The Atomic Resolution Crystal Structure of Atratoxin Determined by Single Wavelength Anomalous Diffraction Phasing*

By using single wavelength anomalous diffraction phasing based on the anomalous signal from copper atoms, the crystal structure of atratoxin was determined at the resolution of 1.5 Å and was refined to an ultrahigh resolution of 0.87 Å. The ultrahigh resolution electron density maps allowed the modeling of 38 amino acid residues in alternate conformations and the location of 322 of 870 possible hydrogen atoms. To get accurate information at the atomic level, atratoxin-b (an analog of atratoxin with reduced toxicity) was also refined to an atomic resolution of 0.92 Å. By the sequence and structural comparison of these two atratoxins, Arg33 and Arg36 were identified to be critical to their varied toxicity. The effect of copper ions on the distribution of hydrogen atoms in atratoxin was discussed, and the interactions between copper ions and protein residues were analyzed based on a statistical method, revealing a novel pentahedral copper-binding motif.

α-Neurotoxins are small three-finger proteins, which can specifically bind to acetylcholine receptors (AChRs) on the postsynaptic membrane and hence inhibit signal transmission across the synapse (1). Two groups of neurotoxins have been characterized in various snake venoms as follows: short chain α-neurotoxin with 60–62 amino acids and four disulfide bonds; and long chain α-neurotoxin with 65–74 amino acids and an additional disulfide bond at the C terminus. Crystal and NMR structures for both groups of α-neurotoxins indicate that they share high structural homology, with three finger-like loops protruding from the palm-like core (2, 3). Because of the potential pharmacological significance for this protein family (4), the binding activity of α-neurotoxins with their acetylcholine receptors has been intensively investigated based on the crystal structure of an acetylcholine-binding protein (AChBP) (5). These studies have shown that both long chain α-neurotoxin and short chain α-neurotoxin have binding activity with AChBP. The complex structural studies for α-bungarotoxin (a long chain α-neurotoxin) and its putative binding peptide in AChBP indicate that the second loop of α-bungarotoxin could insert into the interface of two adjacent subunits of the AChBP homopentamer to realize its binding activity (6, 7). Experimental data suggest that short and long toxins share a similar binding topology to nicotinic receptors (8). Although the interactions between short chain α-neurotoxin and its receptors (AChRs) have been assigned to the insertion of the second loop into the interface of the α7 homopentamer, the binding activity can vary greatly for various short chain α-neurotoxins differing only at several residues (9). No crystal structures to date explain such functional differences clearly, especially at atomic resolution.

In this study, we present atomic resolution crystal structures of two pharmacologically important proteins, atratoxin and atratoxin-b (a homolog of atratoxin but with lower toxicity) from the Naja naja venom. The atomic resolution crystal structure of atratoxin-b was refined to 0.92 Å from the previously reported (10) structure at 1.5 Å resolution determined by molecular replacement. However, the atratoxin structure could not be solved by molecule replacement because there are two planar atratoxin molecules in the crystallographic asymmetric unit. By using erabutoxin A (PDB code 5EBX, the same model used to phase the atratoxin-b structure) as the search model, it was not possible to identify solutions because of the high R factors (~53%), low correlation coefficients (~19%), and no clear contrast between possible solutions and noise. Considering that the protein was crystallized in the presence of copper ions, anomalous diffraction data to 1.5 Å resolution at the copper K-edge (λ = 1.379 Å) were collected in addition to an atomic resolution (0.87 Å) data set collected at a wavelength of 0.916 Å. By using a SAD phasing method developed jointly by the Institute of Physics, Chinese Academy of Sciences, and MacCHESS, Cornell University (11–13), the crystal structure of atratoxin was determined at 1.5 Å resolution and finally refined to an atomic resolution of 0.87 Å. This resolution is higher than any structure published to date on postsynaptic...
neurotoxins (14–23). It is also interesting to note that the correct handedness was determined from only the copper substructure with the program ABS (28). The comparison of these two atomic resolution structures shows clearly the essential residues and their configurations involved in the binding of atratoxin to its target, which could be of importance in protein engineering (24); the correlation between thermal motions of atratoxin at atomic resolution of its NMR structure was revealed by the comparison of the anisotropy of atratoxin and the r.m.s. deviation of multiple NMR structures. In addition, the co-crystallization of copper in the crystal of atratoxin not only aids the phasing of the structure from one wavelength anomalous diffraction data but also causes the dimerization of atratoxin molecules in crystals and could affect hydrogen distributions in the atratoxin. Furthermore, the toxicity of atratoxin was analyzed under the condition of Cu²⁺ binding, providing an alternative way to study the toxicity of the protein.

**EXPERIMENTAL PROCEDURES**

**Determination of Copper Content in Crystals**—In order to determine the ratio between the copper ions and the atratoxin molecule, four crystalline addition crystals were dissolved in 120 μl of 0.1 M HCl solutions. The $A_{400}$ value of the sample solution was obtained by using a DU640 nucleic acid and protein analyzer (Beckman Coulter). The concentration of the protein was calculated from the standard curve of the proteins. The concentration of copper ions was examined by using an inductively coupled plasma atomic emission spectrometer (Thermo Jarrell Ash Corp.). The sample was excited with nitric acid before loading onto the inductively coupled plasma atomic emission spectrometer.

**The Multimeric State of Atratoxin in Copper Solution**—To study the effect of copper ions on the multimeric state of atratoxin, 20-μl protein samples were loaded onto a Protein-Pak™ 300-μm 7.8 × 300-mm column, mounted on a Waters 600 system, pre-equilibrated with 0.02 M NaAc-HAc buffer, pH 4.6 (containing 0.15 M NaCl), and then eluted with the same solution. Various ratios of atratoxin to copper ions were achieved by using incremental protein concentrations of 1, 10, and 50 mg/ml. The monitor wavelength was 280 nm, and the flow rate was 0.5 ml/min.

**Protein Sequence and Gene Cloning**—The sequence of atratoxin was determined by the Edman degradation method as implemented on an Applied Biosystems Procise 491 protein sequencer. The N-terminal and C-terminal amino acid residues were sequenced by using protein from dissolved crystals of atratoxin.

The first strand cDNA synthesis and final amplification of two genes were obtained by using the access reverse transcriptase-PCR method. Typically, vaginal gland total RNA was isolated from the vaginal gland (from the southern mountain region, Anhui Province, China) with SV Total RNA Isolation System (Promega) according to the manufacturer’s protocol. Compared with other neurotoxins, the conservative sequence sites were found by using reverse transcriptase-PCR method. Compared with other neurotoxins, the conservative sequence sites were obtained and used to design two primers as follows: sense, 5'-AGGATAACCCCTCTAATGTC-3' (N terminus), and antisense, 5'-ATGTTGCAATCTGTTGTTGTC-3' (C terminus). PCR products were subcloned into the pGEM-T vector (Promega) and sequenced (Takara, Japan). According to the sequence, the 3'-RACE amplification was performed with the following primers: 5'-CACTACAAGTTGTGTCACGCTT-3' for atratoxin and 5'-AAAGGAAGCTCCGGCCCTGCTTATCT-3' for atratoxin-b. The 5'-RACE amplification was performed with the following primers: atratoxin, 5'-GTTTATTCTCTTTGTTGC-3', 5'-GCCATCTGTCCTGGGATATG-3', 5'-GCCGCGTTCGCTGGATCC-3', 5'-AGGGGATGTGGTTGCCCTTC-3', and 5'-GGCGATGATTGTTGGTTGTGAC-3'; atratoxin-b, 5'-GGTTTTCTCTCTCTATC-3', 5'-GGCTCCTCCTGGAACAGT-3', 5'-CGAAGGGGATGTTGTCCTGGG-3', and 5'-GGCCATGTTGTTGTCG-3'. 3'-RACE and 5'-RACE were used to determine the nucleotide sequence of the 5' and 3' end cDNAs with the Full Race Core Set cDNA amplification kit (Takara, Japan).

**Crystallography and Data Collection**—Atratoxin was purified and crystallized using procedures reported previously (25). Crystals of 0.8 × 0.8 × 1.6 mm were obtained from a protein solution containing 10% ethanol, 10 mM CuCl₂, and 25% (v/v) glycerol for about 20 s and then subjected to cryogenic flash freezing at a temperature of 100 K. Both SAD data and atomic resolution data were collected under the same cryoprotection conditions. The anomalous data set was collected at the MacCHESS F2 station. The x-ray wavelength was optimized to 0.379 Å (equivalent to 8.98 keV) to collect the strongest anomalous signal from copper as indicated by fluorescence scanning. A total of 360 frames was recorded with an oscillation angle of 1° from one crystal. Shortly afterward, the atomic resolution data set to 0.87 Å was collected at MacCHESS F1 station, at a fixed wavelength of 0.916 Å. Data processing for both anomalous data and atomic resolution data was performed with the HKL2000 package (26), and the statistics of data collection and processing are summarized in Table I. The $R_{merge}$ values for anomalous data and atomic resolution data were 14.0 and 6.2%, respectively, indicating that using a shorter wavelength, which usually means less absorption of x-ray to the crystal, may give better crystallographic statistics.

Atratoxin-b was purified and crystallized as reported before (10). Crystals of $0.3 \times 0.3 \times 0.2$ mm were soaked in crystallization mother liquid with 25% (v/v) glycerol for 20 s and then subjected to flash freezing at the temperature of 100 K for cryoprotection. The atomic resolution data of 0.92 Å were collected and processed at MacCHESS F1 station using a similar procedure as described above. Crystallographic statistics for atratoxin-b data are also listed in Table I.

**Phasing with SAD Data for Atratoxin**—To determine the structure of atratoxin from the anomalous scattering of copper atoms, a novel three-step phasing protocol was adopted, enabling the structure to be solved in few hours. The first step was to find substructures of these anomalous scatterers by a direct methods implementation (13). The second step was to find the substructure without trying both possibilities in the following steps. The last step was to resolve phase ambiguity arising from SAD (11, 12).

**Find Heavy Atoms Substructures with the Program SAPI**—The copper anomalous scattering sites were located by the direct methods program SAPI (15), using the magnitudes of the anomalous differences, for reflections up to 2.5 Å resolution. Three potential copper sites were found by SAPI in about half a minute with default running parameters. There was a clear gap between three copper atoms peaks and other peaks in terms of peak height. Two rounds of Karle-recycle calculation made the substructure converge to an $R$ factor of 31.3%, strongly indicating the correctness of the solution.

**The Progress of the Protein Phasing**—The arrangement of anomalous scatterers is non-centrosymmetric then it is also necessary to find their absolute configuration (hand). This is usually done by going through all phasing procedures with two possible configurations and manually choosing one that gives right-hand α-helices in the electron density map. The ABS program, based on the Ps function method (27, 28), has been written to determine the absolute configuration at a much earlier stage, i.e. immediately after the heavy atoms sites are found by a direct methods or a Patterson procedure. For these three copper coordinates, hand was correctly assigned by using anomalous data at about 3.0 Å resolution cut off. The calculation of ABS is very fast and can usually finish in less than a minute.

**Resolution Phase Ambiguity (SAD Phasing) and Improve Phases**—Initial phases for all reflections to 1.5 Å resolution were generated by using the program OASIS (11). The average figure of merit of phases was 0.54. Density modification using the CCP4 program DM (29) was then applied to the resulting phase set, and the average FOM was gradually improved to 0.65. Both electron density maps calculated using phases after OASIS and after OASIS/DM are traceable for the protein main chain. However, the OASIS/DM map gives better protein solvent contrast and clearer density.

**Model Building and Refinement of Atratoxin**—The main chain was built automatically by the program ARP/WARP (30) using diffraction data from 12 to 1.5 Å. The initial R factor was 41.2%. After 50 cycles of refinement, the content of water was reduced steadily from 0.62 to 0.90, and the main chain peptide was successfully traced. In total, 110 residues, pertaining to two chains, were found by the program. Except for a few disordered residues at the molecular surface and the loop area, it was very easy to recognize and to build almost all side chains based on both Fo and Fc maps. Incorrect hydrogen atoms were corrected using the program O (31). Resolution of the model was improved according to the cDNA sequence. Three peaks that appear on the difference map at the contour of 8σ corresponded to the anomalous scatterer peaks found by SAPI and were modeled as copper atoms. The crystallographic $R$ factor of the model built at this stage (including the dummy free atoms) dropped to 18.5%.

The atomic model was further refined with REFMAC (32) with additional restraints provided by REFMAC (32) with the constraints of a common environment (C-terminal residues). At the conclusion of refinement, the $R_{free}$ factor was 22.6% (Fo) and 24.0% (Fc) after solvent building. Then the diffraction data were trans-
formed to SHELX format, and the 5% validation reflections were kept. The refinement continued with SHELX97-2 (33) using conjugate gradient least squares minimization against an intensity-based residual target function that included distance, planarity, and chiral restraints. A bulk solvent correction (SWAT) was carried out. New ordered solvent molecules were found in the difference Fourier map. 2

\[ \text{Fo}/H11002 \] \[ Fc \] and \[ 2\text{Fo}/H11002 \] \[ Fc \] electron density maps were calculated after each step, and the model (protein and other molecules) was checked and rebuilt. The \( R \) and \( R_{\text{free}} \) factors calculated after the first run, with isotropic \( B \) factors, were 21.68 and 24.63%, respectively. Then the anisotropic displacement parameters were introduced into the refinement, reducing the \( R \) and \( R_{\text{free}} \) factors to 17.20 and 20.70%, respectively. At this stage, clear electron density was observed near several side chains, which were subsequently modeled in two conformations by the inspection of both \[ 2\text{Fo}/H11002 \] \[ Fc \] and \[ 2\text{F o}/H11002 \] \[ Fc \] maps. In relation to the side chain disorder, the main chain was also found to adopt two conformations in residues 31 and 32 of molecule A and residues 34 and 35 of molecule B. Two ethanol molecules were added in the subsequent refinement based on the Fourier difference map. Hydrogen atoms were added to the model according to geometrical criteria, decreasing the \( R \) and \( R_{\text{free}} \) factors by 0.5 and 0.9%, respectively. In the post-refinement, nine partial occupancy copper ions and two sodium ions were added, and the structure was refined to convergence again, leading to a final \( R \) factor of 11.96% \( R_{\text{free}} \) of 15.29% for all data in the resolution range 10–0.87 Å.

**Structural Refinement of Atratoxin-b**—For atratoxin-b, the 1.55-Å crystal structure of the same protein (PDB code 1onj) was chosen as the starting model. The refinement continued with SHELX97-2 (33) by using similar procedures as described for atratoxin. The final \( R \) factor and \( R_{\text{free}} \) factor of atratoxin-b after adding riding hydrogen atoms were 11.42 and 13.29%, respectively. Eight residues were modeled as having alternative conformations in the final structure. Other structural statistics are listed in Table I.

**RESULTS AND DISCUSSION**

**Protein Sequence and Gene Cloning**—The N-terminal sequence of intact atratoxin, determined by protein sequencing, is LECHNQQSSQTPTTTGCSSGGTNCYKKRR (this is a correction to the previously reported result (25)). The C-terminal fragment sequence of the enzymatically digested fragments of atratoxin is NGIEINCCCTTDRCNN. By reverse transcriptase-PCR utilizing the N-terminal and C-terminal sequence primers, two genes (atriatoxin and atratoxin-b) were obtained (Fig. 1). Based on the gene sequences, the primers for 3'- and 5'-RACE were designed, and amplification was performed. The cloned atratoxin cDNA is 470 bp in length, encoding a product of 83 amino acid residues. Sequence alignment reveals that the
peptide sequence of atratoxin is identical to that of cobrotoxin (34), but their mRNAs have only 97% identities. There is a peptide sequence of atratoxin is identical to that of cobrotoxin (34), and its protein sequence is the same as that of atratoxin. The overall folds of the two structures are similar. The r.m.s. deviations between C-a atoms of the crystal structure and 11 generated NMR structures range from 1.85 to 2.84 Å. The largest deviations in the NMR structure from the crystal structure generally involve the regions of loop I, loop II, loop III, and one turn (residues 18–22). The superposition of the backbone atoms of the x-ray structure and the mean solution structure is shown in Fig. 3A. Comparison of the backbone torsion angles between the mean solution structure and x-ray structure shows that residues 6, 9, 10, 18–20, 43, 45–48, and 58 have Q angles that deviate more than 60°. These residues are mainly located in the high B factor regions that have high flexibility. The agreement between the structures is best in the regions containing regular secondary structures and worst at the exposed loops and turns. The r.m.s. deviation of the first B-sheet consisting of strands 1 and 2 between the crystal structure of molecule A and the mean solution structure is 0.758 Å (0.756 Å for molecule B) and that of the second B-sheet consisting of strands 3–5 is 0.569 Å (0.754 Å for molecule B).

Atomic resolution data allow the modeling of atomic thermal motions by refining nine anisotropic displacement parameters. The anisotropy is defined as the ratio of the minimum and maximum eigenvalues of the 3x3 anisotropic displacement parameter matrix, where 1 is perfect isotropy and where extremely anisotropic atoms approach an anisotropy of 0. There are correlations between the anisotropy, temperature factor (B_eq), and thermal motions of the atom; the lower value of the anisotropy, the higher the B_eq and flexibility. Therefore, the thermal motions of protein atoms could be reflected by their anisotropy. Because the solution NMR structures of cobrotoxin are available, it is possible to study the flexibility of atratoxin by using the experimental data obtained independently. Fig. 3B shows a plot of anisotropy of the C-a atoms of atratoxin and atratoxin-b as a function of residue number. The highest anisotropy is seen at the loop between strands 2 and 3 and loop II (Fig. 3B), suggesting these regions are more flexible than others. It is interesting that the variations of anisotropy are consistent with the r.m.s. deviations of the 11 NMR solution structures (Fig. 3, B and C); the small anisotropy for B-sheets corresponds to the low r.m.s. deviations, and the highest anisotropy for the loop I, loop II, loop III, and regions between B2 and B3 corresponds to the highest r.m.s. deviations at the same regions.

Further analysis of the anisotropy of atratoxin by the program RASTEP (35) shows that the least anisotropy is at the center of atratoxin (as shown by blue spheres in Fig. 4). Anisotropy gradually increases from the center to the three finger loops as shown by ellipsoids (Fig. 4, red spheres indicate the highest anisotropy). Because more residues in the loop II of atratoxin molecule B have been modeled as alternate conformations, the thermal motion behavior of the loop II of molecule B is different from that of molecule A.

**Comparison with Crystal Structure of Atratoxin-b—**Atratoxin-b is a 61-residue α-neurotoxin with lower toxicity than atratoxin. Sequence alignment of atratoxin and atratoxin-b shows that only 13 residues in atratoxin-b are different from atratoxin (Fig. 1B), suggesting the functions of these various residues in control of their toxicity. The crystal structure of atratoxin-b was refined to 0.92 Å (Table I). The r.m.s. deviation
for C-α atoms between molecule A of atratoxin and atratoxin-b is 1.344 Å and that between molecule B of atratoxin and atratoxin-b is 0.995 Å. The largest differences between the two structures are at exposed loops and a turn (residues 18–22) (Fig. 3A). The tip residue of loop II between the two structures shows a large difference in its orientation (about 90°) (Fig. 5A). Another obvious difference is that residue 19 in atratoxin-b is missing (Fig. 1B), so the turn is shorter than atratoxin. The residue His91 in the crystal structure of atratoxin and the NMR structure of cobrotoxin lies in the energetically disallowed regions; however, His91 of atratoxin-b lies in the energetically favorable region with (Φ and Ψ) values of (−63.3 and −18.3°). The alternative conformations can be discerned clearly in the two atomic resolution structures. The residues adopting alter-
atomic resolutions in atratoxin-b also adopt alternative conformations in atratoxin. It seems that residues flexible in atratoxin-b are also flexible in atratoxin-b.

Affinity Activity of Atratoxin—Atratoxin has the same sequence as cobrotoxin purified from Taiwan N. naja atra (34) and NT1 purified from the venom of Naja kaouthia (9). Atratoxin-b has the same sequence as cobrotoxin-b (36) and NT3 (37). Lethal dose 50% (LD_{50}) is the quantity of pathogens required to cause lethal disease in half of the exposed hosts. In our previous study (10), it has been shown that atratoxin has higher lethal activity than atratoxin-b (LD_{50} for atratoxin and atratoxin-b was about 0.08 and 0.18 mg/kg, respectively). This result was consistent with the lower IC_{50} (the concentration of neurotoxin that reduces the muscle contraction by 50%) of 0.04 \mu g/ml observed for NT1 compared with that of 0.23 \mu g/ml for NT3 (9). There are 13 mutations in atratoxin-b from atratoxin.

Five of them (R28W, R30I, Y35I, R36I, and T37I) in atratoxin-b are also flexible in atratoxin. It seems that residues flexible in atratoxin-b also adopt alternative conformations in atratoxin. It seems that residues flexible in atratoxin-b are also flexible in atratoxin-b. Based on these experimental data, the functionally essential atomic structures at the proposed region (loop II). Structure comparison at loop II reveals that there is a significant conformational difference for the side chain of Arg^{34} between atratoxin and atratoxin-b (Fig. 5A).

The affinity experiments of erabutoxin-A (Ea) reveal Ser^{8}, Lys^{27}, Arg^{33}, and Lys^{47} important elements of cutaremimetic function (38, 39). Structural comparison of atratoxin with Ea also shows the biggest difference is the orientation of the side chain of Arg^{33} between atratoxin and atratoxin-b (Fig. 5A). In Ea, it points to the concave face almost perpendicularly. But in atratoxin, it points to the outside of loop II and almost parallel to the concave face. Superposition of the two \beta-sheets between Ea and atratoxin shows that the position of the C-\alpha of Arg^{33} deviates more than 5 \AA. It has been reported that the affinity of Ea binding to AChRs
rises when residue Ile$^{36}$ is replaced with Arg$^{36}$ (37), and NT1 (atratoxin) has higher affinity to AChRs than NT3 (atratoxin-b) (9). Residue 36 could be the factor affecting their affinity activity; Arg$^{36}$ in atratoxin contributes more to affinity than Ile$^{36}$ in atratoxin-b. Further inspection of the conformations of these two residues in the crystal structure suggests two possible structural explanations for their different affinities. First, Ile$^{36}$ is a hydrophobic residue that has weak interactions with the negatively charged AChR-binding site, whereas Arg$^{36}$ may enhance the ligand-AChR interactions through salt bridge or hydrogen bonds. Second, the substitution of Ile$^{36}$ to Arg$^{36}$ in atratoxin may also change the conformation of another positively charged residue Arg$^{33}$ and allow the loop II in atratoxin to enter deeply into the binding pocket. From Fig. 5A, one can see that the side chain of residues Arg$^{33}$ in atratoxin-b and Ea is very close to the side chain of Arg$^{36}$ in atratoxin after structural alignment. The side chain volume of arginine is larger than that of isoleucine so the volume of residue 36 increases when Arg$^{36}$ replaces Ile$^{36}$. Consequently, the side chain of Arg$^{33}$ moves away from residue 36 and points to the outside of loop II (Fig. 5A). Therefore, the side chain of Arg$^{35}$ in atratoxin moves away from residue 36 and points to the outside of loop II whereas that in erabutoxin-A points to the concave face. An experiment-based model of the α-neurotoxin-α7 receptor complex also shows that affinity benefits from the side chain of Arg$^{35}$ pointing to the outside of loop II (40). Therefore, the crystal structures suggest that residue 36 is critical to varied neurotoxin-AChR affinity.

It has been reported that the neurotoxin-binding sites in

| Motif | Pattern | No. | X                  |
|-------|---------|-----|--------------------|
| HHX   | Tri-coordinated | 107 | His, Cys (most); O, CL (rare) |
| HHCM  | Tetra-coordinated | 81  |                     |
| HHHH  | Tetra-coordinated | 30  |                     |
| HHX   | Tetra-coordinated | 61  | O, C_SG, NO2_O, D_OD1, CL |
| HHXX  | Tetra-coordinated | 27  | G_O, D_OD2, C_SG, Q_OE1, E_OE1, E_OE1, CL |
| HCCX  | Tetra-coordinated | 8   | E_O, M_SD |
| HHHHX | Penta-coordinated | 13  | CL, O               |
| HHHXX | Penta-coordinated | 17  | SO4, O, Y_OH, CL   |
| HXXXX | Penta-coordinated | 6   | G_N, E_OE1, O, Y_OH, ACY-O, C_SG |
AChR contain extensive negatively charged residues (5). The α-neurotoxins have many positively charged residues located at three loops, especially at the second loop, normally thought to be involved in affinity activity of neurotoxin. Above, we have demonstrated that these positive residues may enhance the binding activity through salt bridge and hydrogen bond interactions, and atratoxin shows higher affinity to AChR than atratoxin-b due to the substitution of residue Arg36. Apart from this residue, it has been noted that there are additional 12 mutations in atratoxin, which definitely play a part in stabilizing its crystal structure. As described above, both atratoxin and atratoxin-b share very similar secondary structures, and we cannot find many differences for these two molecules as a whole. However, by using structural models determined at atomic resolutions (0.92 Å for atratoxin-b and 0.87 Å for atratoxin), one can find the effect of sequence on structure in side chains because alternative conformations in the two structures could be modeled easily. In atratoxin, 19 residues of each molecule appear to have more than one conformation; in atratoxin-b, only 8 residues can be modeled as having alternate conformations. The map quality for atratoxin-b is better than that for atratoxin as shown by the appearance of hydrogen densities (Fig. 7). Based on the fact that the crystal structure of atratoxin-b is less flexible than that of atratoxin, and atratoxin shows higher affinity to AChR than atratoxin-b, it would be interesting to postulate the relationship between the toxicity of neurotoxin and its flexibility. By checking the distribution of these 19 alternative residues, it was found that almost all of them are located at the loop areas and the second finger (Fig. 5B). The flexibility of these side chains may help the formation and stabilization of the toxin-AChR complex because these flexible side chains could dock into the receptor protein more easily than rigid side chains do.

Copper Ions—The content of copper ions in the atratoxin structure agrees with the spectrophotometric results. The con-
centration of atratoxin was 0.269 mg/ml by comparison of the $A_{280}$ of the sample and the standard curve of atratoxin. 50 μl of sample solution was nitrated and then adjusted to 2.5 ml. The copper concentration in the sample and in the control was 0.1370 and 0.0032 μg/ml, respectively. Using these results, it can be calculated that there are 2.73 copper ions per atratoxin molecule in the dissolved crystal. This agrees well with the content of copper ions in the refined crystal structure, which is 2.77 Cu²⁺/atratoxin.

By surveying the Protein Data Bank, 299 structures were found to contain copper ions but with various chelation forms (Table II). From the table, it is obvious that most copper-associated proteins prefer to adopt tri-coordination to form an approximate plane or tetra-coordination to form distorted tetrahedral geometry. Compared with the above two chelation forms, a penta-coordinated binding motif is rare in copper-associated proteins structures. The crystal structure of atratoxin reveals a novel pentahedral copper-binding motif (Fig. 6) with copper interacting with a Glu, a His, an Asp/Asn, an Arg residue (for Cu¹ and a water molecule for Cu² and Cu³), and one chloride ion (for Cu¹ and a water molecule for Cu² and Cu³). The binding interaction of the pentahedral motif is very strong with a mean bond length of 2.06 Å.

The Cu²⁺ binding activity of the protein can further influence its binding property to its receptor. Analysis shows that copper does not exist in crude venom. Cu²⁺ in solution does not affect the aggregation of atratoxin (Fig. 7), and atratoxin remains in the form of a monomer. After the addition of copper ions to the atratoxin solution, there was no obvious effect on the aggregation form of atratoxin, and there was no obvious change on the LD₅₀ value using the reported experimental procedure (41). LD₅₀ of atratoxin without Cu²⁺ was about 65 μg/kg, and it hardly changed in Cu²⁺ solution (LD₅₀ = 72.8 μg/kg in 1 mM Cu²⁺ solution). Although chelated copper ions were found in the crystal structure, the copper ions may only bind to the surface of atratoxin in solution. Based on the experimental data and the structure, it could be inferred that residues closely bound with Cu²⁺ are not closely related to the toxicity of the protein. Mutation experiments of Ea (38, 39) have shown that F4A mutant has no effect on the affinity of Ea. It is possible that residue 4 in atratoxin does not contribute to its function. However, D31H, F29L, and E38Q mutants of Ea have been shown to result in decreased binding affinity. In the atratoxin crystal, Asp⁶⁴, His⁶⁴, and Glu⁸⁸ bind copper ions closely. The LD₅₀ of atratoxin with and without Cu²⁺ shows no obvious difference, which agrees with the explanation that His⁶⁴ and Glu⁸⁸ may play little functional role but are important for retaining the local conformation that is necessary for toxicity (39). Therefore, copper ions can only bind to the surface of atratoxin in solution and do not affect its toxicity. The binding of surface residues (Glu², His⁴, His³², Glu³⁸, Asp⁵⁸, Arg⁵⁹, and Asn⁶²) (Fig. 6) to copper ions suggests that these residues may not be the key residues involved in the toxicity of atratoxin.

**Hydrogen Atoms and Hydrogen Bonds**—Ultrahigh resolution diffraction data allowed a high percentage of the hydrogen atoms to be recognized on $F_o - F_c$ difference maps. In the case of atratoxin, a total of 317 hydrogen atoms, including 125 main chain (48% of the possible 262) and 192 side chain hydrogen atoms (32% of the possible 608), could be discerned on difference maps contoured at 1.6σ. All of these peaks had a distance of 1.0 < 3 Å to the nearest non-hydrogen atom. There were 73 and 52 peaks observed for the electron density of the hydrogen atoms riding on the main chain Cα and N atoms, respectively.

By comparison of the hydrogen atoms between the 0.87-Å structure of atratoxin and the 0.92-Å structure of atratoxin-b (Table III), it can be seen that there is a large difference in the number of resolved hydrogen atoms riding on the main chain nitrogen atoms of the two structures. The completeness of hydrogen atoms in atratoxin is one-half that in atratoxin-b, although the average σ value used to find the hydrogens in atratoxin-b is higher than that in atratoxin. Moreover, the completeness of hydrogen atoms of Cα and side chain atoms in atratoxin-b is also higher than atratoxin even with a higher contour level. Although atratoxin was determined at a higher resolution (0.87 Å), which should favor the hydrogen observation, atratoxin-b in fact shows higher hydrogen completeness at 0.92 Å resolution, i.e. the higher resolution of the atratoxin data does not help the observation of hydrogen atoms when compared with the case of atratoxin-b. We also found that the average Wilson B factor for atratoxin and atratoxin-b was 7.32 and 6.52 Å², respectively, suggesting that atratoxin is more flexible than atratoxin-b. This conclusion could be further supported by the fact that there are more residues in atratoxin having alternate conformations than those in atratoxin-b (19 residues in each atratoxin molecule and 8 residues in atratoxin-b). Therefore, there may exist a relationship between hydrogen completeness and the flexibility of the protein; the completeness of hydrogen atoms observed at the same atomic resolution is related to the flexibility of the structure, i.e. the more resolvable the hydrogen atoms, the less flexible the structure.

In short chain neurotoxin structures reported previously, three-strand β-sheets are bound firmly by H-bonds, including six H-bonds between strand 3 and strand 4 and six H-bonds between strand 3 and strand 5 (42). In the atomic resolution structure of atratoxin, the hydrogen atoms could be discerned on $F_o - F_c$ difference maps. Six H-bonds ($σ > 1.1$) were found between strand 3 and strand 5, which is in agreement with the previous report. However, only 5 H-bonds ($σ > 1.1$) could be discerned between strand 3 and strand 4. The hydrogen bond between Lys⁶⁷ N and Glu⁸⁸ O was reported in erabutoxin structure (42). This can be confirmed by the atomic resolution structure of atratoxin-b (PDB code 1VB0) without copper. In

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**TABLE III**

| Linked atom name     | Mean distance (Å) | σ    | No. | Completeness (%) |
|----------------------|-------------------|------|-----|------------------|
| Cα atoms of molecule A | 1.07              | 2.5  | 38  | 61               |
| Cα atoms of molecule B | 1.12              | 2.5  | 35  | 56               |
| Cα atoms in atratoxin-b | 1.02              | 2.7  | 47  | 77               |
| N atoms of molecule A | 0.99              | 2.4  | 28  | 47               |
| N atoms of molecule B | 0.95              | 2.2  | 24  | 41               |
| N atoms in atratoxin-b | 0.95              | 2.7  | 46  | 81               |
| Side chain hydrogen in molecule A | 1.08 | 2.5  | 94  | 32               |
| Side chain hydrogen in molecule A | 1.08 | 2.4  | 98  | 33               |
| Side chain hydrogen in atratoxin-b | 1.05 | 2.5  | 128 | 42               |

a σ means the average contour level used in the $F_o - F_c$ difference map to identify hydrogen atoms.
At this structure, the hydrogen atom of the corresponding residue can be discerned (Fig. 8). However, in atratoxin, the hydrogen atoms of Lys$^{27}$ N in molecule A and B could not be found. The disappearance of this hydrogen may correlate to the Cu$^{2+}$ that chelates Glu$^{38}$ O-H. The electron of Glu$^{38}$ O-H was absorbed by Cu$^{2+}$ so an electron in residue Glu$^{38}$ may be transferred to compensate. The electron transfer reduces the electronegativity of Glu$^{38}$ O, which causes an unstable hydrogen bond interaction between Lys$^{27}$ N and Glu$^{38}$ O and failure to observe the hydrogen density in atratoxin.
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The Atomic Resolution Crystal Structure of Atratoxin Determined by Single Wavelength Anomalous Diffraction Phasing
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