Refinement of the NMR Structures of α-Conotoxin MI Using Molecular Dynamics Simulation with Explicit Solvent Water and a Full Molecular Force Field

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Three NMR structures of α-conotoxin MI, a potent antagonist of the nicotinic acetylcholine receptor, have been refined using molecular dynamics (MD) simulation with explicit water. Although the convergence of the NMR structures of α-conotoxin MI was not sufficient to provide detailed structural features, the average structures obtained from MD simulations converged to one conformation, providing structural characteristics. The resulting structure was also found to be consistent with the results of amide proton-exchange experiments. These results demonstrate that MD simulation with explicit solvent water is very useful in refining NMR structures.

Key words molecular dynamics simulation; force field; solution structure; NMR; acetylcholine receptor; *Conus magus*

Molecular dynamics (MD) calculations with a simulated annealing (SA) protocol have been widely used to build up three-dimensional (3D) structures of biomolecules based on NMR data (NMR structures). In molecular modeling programs such as X-PLOR,[1)] the potential function used in such a calculation includes bond stretching, bond angle, dihedral angle, improper, van der Waals, and restraint terms. The restraint term is derived from experimental data; *e.g.* distance restraint based on the nuclear Overhauser effect (NOE). An electrostatic interaction is not included in order to reduce artifacts due to excessive charge–charge interactions, as the MD calculation is performed *in vacuo*. In addition, van der Waals interactions are usually included as a purely repulsive term. Therefore, the attractive forces for building up 3D structures are based only on restraint terms. As a result, a large number of restraints (above 10 per residue) is required to obtain high-resolution NMR structures.

We have previously used NMR spectroscopy to investigate the solution structure of α-conotoxin MI,[2)] which consists of 14 amino acid residues and which is a potent antagonist of the nicotinic acetylcholine receptor.[3,4)] The ^{1}H-NMR spectrum of α-conotoxin MI showed the presence of some conformers, including one major conformer with a population percentage greater than 80% and some relatively minor ones. Therefore, the NOESY spectrum was so complicated that we could obtain no more than 46 distance restraints, even for the major conformation. We tried the standard SA protocol of X-PLOR to calculate 3D structures with respect to the major conformation using a total of 48 restraints, including dihedral ones. Since the total number of restraints was relatively small (approximately 3.4 per residue), the convergence of the 10 obtained NMR structures was not sufficient to provide detailed structural features such as the presence of a β-turn. Then, for the best NMR structure with the lowest total X-PLOR energy value, an MD simulation with explicit solvent water was carried out. We expected that the hydrogen bonding interactions in solution could be deduced from this MD simulation, as it was carried out using a full molecular force field that includes electrostatic interactions. Finally, in our previous paper, we discussed the major conformation of α-conotoxin MI on the basis of results obtained from the MD simulation.

As described above, in our previous study, we performed only one MD simulation with explicit solvent water in order to discuss the major conformation of α-conotoxin MI. If several MD simulations using other NMR structures are performed, we may obtain different conformations from that obtained previously. In the present study, we carried out two additional MD simulations using other NMR structures in order to determine whether all three simulations, including the previous one, would provide similar results with respect to the major conformation of α-conotoxin MI. In addition, amide proton-exchange experiments were carried out at low temperatures, in order to identify the amide protons involved in hydrogen-bonding interactions. The results are compared with those of MD simulations.

Experimental

MD Simulation with Explicit Solvent Water All calculations were performed with AMBER version 4.1[5)] and its all-atom force field parm94.dat.[6)] Of the 10 NMR structures obtained in our previous study, the three with the lowest energy values with respect to the X-PLOR energy function were selected for the MD simulations. The one with the lowest energy value had been used in our previous study. Although this previous MD simulation was carried out only up to 200 ps, we further continued this up to 650 ps in the present study. The other two NMR structures were used for the first time in the present study. Each of these two structures was solvated by TIP3P waters in a rectangular box. The following procedure was performed for each system. After the systems were minimized until the RMS values of the potential gradient were below 0.5 kcal mol^{-1} Å^{-1}, 10 ps of MD on the solvent only was performed. This was followed by a second energy minimization until the RMS value of the potential gradient was below 0.05 kcal mol^{-1} Å^{-1}. Two 3-ps MDs at 100 K and 200 K were then performed, followed by an MD at 300 K with the NMR-derived restraints. We added potential functions for the NMR-derived restraints to the standard AMBER potential functions in order to create a trajectory to satisfy these experimental data more closely. The potential energy term was quadratic for the distance and the dihedral angle violations smaller than 3 Å and 30°, respectively, and was linear beyond these values. The force constants were 10 kcal mol^{-1} Å^{-2} and 50 kcal mol^{-1} rad^{-2} for the distance and the dihedral angle restraints, respectively. Simulations were run with SHAKE, a 1 fs time step, and constant pressure. A cut-off radius of 9 Å for the van der Waals and electrostatic interactions was applied. The interactions up to 14 Å were only calculated at every 10 steps, when the pair list was updated. The coordinates were recorded at every 100 steps.

Amide Proton-Exchange Experiments NMR experiments were performed on a Varian INOVA600 spectrometer operating at 600 MHz for the

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proton frequency. Lyophilized α-conotoxin MI was dissolved in the D_2O at pH 4.0 and 5 °C. TOCSY[7] spectra were recorded sequentially with time intervals of 40 min at 5 °C.

Results and Discussion

Figure 1A shows a Ramachandran-type plot of backbone ϕ and ψ dihedral angles of three selected NMR structures. As shown in this figure, their convergence as to the backbone conformation were so bad that we could identify no detailed characteristics of the major conformation of α-conotoxin MI. Our interest in the present study was to investigate whether an MD simulation with explicit solvent water could refine these structures. In Fig. 2, the time evolution of the root mean square (RMS) deviation between the initial structure, *i.e.* the NMR structure, and the trajectory structures recorded every 0.1 ps in the MD simulation are displayed for each of three MD simulations. Figure 2A shows an MD simulation using the best NMR structure, which consists of a trajectory up to 200 ps, as performed in the previous paper, and further calculations from 200 to 650 ps carried out in the present study. This simulation converged after approximately 100 ps. The average structure has been calculated for the last 200 ps, *i.e.* from 450 to 650 ps. This average structure was found to be almost identical to the one calculated for 100 to 200 ps used to discuss the major conformation of α-conotoxin MI in the previous paper. The new two MD simulations using other NMR structures were performed up to 800 and 850 ps, respectively. As shown in Figs. 2B and C, these two simulations converged after approximately 450 and 550 ps, respectively. Each average structure has also been calculated using the last 200-ps trajectory structures. Figure 1B shows a Ramachandran-type plot of three average structures obtained after the MD simulations. It is apparent that all three average structures have quite similar backbone conformations, in contrast to the three initial structures shown in Fig. 1A. These results strongly indicate that an MD simulation can effectively refine NMR structures. For example, the backbone dihedral angles of Cys8, which are far apart in the NMR structures (Fig. 1A), have converged to the α-helical region after carrying out the MD simulations (Fig. 1B). All backbone dihedral angles of Gly9 have also converged to the region where only a glycine residue can exist (Fig. 1B). Interestingly, although the backbone dihedral angles of Pro6 had almost converged to the β-sheet region in the NMR structures (Fig. 1A), the MD simulations placed them in the α-helical region (Fig. 1B). This change might indicate that the Pro6 backbone conformation of the NMR structures is not stable in the full molecular force field used for MD simulations. Figure 3A and B display the stereopairs of the three NMR structures and the three average structures obtained after the MD simulations, respectively. As indicated by arrows in Fig. 3, the conformation of the region of Cys8-Tyr12 has been especially refined. The average pairwise atomic RMSD value for the backbone atoms has also been improved from 0.80 to 0.72 Å by carrying out the MD simulations.

Figure 4A shows the 1D ^{1}H-NMR spectrum of α-conotoxin MI observed in D_2O at pH 4.0 and 5 °C. The assignment of amide protons with respect to the major conformation is given. Figure 4B shows the 1D ^{1}H-NMR spectrum recorded 20 min after dissolving α-conotoxin MI in the D_2O solution at pH 4.0 and 5 °C. The amide protons of His5, Cys8, Gly9, Asn11, Tyr12, and Ser13 can still be observed in the D_2O solution. These amide protons are considered to be involved in hydrogen-bonding interactions. Then, for each of the three MD simulations, a hydrogen-bond analysis over the last 200 ps was performed. In all three simulations, five hydrogen bonds, *i.e.*, CO (His5)–NH (Cys8), CO (Pro6)–NH (Gly9), CO (Cys8)–NH (Asn11), CO (Gly9)–NH (Tyr12), and NH (Ser13)–CO (Cys3), were identified. These hydrogen bonds are indicated in Fig. 3B. This result is consistent with those of the amide proton-exchange experiments, except for the amide proton of His5, strongly suggesting that the MD simulation provides more reliable solution structures with respect to the major conformation of α-conotoxin MI. We can now describe detailed characteristics of the major conformation of α-conotoxin MI with a high degree of confidence. The characteristic ϕ and ψ angles of Pro6, Ala7, and Cys8 indicate the location of the 3_{10} helix in this sequence portion (Fig. 1B), since the typical ϕ and ψ angles of the 3_{10} helix are ($-60°$, $-30°$). This result is supported by the presence of two hydrogen bonds, CO (His5)–NH (Cys8), and CO (Pro6)–NH (Gly9), as shown in Fig. 3B. The backbone conformation for residues Gly9-Tyr12 can be described as a type I β-turn, since the ϕ and ψ angles of the middle two residues, Lys10 and Asn11, are similar to those for the typical type I β-turn, *i.e.* ($-60°$, $-30°$) and ($-90°$, 0°), respectively (Fig. 1B). This result also suggested the presence of a hydrogen bond for CO (Gly9)–NH (Tyr12). Figure 5 provides a comparison of the major conformation of α-conotoxin MI with the high resolution X-ray structure of α-conotoxin GI,[8] which belongs to the same α-conotoxin family as α-conotoxin MI. The average RMS deviation between the X-ray structure of α-conotoxin GI and three averaged structures of α-conotoxin MI is 0.89 Å for the backbone atoms. The X-ray structure of α-conotoxin GI shows the presence of a 3_{10} helix for residues Pro5–Cys7 and a type I β-turn for residues Gly8–Tyr11. As described above, these secondary structures were also observed for the corresponding residues in α-conotoxin MI. These results obviously indicate that the two toxins exhibit a common molecular folding, as shown in Fig. 5A. This conformational similarity might explain why the profiles of the neurotoxic activities of the two molecules are almost identical. Interestingly, the ϕ and ψ angles for Gly8 of α-conotoxin GI are also located in the region not allowed for other residues as well as the case of Gly9 of MI (Fig. 5B). Therefore, these glycine residues can be expected to play an important role in the globular molecular folding of the two toxins.

With respect to the slow exchange of the His5 amide proton of α-conotoxin MI, we have obtained some relevant information from the X-ray structure of α-conotoxin GI. It displays the same hydrogen-bond interactions[9] with those of α-conotoxin MI described above. In addition, the X-ray structure of α-conotoxin GI demonstrates the possibility of a hydrogen-bond interaction between the amide proton of His4 and the sulfur atom of Cys7 because the distance between these two atoms is less than 3 Å. His4 and Cys7 of conotoxin GI correspond to His5 and Cys8 of conotoxin MI, respectively. We measured the distance between the amide proton of His5 and the sulfur atom of Cys8 in the MD simulations and found the average distance between the two to be approximately 5 Å for each of the three MD simulations. Be-

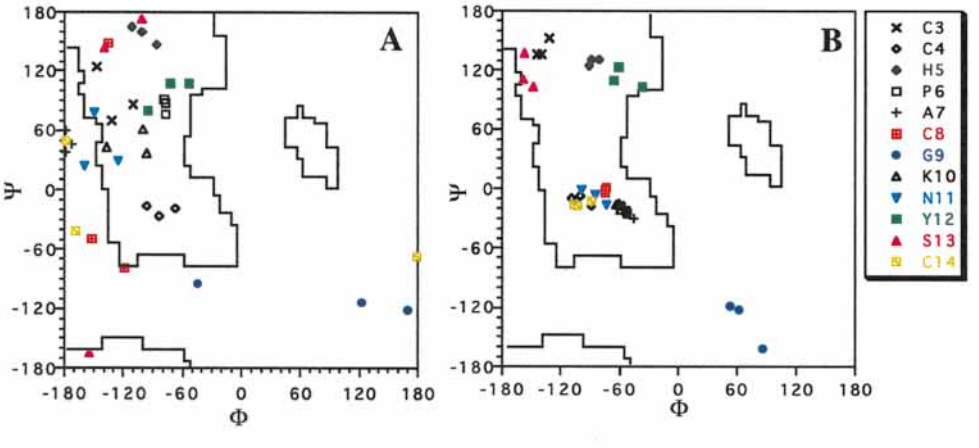

Fig. 1. Ramachandran Plots for the Residues from Cys3 to Cys14 of Three Selected NMR Structures (A) and Three Average Structures Obtained from MD Simulations (B)

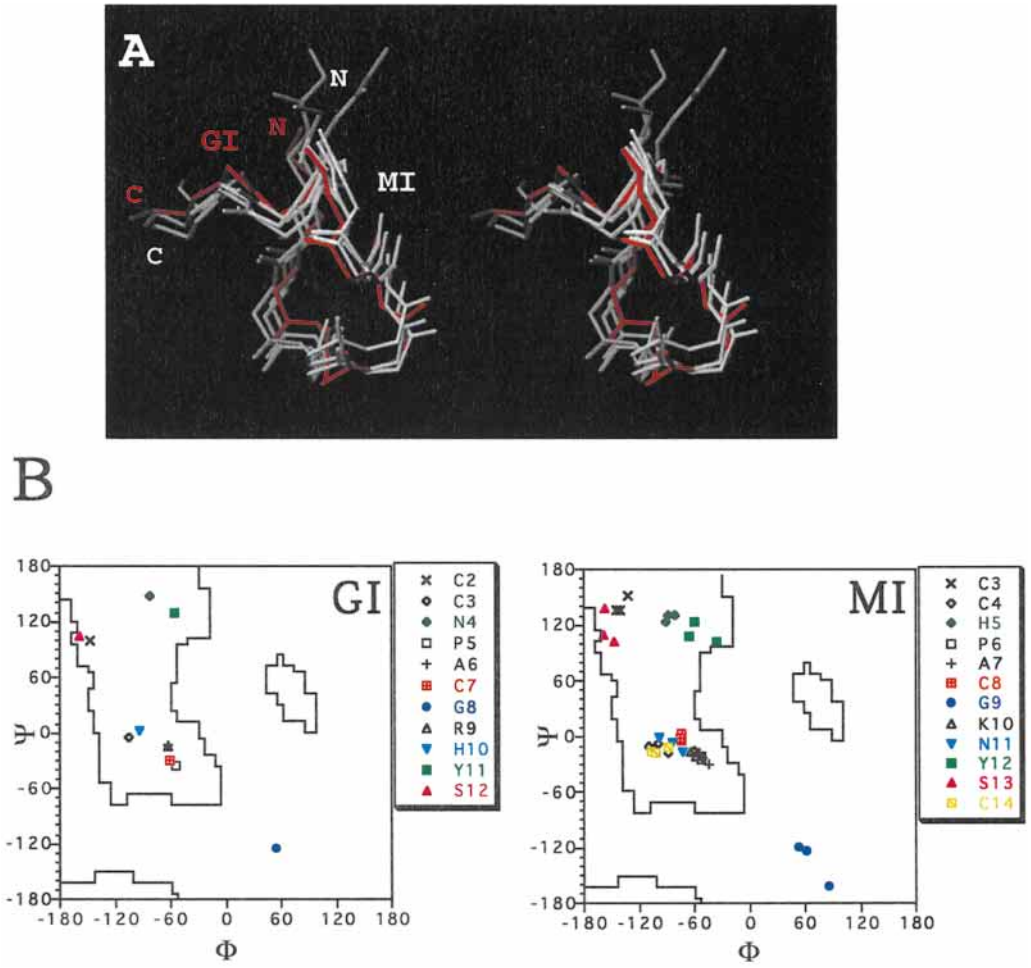

Fig. 5. (A) Stereopairs of the Superposition of X-Ray Structure of α-Conotoxin GI (Red) and Three Average Structures of α-Conotoxin MI Obtained from MD Simulations (White)

Backbone heavy atoms are shown.

(B) Ramachandran Plots for the X-Ray Structure of α-Conotoxin GI and Three Average Structures of α-Conotoxin MI Obtained from MD Simulations

cause this distance is not small enough to be considered a hydrogen bond, it appears that the MD simulations might be unable to produce this hydrogen-bonding interaction, possibly because the lone pairs on sulfur are not used in AMBER parm94 force fields, despite their importance in hydrogen bond directionality.[6] The present force field parameter for the sulfur atom in AMBER parm94 reflects the PDB analysis result, indicating that neutral sulfur works only extremely rarely as a proton acceptor in proteins.[6]

In conclusion, our results demonstrate that MD simula-

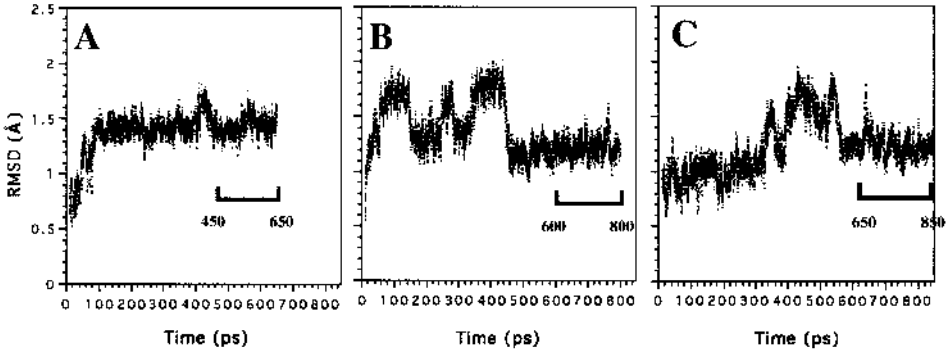

Fig. 2. Backbone Atom RMS Deviation from the Initial Structure as a Function of Simulation Time for Each of Three MD Simulations

Only amino acids 3—14 are taken into account. (A): For the best NMR structure. (B) and (C): For two new NMR structures.

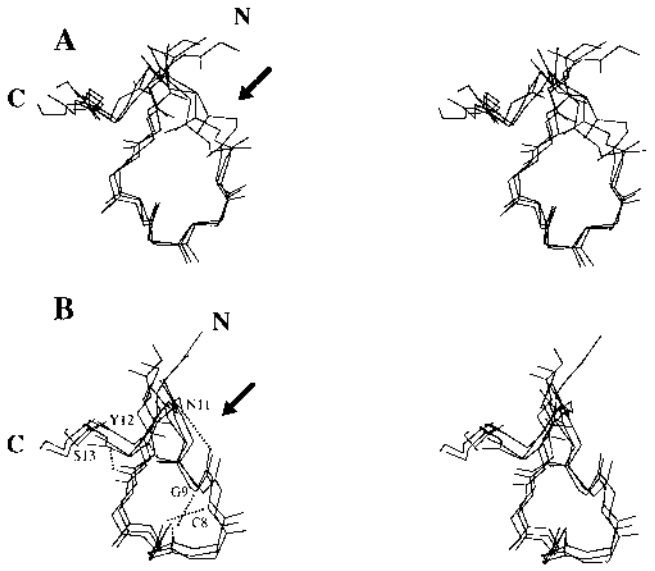

Fig. 3. Stereopairs of the Superposition of Three NMR Selected Structures (A) and Three Average Structures Obtained from MD Simulations (B)

Backbone heavy atoms are shown. Arrows indicate the region of Cys8–Tyr12. Five hydrogen-bonding interactions are displayed by dashed lines (B). Backbone nitrogen atoms of Cys8, Gly9, Asn11, Tyr12, and Ser13 are labeled (B).

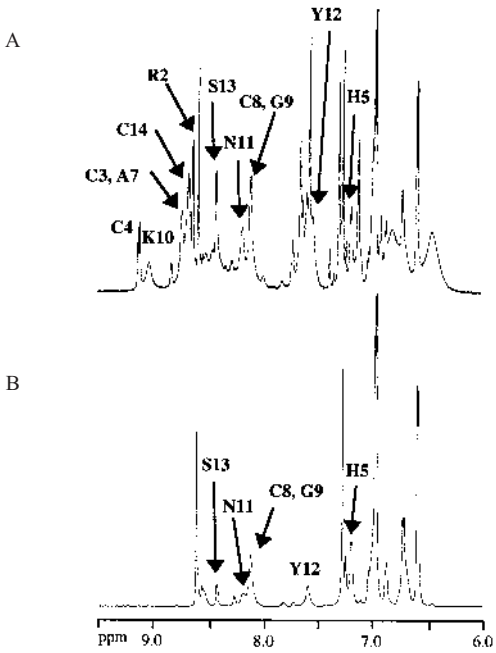

Fig. 4. (A) 1D ^{1}H-NMR Spectrum of α-Conotoxin MI Observed in D_2O at pH 4.0 and 5 °C

The assignment of amide protons with respect to major conformation is given.

(B) 1D ^{1}H-NMR Spectrum Recorded 20 min after Dissolving α-Conotoxin MI in the D_2O Solution at pH 4.0 and 5 °C

tions with explicit solvent water are very useful in refining NMR structures, as we could provide more reliable solution structures with respect to the major conformation of α-conotoxin MI. In addition, it is suggested from our study that a new advanced parameter for the sulfur atom might be necessary in order to produce hydrogen-bond interactions including sulfur atoms in MD simulation with explicit solvent water.

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9) The amino acid sequence of α-conotoxin GI: Glu–Cys–Cys–Asn–Pro–Ala–Cys–Gly–Arg–His–Tyr–Ser–Cys–NH_2. Two disulfide bonds are formed between Cys2 and Cys7 and Cys3 and Cys13. The X-ray structure of α-conotoxin GI shows the following hydrogen bonding interactions, *i.e.* CO (Asn4)–NH (Cys7), CO (Pro5)–NH (Gly8), CO (Cys7)–NH (His10), CO (Gly8)–NH (Tyr11), and NH (Ser12)–CO (Cys2).