoxin AuIB is the best characterized from a functional standpoint and has thus been chosen for detailed structural analysis by NMR. Structural comparison of α-conotoxin AuIB with other neuronal α-conotoxins should provide useful insight into important nAChR subtype determinants, in particular, those that discriminate the β2 and β4 subunits.

### EXPERIMENTAL PROCEDURES

**Peptide Synthesis and Purification—α-Conotoxin AuIB was synthesized using solid-phase chemistry and purified to homogeneity as described previously (6).**

**NMR Spectroscopy—** Samples for NMR studies were prepared in 90% H2O and 10%1H2O or 100% 2H2O, pH 4.1, with a final sample concentration of 6 mM. All NMR experiments were performed using a Varian UNITY INOVA 600 spectrometer at 278 and 298 K. Solvent suppression was carried out using selective, low-powered irradiation of the water resonance during a relaxation delay of 1.5 s. All resonances were referenced to a residual water signal (4.76 ppm at 298 K). Mixing times of 200–400 ms for NOESY and 150 ms for rotating frame NOE experiments were used. For total correlation spectroscopy experiments (19), a 75-ms mixing time was used. JHNH, coupling constants for the backbone torsion angle were measured using phase-sensitive double-quantum-filtered two-dimensional COSY experiments (20). Primitive exclusive COSY (21) was performed in 100% 2H2O to measure the JHNH-coupling constants, which were used in conjunction with the d15 NOEs to provide χ1 torsion angles. Spectral widths were 7.0 kHz in both dimensions. Typical two-dimensional data consisted of 2048 complex points in the t2 dimension and 256 complex t1 increments. Data acquisition, sequential assignments, and spectral interpretation were performed on SPACebation IPX and Ultra 1 Creator workstations (Sun Microsystems) running VnmrX 5.3B software (Varian Associates).

**Computation of Structures—** Two-dimensional NMR data were processed using Felix 95.0 and Insight II 97.0 (Biosym/MSI) on Indigo 2 XL or Indy workstations (Silicon Graphics). NOE interproton distance constraints were derived primarily from the NOESY spectrum obtained at 278K with a mixing time of 200 ms. Before Fourier transformation, free induction decays were reconstructed using linear prediction, apodized with a 60° sine bell-squared window function in both dimensions, and baseline corrected using the FLATT algorithm (22). NOE cross-peak volumes were measured and converted into upper bounds of interproton distances using the distance of 1.8 Å as a reference for nonoverlapping geminal and β-proton cross-peaks. Appropriate pseudolouations of 1.5–2.5 Å were used to constraints. Dihedral angle restraints were inferred from JHNH-coupling constants and were centered on −120° ± 30° for JHNH > 8 Hz and −60° ± 30° for JHNH < 6 Hz. Side chain χ1 torsion angles measured from primitive exclusive COSY experiments were also incorporated in combination with the sequential d15 NOE for stereospecifically assigned β-methylene protons. A total of 156, including 13 long-range and 25 medium-range, distance constraints, together with eight ϕ and two χ2 torsion angles were input for the computation of structures.

Preliminary structures were generated using DGGII (23), NMRRecht, and Insight II 97.0. An extended molecule with two disulfide bridges and a formal charge of zero was constructed. The potentials and partial charges were assigned using consistent valence force fields and were used throughout the computations. Full relaxation matrix analysis using MARDIGRAS version 3.2 (25) was performed on DGGII-generated structures as initial model structures. The three-site jump model for intra- and inter-residue distances was chosen, and the noise level was estimated to be 50% of the un-normalized absolute value of the smallest peak. The rotational correlation time was estimated to be 1.0 ns. Structures were refined with restrained molecular dynamics calculations with Discover 2.97 (Biosym/MSI) and Insight II 95.0. A simulated annealing schedule based on well established protocols (26) was generated and run. The 68-ps protocol consisted of dynamics at 1000 K (50 ps), followed by gradual temperature cooling from 1000 to 300 K (incremental temperature decrease of 100 K each over 18 ps) with staggered geometric increases of NOE, covalent term, and nonbonded force constants. During the final dynamics step at 300 K and subsequent minimization steps, a modified restraint file with decreased force constants for side chain interproton distances was used to allow further conformational dynamics. Energy minimization was performed 2-fold, first with a quadratic potential and then with a Lennard-Jones nonbonded potential. A total of 50 simulated annealing rounds were run.

**Assessment of Structure Quality and Visualization of Structures—**

The Rg and Rfactors (27) were calculated using CORMA version 5.2 (28) for the final ensemble of 20 structures. In addition, the stereochemistry of the computed structures were analyzed with PROCHECK version 3.5 (29). The three-dimensional display of structures was performed either with Insight II 97.0 (Biosym/MSI) or GRASP version 1.36 (30).

### RESULTS

**Resonance Assignment and Secondary Structure—** The complete 1H resonance assignment for α-conotoxin AuIB was achieved in a straightforward manner using double-quantum-filtered two-dimensional COSY, total correlation spectroscopy, and NOESY and rotating frame NOESY spectra following the standard sequential assignment procedure (31). Initial assignment of the amino acids was made along the NH resonances in double-quantum-filtered two-dimensional COSY and total correlation spectroscopy spectra. Apart from Thr11, where the Hα and Hβ protons showed overlapping, all Hα and side chain protons were unambiguously resolved, permitting the tracing of sequential Hα-NH(α + 1) connectivities. The connectivity was broken at three proline residues (Pro6, Pro7, and Pro13), for which resonance assignment was achieved using strong d15(Tyr3), d15(Pro6), d15(Pro7), and d15(Acm12, Pro13) cross-peaks that indicate all prolines have the trans conformation.

Fig. 1 is a summary of sequential and medium-range NOEs used for the resonance assignment, the χ2 values, the temperature factors for NH protons (AδNiiH), and chemical shift indices (32). The stretches of d15(i, i + 3), d15(i, i + 4), and d15(i, i + 4) NOEs between Pro6 and Acm12 make the presence of the 5-helix evident before the detailed structure calculations.

**Structure Calculations—** Fig. 2 shows the superposition of a final ensemble of 20 lowest energy structures. The backbone and heavy atom RMSD values for the entire chain were 2.89 and 2.72 Å, respectively. Omitting the flexible N-terminal Gly1 residue, the backbone RMSD value improves considerably to 0.092 Å, with the heavy atom RMSD being 0.707 Å. As shown in Table II, overall structural statistics concerning experimental restraints, covalent geometry, and structural convergence for α-conotoxin AuIB are extremely good. When subject to quality evaluation by PROCHECK (29), backbone dihedral angles of all non-Gly and non-Pro residues were found to reside within the most favored region of the Ramachandran plot (Table II).

**Three-dimensional Structure of α-Conotoxin AuIB—** The backbone fold of α-conotoxin AuIB has the shape of the Greek...
letter ω, which is characteristic of all α4/7 α-conotoxins (Fig. 3). The N-terminal Cys2–Tyr4 residues constitute a type I β-turn. This is followed by a two-turn α-helix (Tyr5–Asn12), which forms the bottom portion of the α2 fold. The C terminus (Asn12–Cys15) forms a bend at Pro13 to properly complete the Cys3–Cys15 disulfide bridge. Of the two disulfide bridges present in α-conotoxin AuIB, the angles for the Cys3–Cys15 pair were determined experimentally and used to calculate a left-handed spiral conformation. The final ensemble of the Cys2–Cys8 disulfide bridge was converged well to a single, left-handed form. These disulfide conformations are in good agreement with those observed in high-resolution x-ray structures of other α4/7 members such as α-conotoxins PnIA (12) PnIB (13) and [Tyr15]EpI (16). As shown in Fig. 3, the inherent absence of a tyrosine between Asp14 and Cys15 in α-conotoxin AuIB does not influence the overall conformation of the Cys3–Cys15 disulfide bridge to any appreciable extent. However, this deletion renders the local backbone fold near Asp14 and Cys15 in α-conotoxin AuIB noticeably different from that in other α4/7 members (see Fig. 5A). Two consecutive β turns found at their C termini of other α4/7 members are absent in α-conotoxin AuIB.

DISCUSSION

Structural Comparison with Other α4/7 α-Conotoxins—The α4/7 subfamily of α-conotoxins share a common ω backbone fold, as demonstrated in Fig. 3. The pairwise backbone RMSDs of α-conotoxin AuIB with other α4/7 members are 0.97 Å with MII (Protein Data Bank file name, 1M2C), 0.61 Å with PnIA (1PEN); 0.70 Å with PnIB (1AKG), and 0.52 Å with [Tyr15]EpI (1A6M). A common backbone scaffold shared by the α4/7 members would presumably provide an efficient means for combinatorial presentation of functional side chain moieties that specifically interact with the receptor (5). As shown in Table I, when excluding the neuromuscular-targeting α-conotoxin EI, the N-terminal disulfide loop of all α4/7 subfamily members contains an S(P/V) sequence (ν is a variable residue) except for α-conotoxin EpI, which is not particularly specific for one subtype. Ligand binding to the α subunit of nAChR is known to be primarily mediated by hydrophobic aromatic interactions (33–35). Surface characteristics of the putative S(P/V) region in α4/7 α-conotoxins shown below by GRASP suggest that the mostly hydrophobic S(P/V) sequence may represent a common α2 subunit recognition face in neuronal α4/7 members.

The variability in sequence across the C-terminal seven-residue loop is greater, in particular, for the first three residues. The amino acid types for the last four residues in the

letter ω, which is characteristic of all α4/7 α-conotoxins (Fig. 3). The N-terminal Cys2–Tyr4 residues constitute a type I β-turn. This is followed by a two-turn α-helix (Tyr5–Asn12), which forms the bottom portion of the α2 fold. The C terminus (Asn12–Cys15) forms a bend at Pro13 to properly complete the Cys3–Cys15 disulfide bridge. Of the two disulfide bridges present in α-conotoxin AuIB, the angles for the Cys3–Cys15 pair were determined experimentally and used to calculate a left-handed spiral conformation. The final ensemble of the Cys2–Cys8 disulfide bridge was converged well to a single, left-handed form. These disulfide conformations are in good agreement with those observed in high-resolution x-ray structures of other α4/7 members such as α-conotoxins PnIA (12) PnIB (13) and [Tyr15]EpI (16). As shown in Fig. 3, the inherent absence of a tyrosine between Asp14 and Cys15 in α-conotoxin AuIB does not influence the overall conformation of the Cys3–Cys15 disulfide bridge to any appreciable extent. However, this deletion renders the local backbone fold near Asp14 and Cys15 in α-conotoxin AuIB noticeably different from that in other α4/7 members (see Fig. 5A). Two consecutive β turns found at their C termini of other α4/7 members are absent in α-conotoxin AuIB.

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The variability in sequence across the C-terminal seven-residue loop is greater, in particular, for the first three residues. The amino acid types for the last four residues in the
same loop can reasonably be represented by (P/S/D/N/Y/L)/C. Because this C-terminal sequence is shared, it is unlikely that this region is responsible for the different nAChR subtype specificities exhibited by each toxin. For example, both α-conotoxins AuIA and AuIC possess Tyr\(^{15}\) (Table I), yet their α₃β₄ subtype selectivity is only slightly weaker than α-conotoxin AuIB (6). Therefore, Gly\(^{14}\) in α-conotoxin AuIC and the missing Tyr\(^{15}\) α-conotoxin AuIB are not likely to be essential for defining the α₃β₄ subtype selectivity. As noted above, the missing Tyr\(^{15}\) from the PDYC sequence in α-conotoxin AuIB results in a different local backbone C-terminal fold from other α4/7 members. Using a-conotoxin PnIA as a reference, divergence in the α-conotoxin AuIB backbone trace begins with Pro\(^{13}\), where the Pro rings of both α-conotoxins PnIA and AuIB display perpendicular orientations. A strong backbone bend follows, placing the Asp\(^{14}\) Cα of α-conotoxin AuIB closer to the Tyr\(^{15}\) Cα than the Asp\(^{14}\) Cα of α-conotoxin PnIA (Fig. 3). It remains to be seen whether the observed spatial shift of the Asp\(^{14}\) side chain contributes to the higher antagonistic activity of α-conotoxin AuIB than AuIA/C.

Fig. 4 shows electrostatic potential surfaces generated by GRASP (30) for α-conotoxins AuIB, PnIA, [Tyr\(^{15}\)]EpI and MII. The viewing direction is kept same as that in Fig. 3. The positively charged N terminus (upper left region) is observed for all four toxins. The upper right hydrophobic bulge corresponds to Tyr\(^{15}\) of α-conotoxins PnIA and [Tyr\(^{15}\)]EpI or Leu\(^{15}\) in α-conotoxin MII. Note that the negatively charged Asp\(^{14}\) of α-conotoxin AuIB is found at the corresponding location. A deep cleft is observed in the left side of MII, but the corresponding site in α-conotoxins AuIB and EpI is filled with Tyr\(^{6}\) and Asp\(^{5}\), respectively. The conspicuous hydrophobic bulge formed by Leu\(^{10}\) in the middle of MII is absent in other α4/7 members, suggesting that Leu\(^{10}\) in MII is important for its β₂ subunit selectivity (see below). Significance of the residue at position 10 in the α4/7 subfamily is further illustrated by the following observation. α-Conotoxins PnIA and PnIB differ in only two positions, yet their selectivities are markedly different (Table I). Interestingly, substituting Ala\(^{10}\) by Leu turns the α₃β₂ subtype-specific PnIA into an α₃ subtype-specific toxin, suggesting that only position 10 is necessary to differentiate the two toxins (1).

Comparison of α-Conotoxins AuIB and MII—As shown in Fig. 5, more specific comparison can be made between two members of the α4/7 subfamily, AuIB and MII, because the former is highly selective toward the α₃β₄ nAChR subtype, whereas the latter is selective for the α₃β₂ subtype. MII is chosen as the α₃β₂ subtype-specific toxin, because α-conotoxin PnIA, another α₃β₂-targeting toxin, is much less selective than α-conotoxin MII toward the same receptor subtype (1). At present, two different NMR structures of MII are available, one determined in aqueous solution (Ref. 15; 1M2C) and the other in 30% 2,2,2-trifluoroethanol (Ref. 16; 1MII). Because the structure of α-conotoxin AuIB was determined in aqueous solution without 2,2,2-trifluoroethanol, structural comparisons were made with the former. In fact, backbone comparisons of α-conotoxin AuIB with both structures indicate that the former agrees better when superimposed (pairwise RMSDs, 0.97 Å for 1M2C and 1.29 Å for 1MII). Note in Fig. 5A the aforementioned
local backbone difference near residues 13–15 between \( \alpha \)-cono-toxins MII and AuIB. This difference is also clearly visible when the structure of \( \alpha \)-conotoxin MII determined in 2,2,2-trifluoroethanol is used for comparison, because the structures of \( \alpha \)-conotoxin MII in two solvent conditions are essentially the same.

If a comparative surface exposure approach previously used for \( \alpha \)-conotoxin EI is applied to compare the two toxins (Fig. 5B), three residues of \( \alpha \)-conotoxin MII become exposed out of the AuIB surface, Leu10, Glu11, and His12. The former two were shown to be important for distinguishing neuromuscular from neuronal nAChR. Note that GRASP analysis above suggested Leu10 to be important for the \( \beta_2 \) subunit selectivity of \( \alpha \)-cono-toxin MII. The third residue, His12, is unique in that no other \( \alpha_4/7 \) members have such an aromatic bulky residue at the same position (Table I). Intriguingly, His12 is buried underneath bulky side chains of Arg9 and His10 of neuromuscular \( \alpha \)-conotoxin GI and hence is not surface-exposed when \( \alpha \)-conotoxins GI and MII are superimposed. These results suggest that His12 in \( \alpha \)-conotoxin MII is important for distinguishing two different neuronal nAChR subtypes, \( \alpha_2\beta_4 \) and \( \alpha_2\beta_2 \), but makes no contribution to discriminating the neuronal nAChR subtype against the neuromuscular subtype.

The surface-exposed Ser13 of \( \alpha \)-conotoxin MII is not considered important, because it stays outside of the AuIB surface because of the different local backbone topology pointed out earlier. This residue is not exposed if \( \alpha \)-conotoxin MII is super-imposed with other \( \alpha_4/7 \) members such as PnIAB or Epl. Rather interesting is that there are no prominently protruding AuIB residues out of the MII surface, except for the hydroxyl tip of Tyr4 and the carboxylate side chain of Asp14. An alanine-scanning study on \( \alpha \)-conotoxin AuIB should provide answers on the significance of these residues for the \( \beta_2 \) subtype specificity.

The structural details of \( \alpha \)-conotoxin binding with nAChR are currently incomplete. Despite the overall differences or local similarity among these toxins, it is certainly difficult to unambiguously define the respective subtype selectivities based only on such structural features (18). Binding determinants within the \( \alpha_4/7 \) subfamily are subtle and may not be immediately discernible, as can be judged from the fact that the overall structural fold is same, whereas only types of amino acids are varied. Hence, structural comparison of different \( \alpha \)-conotoxins needs to be augmented by additional information from site-directed mutagenesis and chimeric receptor engineering (3, 36, 37). Cumulative results support that both \( \alpha \) and non-\( \alpha \) subunits of the neuronal nAChR contribute to the binding of agonists and antagonists (38–42), although the extent of contribution from different subunits to ligand binding needs to be better-characterized.

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