NMR Structure Determination of a Novel Conotoxin, [Pro 7,13] αA-Conotoxin P_{IVA}[†,‡]

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ABSTRACT: A high-resolution solution conformation of a novel conotoxin, [Pro 7,13] αA-conotoxin P_{IVA}, GCCGSYPNAACHPCSCKDROSYCGQ-NH_2, has been determined by two-dimensional ^{1}H NMR methods and distance geometry calculations. The total of 324 NOE-derived interproton distance restraints including 33 long-range NOE restraints as well as 11 ϕ and 7 χ^1 torsion angle restraints was used for computation of structures. Back-calculation from the experimental NOE spectrum has provided 49 new NOE restraints and yielded the final *R*-factors of $R_a = 0.641$ and $R_b = 0.157$. The final RMSD values are 0.90 and 1.16 Å for the backbone and the heavy atoms, respectively. The C-terminal half of the molecule involving the residues 12−24 is extremely well-defined with a backbone RMSD value of 0.56 Å, whereas the N-terminal 3−11 disulfide loop is relatively flexible, possessing a backbone RMSD value of 1.09 Å. The [Pro 7,13] αA-conotoxin P_{IVA} does not contain any significant secondary structure although the 21S-24G nearly completes one turn of a 3_{10} helix. The overall protein fold is largely maintained by the three disulfide bridges of 2−16, 3−11, and 14−23. The presence of the three disulfide bridges imposes geometric constraints that force the molecule to form six continuous bends involving the following residues: 3C-5S, 7P-10A, 12H-14C, 15S-17K, 17K-19R, and 21S-25Q. The overall shape of the [Pro 7,13] αA-conotoxin P_{IVA} can be described as an "iron". Residues 15S-19R form a loop that protrudes out of the "bottom plate" formed by the rest of the protein and constitute the handle of the iron. The N-terminal tip of the molecule is relatively immobile due to attractive electrostatic interactions between the γ-hydroxyl group of 20 Hyp and the phenolic hydroxyl group of 22Y. The flexible 3−11 disulfide loop consists mostly of hydrophobic residues, while the best-defined 14−23 disulfide loop contains the highly charged hydrophilic 15S-19R "handle" domain exposed to the exterior of the protein. Binding to nicotinic acetylcholine receptor can be mediated through two different types of interactions: one involving the aromatic hydrophobic residues such as 6Y and 12H and the other involving the positively charged hydrophilic side chain of the 19R. The side chain of the 19R in the [Pro 7,13] αA-conotoxin P_{IVA} and that of the 9R of the α-conotoxin G_I, and also the side chains of the 12H and 6Y in the former and those of 10H and 11Y in the latter can be aligned to point to the same direction when the corresponding backbone atoms are superimposed to an RMSD value of 2.5 Å.

The best-understood of all ligand-gated ion channels is the nicotinic acetylcholine receptor (nAChR) at the muscle end plate (Czajkowski et al., 1993; Galzi & Changeux, 1994; Toyoshima & Unwin, 1988, 1990; Unwin, 1989, 1993). Much of the present detail regarding receptor structure is inferred from electron microscopic (EM) images obtained at 9 Å resolution (Unwin, 1989, 1995) and more recently at 7.5 Å (Unwin, 1996). Such EM images have been extremely useful in providing a suggestive picture regarding structural differences between the closed and open states of the receptor, the location of possible secondary structural domains, and possible ligand binding sites. In combination with molecular modeling, insight into the mechanism of channel gating can be obtained (Galzi & Changeux, 1994; Kerr et al., 1993; Unwin, 1989, 1995). Nevertheless, much more detailed information in terms of the three-dimensional structure and function is needed in order to get a better understanding of ligand−receptor interactions. Obtaining high-resolution structures of the receptor at an atomic resolution is beyond the limit of the current physicochemical detection techniques such as X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy. An alternative approach to the study of ligand−receptor interactions is to investigate the high-resolution structures of various ligands which are molecules of less complexity than the receptor by systematically collecting relevant information regarding ligand−receptor interactions. Modern NMR spectroscopic

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[‡] Atomic coordinates for 12 converged structures of αA-conotoxin P_{IVA} have been deposited with Protein Data Bank, Brookhaven National Laboratories, Long Island, NY 11973, under the accession code 1P1P.

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Table 1: Comparison of the Sequence of the [Pro 7,13] αA-Conotoxin P_{IVA}[a] with Previously Characterized α-Conotoxins

conotoxin	sequence[b]
αA-P_{IVA}	G**CC**GSYONAA**C**HO**CSC**KDROSY**C**GQ*
α-G_I	E**CC**NPA**C**GRHYS**C***
α-G_{IA}	E**CC**NPA**C**GRHYS**C**GK*
α-G_{II}	E**CC**HPA**C**GKHFS**C***
α-M_I	GR**CC**HPA**C**GKNYS**C***
α-S_I	I**CC**NPA**C**GPKYS**C***
α-S_{IA}	Y**CC**HPA**C**GKNFD**C***
α-Pn_{IA}	G**CC**SLPP**C**AANNPDY**C***
	CC C C

[a] [Pro 7,13] αA-conotoxin P_{IVA} is the under-hydroxylated derivative of αA-conotoxin P_{IVA} at prolines 7 and 13. [b] The asterisk indicates C-terminal amidation, and the O indicates *trans*-4-hydroxyproline.

methods have been a powerful tool to this end (Davis et al., 1993; Farr-Jones et al., 1995; Kobayashi et al., 1989; Lancelin et al., 1991; Pallaghy et al., 1993; Pardi et al., 1989).

One group of particularly promising ligands for studying ion channels is the conotoxin. Conotoxins are small conformationally-constrained peptides, mostly 12 to 27 amino acids with two or three disulfide bonds (Myers et al., 1993; Olivera et al., 1985, 1990; Woodward et al., 1990), produced by the venomous marine snails (Fainzilber et al., 1994; Hopkins et al., 1995; Martinez et al., 1995; Myers et al., 1993; Olivera et al., 1990; Ramilo et al., 1992; Stroud et al., 1990) belonging to the gene *Conus* (cone shells). Although the cone snails appear to use a combinatorial peptide library strategy to rapidly evolve peptides with novel pharmacological specificity (Olivera et al., 1995), most conotoxins share one of a small group of highly-conserved structural frameworks. These conserved frameworks comprise the cysteine residues which form disulfide bonds. Every *Conus* venom has a large complement of pharmacologically diverse conotoxins; these are the major weapon that the snail uses for capturing prey. Some conotoxins may also be used to defend against predators or deter competitors. Since conotoxins often have remarkable discrimination between closely related subtypes of a family of ion channels or receptors, the availability of a large homologous peptide series targeting the same ligand binding site and sharing a rigid structural framework provides an attractive tool kit for intensively probing a particular ligand binding site.

Conotoxins from different *Conus* species that bind to homologous sites on a receptor complex generally exhibit the same disulfide framework. Indeed, even pharmacologically diverse conotoxin families that target to different binding sites can share the same structural framework. For example, the ω-conotoxins, peptides which block voltage-gated calcium channels (Farr-Jones et al., 1995; McClesky et al., 1987; Sevilla et al., 1993; Skalicky et al., 1993; Tsien et al., 1991; Valentino et al., 1993; Wheeler et al., 1994; Zhang et al., 1993) have the same disulfide-bonding pattern as the δ-conotoxins, a family of peptides which inhibit inactivation of voltage-gated sodium channels (Shon et al., 1994). The δ-conotoxins have been found in both fish-hunting and snail-hunting *Conus* species; although some δ-conotoxins diverge as much as 70% in non-cysteine amino acids, all members of the δ-conotoxin family share a single conserved disulfide framework.

Thus, it was a somewhat surprising discovery that more than one structural framework is found in conotoxins which are competitive inhibitors of the nicotinic acetylcholine receptor (Hopkins et al., 1995; Martinez et al., 1995; Myers et al., 1991; Olivera et al., 1985). The very first conotoxin characterized, α-conotoxin G_I, whose three-dimensional structure has been studied in detail by NMR techniques (Kobayashi et al., 1989; Pardi et al., 1989), typifies the major conotoxin family targeted to the ligand binding site of the nicotinic acetylcholine receptor, the α-conotoxins. All α-conotoxins share the two-disulfide pattern shown in Table 1; homologous peptides have been reported from the venoms of a number of different *Conus* species. However, the major peptide purified from *Conus purpurascens* venom which inhibits the nicotinic acetylcholine receptors competitively at the ligand site has an entirely different disulfide framework and exhibits no obvious homology to the α-conotoxins. We have referred to this new family of peptides as the αA-conotoxins (Hopkins et al., 1995).

Clearly, having structural information on the entirely different disulfide-bonded framework found in the αA-conotoxins should provide novel opportunities for probing the ligand binding sites of the nicotinic acetylcholine receptor, with a possibility of defining residues that could not be probed using the α-conotoxin framework. However, before this new group of conotoxins can be widely used to probe the target site, the basic conformation of the structural framework has to be obtained. To date, high-resolution three-dimensional structures have been obtained for only a limited number of conotoxins: α-conotoxin G_I (Kobayashi et al., 1989; Pardi et al., 1989), μ-conotoxin G_{IIIA} (Lancelin et al., 1991; Ott et al., 1991), ω-conotoxin G_{VIA} (Davis et al., 1993; Pallaghy et al., 1993; Sevilla et al., 1993; Skalicky et al., 1993), ω-conotoxin M_{VIIC} (Farr-Jones et al., 1995), ω-conotoxin M_{VIIA} (Kohno et al., 1995), and α-conotoxin Pn_{IA} (Hu et al., 1996). All of the structures were determined in solution by NMR spectroscopy except one (Hu et al., 1996). The α- and αA-conotoxins show excellent promise as probes for receptor or ion channel complexes. In this article, we report the three-dimensional structure of the [Pro 7,13] αA-conotoxin P_{IVA} using two-dimensional ^1H NMR techniques, in combination with distance geometry and NOE back-calculation and compare the results with the structures of the other α-conotoxins. The [Pro 7,13] αA-conotoxin P_{IVA} is an equipotent analog of the αA-conotoxin P_{IVA} with proline substitution at the positions 7 and 13 and is present as a major component of *C. purpurascens* venom (Hopkins et al., 1995).

MATERIALS AND METHODS

(A) Protein Purification. [Pro 7,13] αA-conotoxin P_{IVA}, purified from the venom of *C. purpurascens*, was synthesized as previously described (Hopkins et al., 1995).

(B) CD Spectropolarimetry. CD spectra of 20 μM [Pro 7,13] αA-conotoxin P_{IVA} prepared in 100% water at three pH values (3.5, 4.5, and 6.0) were measured at 25 °C using a Jasco J720 model spectropolarimeter. All of the spectra were the average of at least eight scans. Results (data not shown) indicate that the protein does not contain any significant secondary structures, and the overall structure remains the same within the pH range 3.5−6.0.

(C) NMR Experiments. Samples for the NMR studies were prepared in 90% H_2O/10% ^2H_2O or in 100% ^2H_2O with

a final concentration of approximately 5 mM at pH 3.4. The pH was measured as a direct reading from a combination microelectrode calibrated at two reference pHs. All NMR experiments were performed in a phase-sensitive mode (States et al., 1982) using a Varian UNITY 500 spectrometer at two temperatures, 14 and 25 °C, in order to obtain unambiguous resonance assignment. The typical 90° pulse width used for ^{1}H was 7 μs except for the spin-locking period of clean TOCSY experiments in which it was adjusted to 16 μs (Griesinger et al., 1988). Solvent suppression was carried out using selective, low-power (approximately 60 Hz field strength) irradiation of the water resonance during the relaxation delay of 1.5 s (Han & La Mar, 1986). Solvent suppression was also applied during the mixing period in the case of the NOESY (Jeener et al., 1979) or ROESY (Bothner-By et al., 1984; Griesinger & Ernst, 1987) experiments. All peaks were referenced to a residual water signal (4.76 ppm at 25 °C). Mixing times of 80−400 ms for NOESY and of 80−150 ms for ROESY, respectively, were used. For TOCSY experiments, mixing times of 22−82 ms were applied.

In addition, the total of 11 $^3J_{HN\alpha}$ coupling constants for the backbone torsion angle was measured from phase-sensitive DQF-COSY experiments (Rance et al., 1983). In order to obtain $^3J_{\alpha\beta}$ coupling constants for the χ^1 torsion angle, the PE-COSY experiment (Muller, 1987) was performed in 100% ^2H_2O. The $^3J_{\alpha\beta}$ values were used in conjunction with the $d_{N\beta}$ to provide seven χ^1 torsion angles. Spectral widths were 5 kHz in both dimensions. Typical 2D data consist of 2048 complex points in t_2 dimension with 512 complex t_1 increments except for the DQF-COSY and PE-COSY experiments where these numbers were doubled to give a final digital resolution of 1.2 Hz in the F2 dimension after a zero-filling. In order to monitor presence of slowly exchanging NH protons a ^{1}H spectrum was obtained within 30 min after the fully protonated protein was dissolved in 100% ^2H_2O. All of the amide NHs exchanged rapidly and disappeared by the time the spectrum was obtained.

(D) Calculation of Structures. Following methods for calculation of structures, which are in principle similar to the published procedures (Davis et al., 1993; Pallaghy et al., 1993), were used using distance geometry (DGII) (Biosym Technologies Inc., San Diego, CA) based on the metric matrix. Interproton distance constraints were derived primarily from the NOESY spectrum recorded with a mixing time of 200 ms obtained at 14 °C. The FELIX (Biosym Technologies Inc., San Diego, CA) program was used for quantification of the NOE crosspeak volumes and for converting them into upper bounds of interproton distances. Interproton distances were input initially only when they are also clearly observable both in the NOESY and the ROESY spectra obtained with mixing times of 80−150 ms. Additional NOEs corresponding to long-range interproton distances were measured from the NOESY spectra recorded with a mixing time of 400 ms and used as upper distance restraints. Automatic pseudo-atom constraint corrections (Wüthrich et al., 1983) and floating chirality (Weber et al., 1988) for non-stereospecifically assigned protons are implemented in DGII. As a distance reference, volumes of the five non-overlapping geminal β-proton crosspeaks were averaged and correlated with the appropriate geminal distance of 1.8 Å. Such a calibration was found to yield distance ranges, in Å, for intraresidue and sequential interactions consistent with the theoretically expected ranges. In order to allow for volume integration errors and possible conformational averaging, 0.5 and 1.0 Å additions for constraints involving only backbone protons, and at least one side chain proton were used, respectively. Lower bounds for interproton distances were set to 1.8 Å. In addition to the interproton distance restraints, dihedral angle restraints were measured from $^3J_{HN\alpha}$ coupling constants, centered on −120° (±30°) for $^3J_{HN\alpha} > 8$ Hz and −60° (±30°) for $^3J_{HN\alpha} < 6$ Hz. Also included in the calculation were χ^1 torsion angles measured from the PE-COSY in combination with the sequential $d_{\alpha\beta}$ distances when the β methylene protons were stereospecifically assignable.

For the back-calculation of NOESY spectra using BKCALC (included in FELIX), a mixing time of 200 ms, a correlation time of 1 ns, and a *z*-leakage rate of 2 Hz with a distance cutoff of 7 Å were used. A total of three rounds of back-calculation were carried out. After each round of back-calculation, new distance constraints were generated from a given structure, compared with the experimental NOEs, and edited appropriately. The initial constraint set for the [Pro 7,13] αA-conotoxin P_{IVA} consisted of 275 interproton distances inferred from the crosspeaks that could be assigned unambiguously in both dimensions. Comparison of the back-calculated spectra of 12 best refined structures after the first round of refinement with the experimental spectra yielded 31 additional constraints and analysis of the back-calculated spectra after the second round of refinement yielded another 18 NOE crosspeak assignments. At the final stage of refinement the total number of NOEs, including 49 obtained by back-calculation, was 324, which consisted of 143 intraresidue distances, 148 short-range ($|i - j| < 5$) and 33 long-range ($|i - j| \geq 5$) inter-residue distances.

RESULTS

(A) Resonance Assignment. Complete ^{1}H resonance assignment for the [Pro 7,13] αA-conotoxin P_{IVA} was achieved by a combined use of DQF-COSY, TOCSY, and NOESY/ROESY following the sequential resonance assignment procedure (Billeter et al., 1982). Initial assignment of the spin systems to individual amino acids was done along the amide NH resonances. At this point unambiguous assignment of some amino acids such as 19R, 12H, and 17K were already possible taking the advantage of their back-transfer crosspeaks. Once the individual spin systems were classified according to the characteristic spin systems, sequential NOE crosspeaks were used to finalize the assignment procedure. At 14 °C, all of the sequential $d_{\alpha N}$ type crosspeaks were clearly visible allowing the sequential resonance assignment except for the 3C and 6Y, where the NH−C_αH correlation peaks were barely visible at this temperature due to the water resonance. Also for 18D, NH−C_αH crosspeak was completely buried under the solvent resonance at 14 °C. Data obtained at 25 °C were used for completing the assignment for these residues. Figure 1 shows the fingerprint region of a NOESY spectrum, and shown in Figure 2 is the summary of sequential NOEs used for resonance assignment along with $^3J_{NH\alpha}$. Small minor crosspeaks (approximately 10% of the major crosspeaks) were present in the spectra, which seem to originate from a *cis*-isomer due to the three prolines,7P, 13P, and the hydroxyproline (20 Hyp), in the [Pro 7,13] αA-conotoxin P_{IVA}. Resonance assignment and subsequent structure cal-

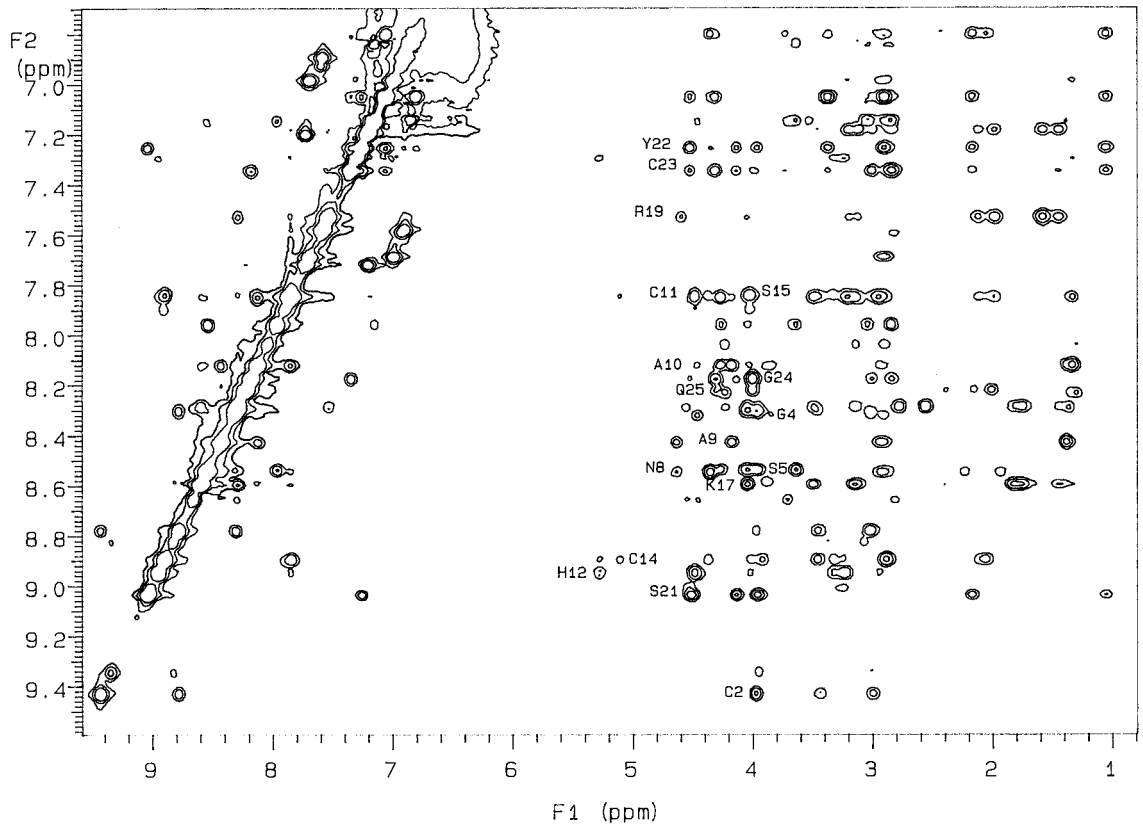

FIGURE 1: Portion of a NOESY spectrum of 5 mM [Pro 7,13] αA-conotoxin P_{IVA} obtained with a 200 ms mixing time in 90% H_2O/10% ^2H_2O, pH 3.4 at 14 °C. Selected residues are labeled along their amide NH resonances.

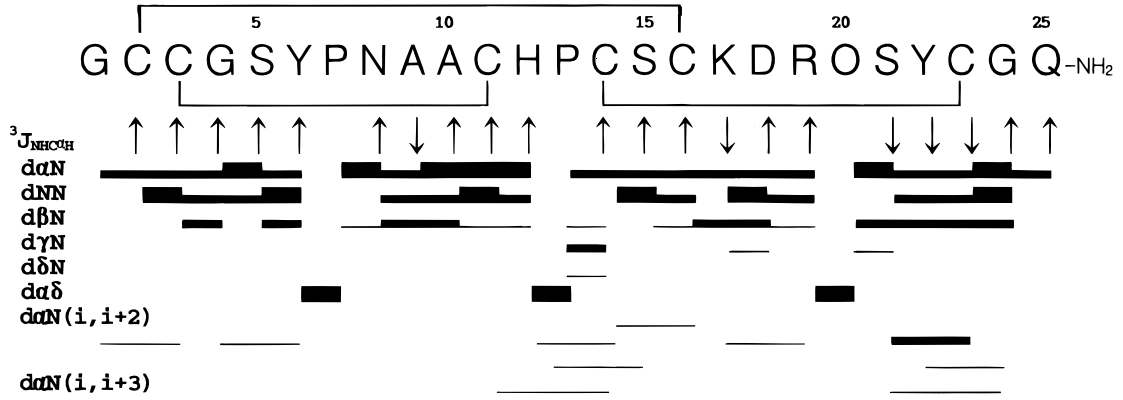

FIGURE 2: Sequence of the [Pro 7,13] αA-conotoxin P_{IVA} and a summary of short-range inter-residue NOEs obtained at 14 °C with a mixing time of 200 ms.

culations we report here are for the major form (*trans*-isomer) of the [Pro 7,13] αA-conotoxin P_{IVA} where all of the prolines show sequential $d_{\alpha\delta}$ type crosspeaks in the NOESY spectrum.

(B) Description of Structures. In Figure 3, the superposition of 12 best structures of the [Pro 7,13] αA-conotoxin P_{IVA} is shown. The overall shape of the [Pro 7,13] αA-conotoxin P_{IVA} can be described as an "iron" in which the "handle" of the iron is formed by the residues 15S-19R while the rest of the residues form the flat "bottom plate" of the iron with the C-terminus of the protein as the forwarding tip. The highly charged hydrophilic loop of 15S-19R which forms "handle" domain is exposed to the exterior of the protein. The N-terminus of the protein is located on the right-hand side of the bottom plate looking down the iron. The N-terminus is relatively immobile due to the attractive electrostatic interactions between the hydroxyl group of the 20 Hyp and that between the phenolic hydroxyl group of the 22Y. Typically, both N- and C-termini of a protein in solution are flexible, having large RMSD values although in some cases relatively immobile protein termini were observed (Clore et al., 1987; Han et al., 1996). The presence of hydroxyproline or proline in residues 7 and 13 does not affect the biological activity of the αA-conotoxin P_{IVA} (Hopkins et al., 1995).

The stability of the [Pro 7,13] αA-conotoxin P_{IVA} is primarily governed by the three disulfide bridges with very little stabilization contribution originating from the secondary structures. The presence of the three disulfide bridges imposes geometric constraints that force the molecule to form continuous bends. Six bends are formed involving the following residues: 3C-5S, 7P-10A, 12H-14C, 15S-17K, 17K-19R, and 21S-25Q. Among these, the two continuous bends, 15S-17K and 17K-19R, constitute the 15S-19R loop, the "handle" of the iron. One noteworthy observation is the

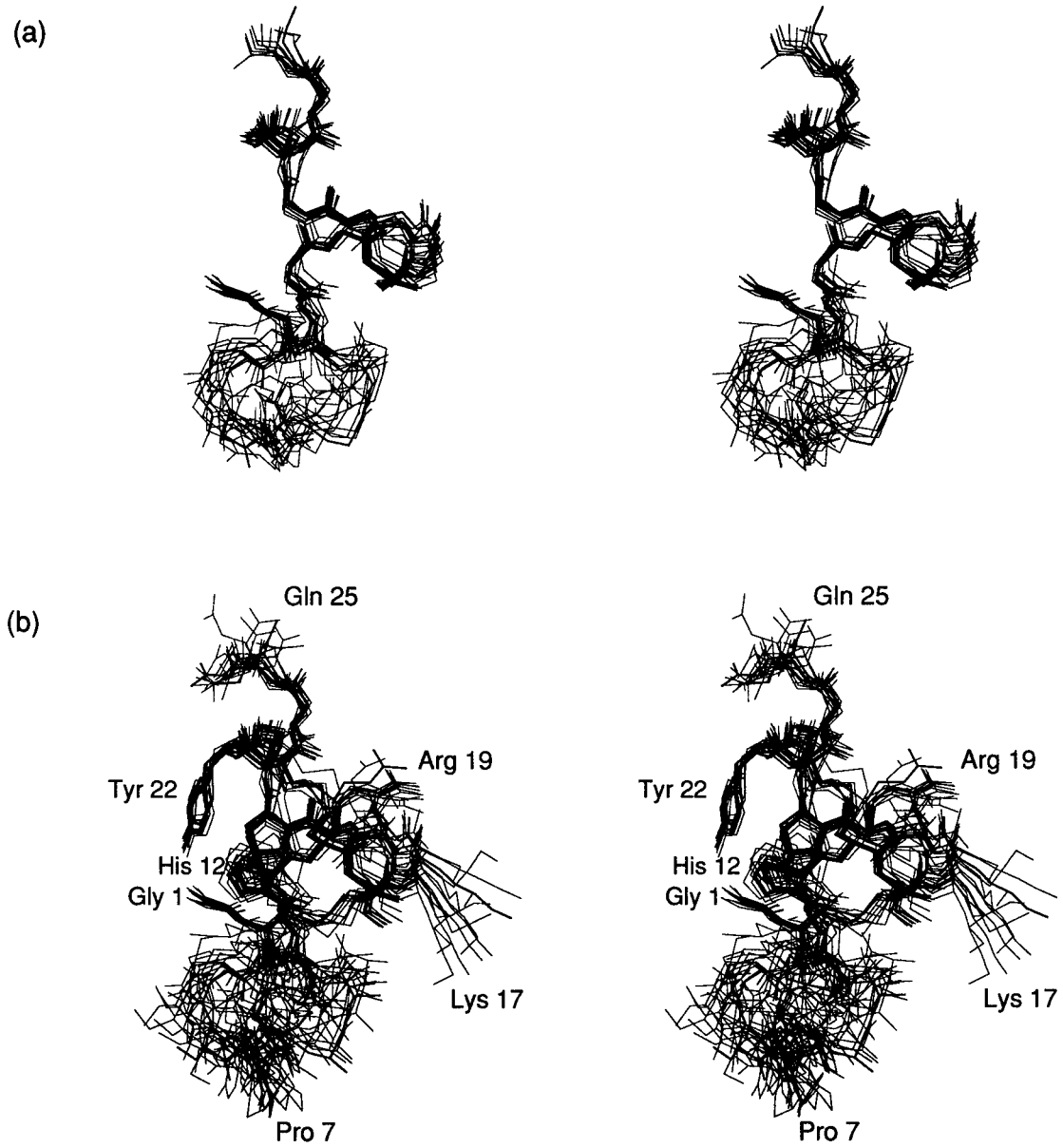

FIGURE 3: Superposition of the 12 refined structures of the [Pro 7,13] αA-conotoxin P_{IVA} (a) backbone only and (b) heavy atoms, respectively. The average structure is shown with a thick line, and selected residues are labeled.

relative orientation of the phenolic ring of the 22Y that lies in parallel with the "bottom plate" of the iron. Such an orientation is maintained because the phenolic hydroxyl group of 22Y is located within 5 Å from the hydroxyl group of the neighboring 20 Hyp and the positively charged N-terminus of the protein, enabling attractive electrostatic forces to pull the phenol ring of 22Y toward the bottom plate. Finally, the imidazole ring of 12H slightly protrudes out of the bottom plate in a direction opposite to the 15S-19R loop.

No appreciable amount of secondary structure is present in the [Pro 7,13] αA-conotoxin P_{IVA} as is also observed by the CD spectra and the H−D exchange experiments where all the backbone amide NHs have exchange half-life of less than an hour. One exception is the 3_{10} helix formed by the residues 21S-24G. The temperature dependence of the backbone amide protons is greater than 6 ppb/deg for most residues except for the two residues (15S and 23C) which show extremely small temperature dependence (1 ppb/deg). The presence of the 3_{10} helix formed by the 21S-24G is consistent with the prediction made by the STRIDE (Eisenhaber & Argos, 1993; Eisenhaber et al., 1995; Frishman & Argos, 1995) and by comparing the $C_\alpha H$ chemical shifts (Wishart et al.,1991). Such a result contrasts to the case of non-α type conotoxins such as ω-conotoxins G_{VIA} and M_{VIIC} which have a well-defined anti-parallel triple-stranded β-sheet with several slowly exchanging amide NHs (Davis et al., 1993; Pallaghy et al., 1993; Sevilla et al., 1993; Skalicky et al., 1993).

Figure 4a shows the summary of the distribution of the NOE restraints as a function of residue number, and Figure 4b shows the RMSD for individual residues. The angular order parameters (Hyberts et al., 1992; Thomas et al., 1991) are shown in Figure 4c−e. The overall RMSD for the backbone and the heavy atoms are 0.90 and 1.16 Å, respectively. In the final structures averaged distance violation for all restraints is 0.08 Å.

The quality of the NMR protein structures is also assessed by the *R*-factors (Thomas et al., 1991), which show the residual error between the real structure and the current model. Even though both R_a and R_b are calculated by summing over all well-resolved crosspeaks of the experimental spectrum, the R_a is more strongly influenced by the

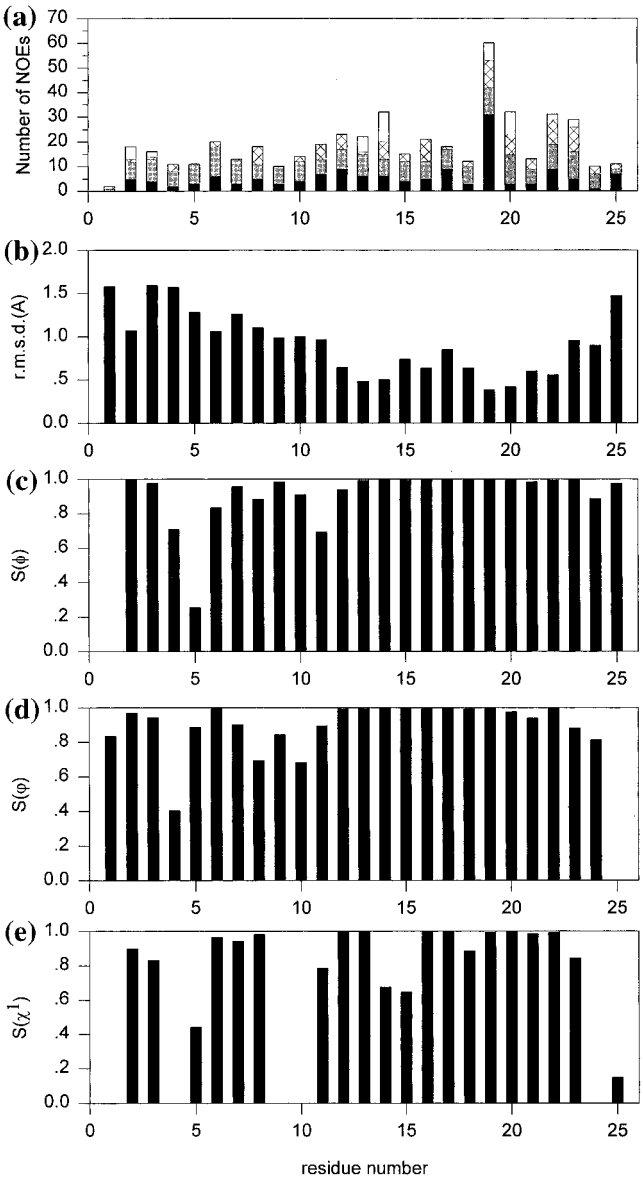

FIGURE 4: Plots as a function of the residue number of (a) the number of NOE constraints used in the structure refinement, (b) the average RMSD for the backbone heavy atoms, and (c−e) the angular order parameters for the backbone and first side chain dihedral angles.

short-range NOEs than the R_b. We have obtained values of 0.641 and 0.157 for R_a and R_b, respectively.

DISCUSSION

We have presented a high-resolution solution conformation of a novel conotoxin, [Pro 7,13] αA-conotoxin P_{IVA} purified from milked *C. purpurascens* venom. The presence of proline or hydroxyproline at positions 7 and 13 did not significantly affect biological activity (Hopkins et al., 1995). The structure of the [Pro 7,13] αA-conotoxin P_{IVA} described here is the third among the structures determined for the acetylcholine receptor-targeted conotoxins. The first was that of the α-conotoxin G_I obtained by NMR (Kobayashi et al., 1989; Pardi et al., 1989). The second was that of the α-conotoxin PnIA, recently determined by X-ray crystallography (Hu et al., 1996).

αA-conotoxin P_{IVA} is novel in several ways: first, its amino acid sequence is nearly twice as long as the α-conotoxins, which are typically less than 15 residues long (Table 1). Second, three disulfide bonds are present within αA-conotoxin P_{IVA} while there are only two in the α-conotoxins. Third, αA-conotoxin P_{IVA} exhibits no obvious sequence homology with other paralytic α-conotoxins from fish-hunting *Conus*, most of which share the consensus sequence $CC(X_1)PACG(X_2X_3X_4)SC$, where X_1 and X_3 are either N or H, X_2 is a residue with a long positively charged side chain such as R or K, and X_4 is aromatic residue Y or F. Thus, it is not apparent how the αA-conotoxin P_{IVA} manifests the same biological activity as the other α-conotoxins.

Two non-equivalent ligand binding sites exist within the acetylcholine receptor (Pedersen & Cohen, 1990; Middleton & Cohen, 1991; Sine & Claudio, 1991; Sine, 1993; Kreienkamp et al., 1994); one at the $\alpha-\gamma$ subunit interface and the other at the $\alpha-\delta$ subunit interface. For the acetylcholine receptor from the *Torpedo* electric organ, some ligands (Blount & Merlie, 1989; Groebe et al., 1995; Hann et al., 1994; Kreienkamp et al., 1992, 1994; Pedersen & Cohen, 1990; Utkin et al., 1994) preferentially bind to the former while the others (Martinez et al., 1995) bind preferentially to the latter. High-affinity binding to the $\alpha-\gamma$ subunit interface has been attributed to aromatic residues such as tyrosines of both α (Sine et al., 1994) and γ subunits (Sine, 1993; O'Leary et al., 1994), while negatively charged residues of the δ subunit (Czajkowski et al., 1993) have been shown to be important for binding of ligands to the $\alpha-\delta$ subunit interface. Three residues have been found that distinguish the high-affinity $\alpha-\gamma$ binding site from the low-affinity $\alpha-\delta$ binding sites (Sine et al., 1995). The conversion of the low-affinity $\alpha-\delta$ binding site to the high-affinity $\alpha-\gamma$ binding site was achieved by mutating these three residues in the δ subunit (Sine, 1993).

While the presence of aromatic and negatively charged loci within the acetylcholine receptor is believed to be important for ligand binding (Czajkowski et al., 1993; Sine, 1993; O'Leary et al., 1994; Sine et al., 1995) critical elements within the ligands for receptor binding are aromatic, hydrophobic, as well as some type of positive charged loci (Kobayashi et al., 1989; Pardi et al., 1989; Fraenkel et al., 1994; Fu & Sine, 1994; Sussman et al., 1991). Although the number of such loci and the physicochemical characteristics of each residue may vary from one ligand to the other, a perturbation of any of these loci produces a noticeable reduction in the biological activity. As an example, α-conotoxin G_I was proposed to possess two aromatics and two positive loci (Hider, 1985; Kobayashi et al., 1989). 11Y is one of the aromatic residues, and its replacement by a leucine resulted in 20-fold reduced potency of the toxin (Almquist et al., 1990). 9R is one of the two positive residues. A role of this arginine in the overall structure of the α-conotoxin G_I (Pardi et al., 1989) was noted. Affinity labeling study has also shown that the activity is critically dependent upon this residue (Florance et al., 1986). In fact, comparison of the known sequences for the α-conotoxins (Table 1) points out the importance of this arginine or, more generally, the positively charged residue at the X_2 position: S_I is only minimally active in mammals (Zafaralla et al., 1988), but S_{IA}, an analog of S_I with a lysine at the X_2 position, regains its activity in mammals.

As has been mentioned earlier, the usefulness of the αA-conotoxin P_{IVA} stems from the fact that it allows us to monitor the ligand binding sites within the acetylcholine receptor that cannot be probed using the other α-conotoxins.

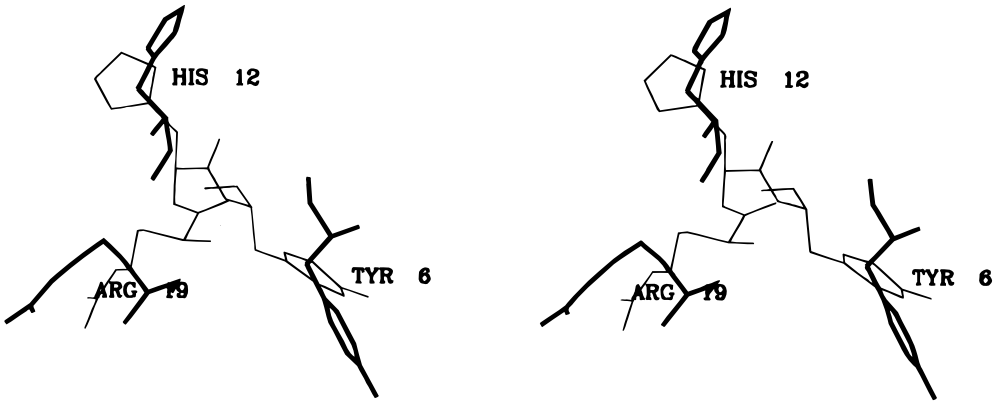

FIGURE 5: Superposition of the side chains of 9R, 10H and 11Y of the α-conotoxin G_I (Pardi et al., 1989) and those of 6Y, 12H, and 19R of the [Pro 7,13] αA-conotoxin P_{IVA}. The thick lines are for the latter.

On the other hand, lack of apparent sequence homology with the other α-conotoxins makes it difficult to understand how αA-conotoxin P_{IVA} could bind to the same acetylcholine receptor. Sequence comparison such as the above and affinity labeling as well as mutagenesis studies of the receptor do suggest that some aromatic and positively charged residues within the [Pro 7,13] αA-conotoxin P_{IVA} must be involved in receptor binding as in the other α-conotoxins.

Now, the high-resolution structure of the αA-conotoxin P_{IVA} provided by this investigation reveals how, at an atomic level, this novel conotoxin would bind to the acetylcholine receptor and compete against the other α-conotoxins and α-bungarotoxin. As can be seen Table 1, the specificity of binding to the receptor most strongly depends upon what types of residues are present at the positions X_2 and X_4. In the case of the α-conotoxin G_I these residues are 9R and 11Y, respectively. While the αA-conotoxin P_{IVA} does not have such residues at the neighboring positions as in the α-conotoxin G_I the two residues at the positions 19 and 6 are in fact R and Y. Figure 5 shows the superposition of the side chains of the 9R, 10H, and 11Y in the α-conotoxin G_I with those of the 19R, 12H, and 6Y in the [Pro 7,13] αA-conotoxin P_{IVA} when the backbone atoms of the three residues are aligned with an RMSD of 2.5 Å. Even though there are three high-resolution structures of the α-conotoxins available to date including this report, only two of these, one for α-conotoxin G_I and the other for the [Pro 7,13] αA-conotoxin P_{IVA} from the present work, are compared because the PnIA, whose crystal structure has been recently determined, is from a non-piscivorous *Conus*. In both molecules, the side chains of these residues point toward the same face, perhaps toward the ligand binding surface of the receptor, and form a triangle with the length of each side being in the range of 12−15 Å. We propose that such a triangular structural motif formed by these three residues is a "binding core" for the acetylcholine receptor targeting conotoxins. Such a motif is in agreement with the general view that the acetylcholine receptor targeting ligands possess aromatic as well as positively charged residues, positioned at an appropriate distance.

One interesting feature in the structure of the [Pro 7,13] αA-conotoxin P_{IVA} is the contrast between two parts of the molecule: the rigid C-terminal end which has a small backbone RMSD value of 0.56 Å and the relatively flexible N-terminal portion of the molecule with a corresponding RMSD of 1.09 Å. As shown in Figure 3, the latter may be equilibrating between a number of different conformations in solution. Such inherent flexibility of the N-terminal half of the αA-conotoxin P_{IVA} could be important for receptor binding. Upon interaction with macrosite residues on the receptor, this flexible region could be stabilized into a rigid conformation, resulting in conformational averaging between the flexible receptor-free solution structure and the bound conformation (Behling et al., 1988; Fraenkel et al., 1994). Conformational changes have also been observed in the α-conotoxin M_I, where at least two quasistable states equilibrate with each other in solution. In the case of the α-conotoxin G_I the rigidity of the C-terminus was found to correlate with its toxicity (Almquist et al., 1990). Conformational change of small peptidic ligands upon binding have been observed in other systems (Altschuh et al., 1992; Fesik et al., 1990, 1991; Neri et al., 1991; Weber et al., 1991). The issue of conformational flexibility or rigidity and its significance for receptor binding by peptides of the size of the α-conotoxins needs to be addressed further.

Ligand-receptor binding mechanism for the acetylcholine receptor is complex. Not only there are two ligand binding sites at the $\alpha-\gamma$ and $\alpha-\delta$ subunit interfaces with different affinities but also different types of ligands exhibit differential preference for the two sites (Blount & Merlie, 1989; Groebe et al., 1995; Hann et al., 1994; Kreienkamp et al., 1992, 1994; Martinez et al., 1995; Pedersen & Cohen, 1990; Utkin et al., 1994). Furthermore, such differential preference for the two ligand binding sites varies depending upon the origin of the receptor, *Torpedo* or mammlian (Groebe et al., 1995; Hann et al., 1994; Utkin et al., 1994). Although acetylcholine, *d*-tubocurarine, α-bungarotoxin as well as α-conotoxins all compete in the receptor binding assays, there is no direct physical evidence that these ligands with differing molecular sizes and physicochemical properties interact with the same amino acid residues of the receptor; the "macrosite" binding model (Olivera et al., 1991) suggests that different ligands could have different binding loci. The fact that mutation of the three residues within the receptor responsible for high-affinity ligand binding does not affect the binding of α-bungarotoxin (Sine, 1993) indicates that this α-neurotoxin with a much larger size than the common cholinergic ligands may not share the same overlapping binding loci with the small ligands. Indeed, even the possibility of allosteric interaction (Kreienkamp et al., 1992) cannot be eliminated. Large conformational change accompanying channel opening at a site distant from the ligand binding implies that more

than one type of ligand binding site could exist. Before an attempt is made to understand specific binding characteristics of individual acetylcholine receptor agonists or antagonists, much more detailed structural information along with the relevant biochemical data has to be available. This report constitutes only the first step in this direction. The αA-conotoxin P_{IVA} is the first of the αA-conotoxins which have been discovered in *Conus* venom. Two additional αA-conotoxins have been identified so far (J. S. Martinez and J. M. McIntosh, unpublished results). The solution structure of the αA-conotoxin P_{IVA} that is described in this report provides a framework for structure−function studies for the entire family of α-conotoxins.

SUPPORTING INFORMATION AVAILABLE

^{1}H resonance assignments for the [Pro 7,13] αA-conotoxin P_{IVA} at 14 °C and pH 3.4 (1 page). Ordering information is given on any current masthead page.

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