for a relatively strong NOE between these protons and a second NH proton, for which such a situation could also give rise to an HRNOESY cross-peak linking these two NH proton resonances. For none of the HRNOESY cross-peaks of α-bungarotoxin has there been any ambiguity of this nature, although in some cases the β-proton to NH HOHAHA cross-peaks was relatively strong.

In conclusion, the HRNOESY experiment has proven quite useful in resolving assignment ambiguities for NOESY cross-peaks involving α-proton resonances which overlap with other resonances. In particular, for α-bungarotoxin it has made possible the assignment of 13 NOESY cross-peaks not assignable previously due to overlap, even after taking advantage of the NH chemical shift temperature dependence. Only one of these NOESY cross-peaks was due to a nonsequential NOE. The sensitivity improvement and reduction of diagonal peak dispersion components, as compared to the original relayed NOESY experiment, have made this experiment more useful for obtaining resonance assignments in larger proteins, where coincidence of chemical shifts will occur more often. HRNOESY is especially useful for those proteins containing β-sheets where the NH to α-proton NOEs and J couplings are favorable for the regular appearance of relayed cross-peaks.

Registry No. α-Bungarotoxin, 11032-79-4.

REFERENCES

Aue, W. P., Bartholdi, E., & Ernst, R. R. (1976) *J. Chem. Phys. 64*, 2229.

Basus, V. J., Billeter, M., Love, R. A., Stroud, R. M., & Kuntz, I. D. (1988) *Biochemistry* (first paper of three in this issue).

Bax, A., & Freeman, R. (1981) *J. Magn. Reson. 44*, 542.

Bax, A., & Davis, D. G. (1985a) *J. Magn. Reson. 63*, 207.

Bax, A., & Davis, D. G. (1985b) *J. Magn. Reson. 65*, 355.

Billeter, M., Braun, W., & Wüthrich, K. (1982) *J. Mol. Biol. 155*, 321.

Braunschweiler, L., & Ernst, R. R. (1983) *J. Magn. Reson. 53*, 521.

Davis, D. G., & Bax, A. (1985) *J. Am. Chem. Soc. 107*, 2821.

Eich, G., Bodenhausen, G., & Ernst, R. R. (1982) *J. Am. Chem. Soc. 104*, 3732.

Hartmann, S. R., & Hahn, E. L. (1962) *Phys. Rev. 128*, 2042.

Klevit, R. E., & Drobny, G. P. (1986) *Biochemistry 25*, 7770.

Kosen, P. A., Finer-Moore, J., McCarthy, M., & Basus, V. J. (1988) *Biochemistry* (third paper of three in this issue).

Marion, D., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967.

Wagner, G. (1984) *J. Magn. Reson. 57*, 497.

Wüthrich, K., Wider, G., Wagner, G., & Braun, W. (1982) *J. Mol. Biol. 155*, 319.

Structural Studies of α-Bungarotoxin. 3. Corrections in the Primary Sequence and X-ray Structure and Characterization of an Isotoxic α-Bungarotoxin†

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ABSTRACT: The most plausible set of chemical shift assignments for α-bungarotoxin as deduced from the combined use of two-dimensional J-correlated and two-dimensional nuclear Overhauser effect ^{1}H nuclear magnetic resonance (NMR) spectroscopy was in conflict with the accepted amino acid sequence between residues 8 and 12 and residues 66 and 70 [Basus, V. J., Billeter, M., Love, R. A., Stroud, R. M., & Kuntz, I. D. (1988) *Biochemistry* (first paper of three in this issue)]. Furthermore, NMR spectra of α-bungarotoxin, purified by conventional methods, evidenced a second species at the level of approximately 10% total protein. The minor component was separated from α-bungarotoxin by Mono-S (cationic) chromatography. Sequencing of Mono-S-purified α-bungarotoxin and one of its tryptic peptides showed that the correct sequence for α-bungarotoxin is Ser-Pro-Ile at positions 9–11 and Pro-His-Pro at positions 67–69. The electron density map of α-bungarotoxin [Love, R. A., & Stroud, R. M. (1986) *Protein Eng. 1*, 37] was refined with the new sequence data. Improvements in the structure were found primarily for residues 9–11. Sequence analysis of two overlapping tryptic peptides proved that the minor species differed from α-bungarotoxin by replacement of a valine for an alanine at position 31. This new toxin, α-bungarotoxin$^{(Val\text{-}31)}$, binds to the acetylcholine receptor with an affinity that is comparable to that of α-bungarotoxin.

α-Bungarotoxin is a 74-residue protein belonging to the homologous family of long (snake) neurotoxins [reviewed by Karlsson (1979), Low (1979), Bystrov et al. (1983), and Dufton and Hider (1983)]. Because these neurotoxins are readily available and because they bind tightly to the nicotinic acetylcholine receptor of vertebrate muscle and electric eel organ, α-bungarotoxin and others of this toxin family have been extensively utilized in physiological studies directed at understanding neuromuscular transmission in general and in biophysical studies directed at the structure and mechanism of the acetylcholine receptor [e.g., Changeux et al. (1970), Albuqueruqe et al. (1979), Bystrov et al. (1983), and Surin et al. (1983)]. In the future, a toxin–receptor complex may find use as a direct model system for high-resolution membrane protein–protein structural studies.

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A more complete understanding of the functional role of α-bungarotoxin would be greatly aided by knowledge of its structure. Recently, the crystal structure of α-bungarotoxin was solved to 2.5 Å (Love & Stroud, 1986). However, it proved difficult to obtain crystals suitable for X-ray crystallography. Also, the X-ray structure differed significantly from both the crystal and solution structures of other homologous neurotoxins (Walkinshaw et al., 1980; Endo et al., 1981; Inagaki et al., 1982; Bystrov et al., 1983; Dufton & Hider, 1983; Saenger et al., 1983) and from a partial analysis of the solution structure of α-bungarotoxin (Endo et al., 1981; Inagaki et al., 1985).

A complementary approach to crystallography, developed within the last 5 years, is high-resolution two-dimensional ^1H NMR[1] spectroscopy [reviewed by Wüthrich (1986)]. As an initial step toward solving the complete solution structure by NMR spectroscopy, the assignment of all α-bungarotoxin backbone and most of the side-chain proton resonances has been completed, and a large number of through-space connectivities have been collected by 2D nuclear Overhauser spectroscopy (NOESY) (Basus et al., 1988). During the course of this work, it became apparent that the published sequences between residues 8 and 12 and residues 66 and 70 [Mebs et al. (1971, 1972); a sequential numbering system is used in this series of papers] were inconsistent with the observed NOESY cross-peaks when the procedures of Wüthrich and colleagues for assignment of sequence connectivities were followed (Billeter et al., 1982; Wagner & Wüthrich, 1982; Wüthrich et al., 1982, 1984). In this paper we provide evidence, both spectral and chemical, that the correct sequence of α-bungarotoxin is Ser-Pro-Ile at positions 9–11 and Pro-His-Pro at positions 67–69. Additionally, a newly identified isotoxin, which we named α-bungarotoxin$^{(Val\text{-}31)}$, comigrated with α-bungarotoxin when classical chromatography techniques were used for the purification of α-bungarotoxin.

Materials and Methods

Materials. Lyophilized snake venom from *Bungarus multicinctus* was obtained from Miami Serpentarium laboratories and from Sigma Chemical Co. Most of the work described in this paper utilized the venom obtained from Sigma Chemical Co., except for the spectrum shown in Figure 1. TPCK-treated trypsin was obtained from Sigma Chemical Co. Bovine pancreatic trypsin inhibitor was purchased from Mobay Chemical Corp. Constant-boiling HCl was a product of Pierce Chemical Co. HPLC-grade H_2O and TFA were products of Aldrich Chemical Co. Iodoacetic acid was recrystalized from benzene.

Purification. α-Bungarotoxin was purified according to Love and Stroud (1986) except that Sephadex CM-25, not Whatman CM-52 cellulose, was used. The protein preparation was desalted over Sephadex G-25 in 0.5% ammonium bicarbonate, pH 7. Following lyophilization, the preparation was further purified by chromatography on the Pharmacia Mono-S 10/10 preparative system with a 30–50% gradient of 2 M ammonium acetate, pH 4, at a flow rate of 0.8 mL/min for 53 min. Starting conditions were 50 mM in ammonium acetate, pH 4. α-Bungarotoxin and α-bungarotoxin$^{(Val\text{-}31)}$ were separated under these conditions; each was desalted over Sephadex G-25 as above and stored in lyophilized form at −20 °C.

Peptide Mapping and Sequencing. Protein (2.5×10^{-4} M) was dissolved in 0.2 M Tris-HCl, pH 8.7, 6 M guanidine hydrochloride, 10 mM ethylenediaminetetraacetic acid, and 0.015 M dithiothreitol. The reduction of protein disulfides by dithiothreitol proceeded for 0.5 h and was followed by the addition of 0.15 volume of 0.5 M iodoacetic acid, 0.05 M KOH, and 0.25 M Tris-HCl, pH 6.8, to alkylate the thiols. After an additional 0.5 h in the dark, the S-carboxymethylated protein was desalted using Sephadex G-25 in 0.01 M HCl. Part of the S-carboxymethylated protein was used for amino acid analysis and the remainder for tryptic digestion. Additionally, in the case of α-bungarotoxin, the S-carboxymethylated protein was submitted directly for sequencing of the amino-terminal portion of the protein.

For tryptic digestion, the S-carboxymethylated protein was dissolved in 0.1 M ammonium bicarbonate, pH 8.2, at a concentration of 1 mg/mL, and 1% by weight of TPCK-treated trypsin added. After 2 h at 37 °C, an additional 1% trypsin was added. At the end of 2 h, the reaction was terminated by lyophilization. Peptide digestion mixtures were dissolved in 0.1% TFA/HPLC-grade H_2O and separated on the Pharmacia PEP RPC HR 5/5 system with a 15–25% gradient of 0.1% TFA/HPLC-grade acetonitrile at a flow rate of 0.4 mL/min for 15 min and 0.5 mL/min for an additional 29 min. The separation was monitored by absorbance at 214 nm.

Automated Edman sequencing (Hunkapiller et al., 1983) was performed on an Applied Biosystems Inc. 470A gas-phase sequencer equipped with an on-line 120A PTH analyzer at the Biomolecular Resource Center, UCSF.

Acetylcholine Receptor Binding Affinity. The binding affinity of α-bungarotoxin$^{(Val\text{-}31)}$ for the acetylcholine receptor was accessed by a competition assay with ^{125}I-labeled bungarotoxin. α-Bungarotoxin$^{(Val\text{-}31)}$ or α-bungarotoxin, at concentrations ranging between 0.75 nM and 1000 nM, was incubated with affinity-purified acetylcholine receptor reconstituted in dioleoylphosphatidylcholine, in 100 μL of 10 mM sodium phosphate, pH 7.4, containing 0.1% Triton X-100 for 30 min at room temperature. Below 10 nM toxin, receptor concentration was 0.31 nM; above 10 nM toxin, receptor concentration was 1.89 nM. At the end of the incubation period, 5 μL of ^{125}I-labeled bungarotoxin, which is at least a 10-fold molar excess over toxin binding sites, was added and incubation continued for 10 min.

The reaction mixtures were applied to DE-81 ion-exchange filters and washed twice with 500 mL of phosphate/Triton X-100 (Schmidt & Raftery, 1973). Only receptor-bound toxin should remain adsorbed to the filter, as the positively charged bungarotoxin is repelled. The ^{125}I-labeled bungarotoxin–receptor complex absorbed to the filter was measured in a γ counter. Nonspecific adsorption of radiolabel was measured by excluding receptor from the reaction mixture, and 100% binding of radiolabeled bungarotoxin was measured in the absence of unlabeled bungarotoxin.

Miscellaneous Techniques. For amino acid analysis, the S-carboxymethylated proteins were hydrolyzed in constant-boiling HCl in vacuo for 20 h at 110 °C. Analysis was performed on a Beckman 119 CL analyzer. Tryptic peptides were

[1] Abbreviations: 1D, one dimensional; 2D, two dimensional; COSY, *J*-correlated spectroscopy; CM, carboxymethyl; FPLC, fast purification liquid chromatography; HOHAHA, 2D homonuclear Hartmann–Hahn spectroscopy; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; PTH, phenylthiohydantoin; rms, root mean square; TFA, trifluoroacetic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane. The one-letter system is used for the following amino acids, with the single-letter abbreviation given in parentheses: alanine (A), cysteine (C), aspartic acid (D), glutamic acid (E), phenylalanine (F), glycine (G), histidine (H), isoleucine (I), lysine (K), methionine (M), asparagine (N), proline (P), threonine (T), valine (V), and tryptophan (W).

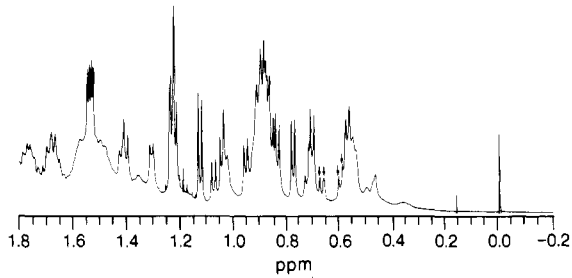

FIGURE 1: Spectral region upfield of the water resonance in a 1D 500-MHz ^1H NMR spectrum of α-bungarotoxin purified only by Sephadex CM-25. Solution conditions are pH 4.8 (not corrected for 10% D_2O), 45 °C, and no salts present except as needed to adjust the pH. The venom from which this sample of α-bungarotoxin had been isolated was obtained from Miami Serpentarium laboratories. The two sets of methyl doublets belonging to the contaminating α-bungarotoxin $^{(Val-31)}$ are marked by arrows. The sharp singlet at 0 ppm is due to sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate, which served as an internal reference.

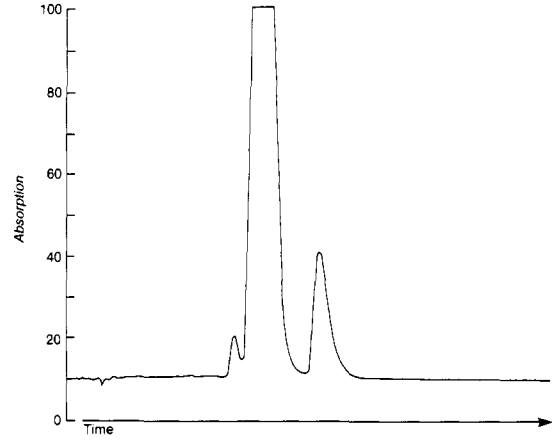

FIGURE 2: Mono-S chromatography of α-bungarotoxin after Sephadex CM-25 chromatography. The 100% scale expansion is 0.5 absorbance unit at 280 nm, which emphasizes the appearance of the minor peaks. Other experimental conditions are described in the text.

identified by amino acid analysis with the Waters Pico-tag system.

Polyacrylamide gel electrophoresis utilized the nondenaturing low-pH gel system of Reisfeld et al. (1962) with a 12% separating gel. The gels were stained with 0.1% Coomassie brilliant blue R-250 in 10% trichloroacetic acid and 10% sulfosalicyclic acid and destained in 5% methanol and 7.5% acetic acid. Sephadex G-50, 50 mesh, chromatography was performed with a 1.5 × 98 cm column equilibrated with 50 mM ammonium acetate, pH 6.75, and standardized with bovine serum albumin, cytochrome *c*, and bovine pancreatic trypsin inhibitor. Reverse-phase chromatography used the Pharmacia PEP RPC HR 5/5 column with a 0–50% gradient of 0.1% TFA/HPLC-grade acetonitrile at 50 °C and a flow rate of 0.5 mL/min for 20 min.

NMR Spectroscopy. NMR spectra were acquired on a General Electric GN 500-MHz spectrometer as described (Basus et al., 1988). The homonuclear Hartmann–Hahn (HOHAHA) spectrum (Bax & Davis, 1985) of α-bungarotoxin$^{(Val-31)}$ was acquired with a mixing time of 60 ms.

RESULTS

Identification of α-Bungarotoxin$^{(Val-31)}$ by NMR Spectroscopy and Its Separation from α-Bungarotoxin. When assignment of the α-bungarotoxin NMR resonances was initiated, the sample studied, obtained from Miami Serpentarium, had been purified only by Sephadex CM-25 chromatography. It soon became apparent that a second species was present, since minor peaks in both 1D and 2D NMR spectra could be discerned. This is illustrated in Figure 1. In that figure, the arrows point to the valine methyl resonances belonging to what was later identified as α-bungarotoxin$^{(Val-31)}$.

Quik and Lamarca (1982) had shown that the toxin, II-S1 of M_r 15 000, copurified upon cation-exchange chromatography with α-bungarotoxin. However, in our hands, chromatography on Sephadex G-50, after Sephadex CM-25, showed only one peak (which migrated between cytochrome *c*, M_r 12 400, and pancreatic trypsin inhibitor, M_r 6500). Thus, the possibility that the minor component was II-S1 toxin or some other species with a molecular weight different from that of α-bungarotoxin was eliminated.

The minor NMR resonances were present at pH 4.8 between 4 and 80 °C. No increases in the cross-peak's line widths were observed when the temperature was raised. It therefore seemed more reasonable that a second homologous bungarotoxin was present and less likely that there were two slowly interconverting conformations of α-bungarotoxin. Indeed, the protein preparation could be further fractionated by Mono-S chromatography as shown in Figure 2. A very minor species accounting for no more than 1–2% of the total protein eluted just before the major fraction. This species was not examined further. A second minor species eluted after the main fraction, accounted for 10% of the total, and was subsequently identified as α-bungarotoxin$^{(Val-31)}$. Characterization of this isotoxin is discussed in the final section under Results.

Correction of the α-Bungarotoxin Sequence. A crucial step in any NMR structural study is identification (by the sequential assignment procedure) of each scalar-coupled spin system with the corresponding residue of known sequence number. Because the backbone amide bond disrupts direct connectivities between residues, the assignment procedure relies on two pieces of information in addition to the identified spin systems. Both 2D NOE data, for through-space connectivities, and the protein sequence itself are necessary.

Sequential assignments of α-bungarotoxin were established by identifying the sequential NOE connectivities of a given residue's amide proton to the preceding residue's α-proton, amide proton (or in the case of prolines, δ-proton), and/or β-proton as discussed in the first paper in this series (Basus et al., 1988). (For the remainder of this discussion, substitute the methylene δ-protons for the amide proton in the case of prolines.) The mixing time of the NOESY experiment was 160 ms, which is long enough that a cross-peak, due to cross-relaxation between two protons within approximately 4.5 Å of each other, should be present in the NOESY spectrum. As mentioned by Basus et al. (1988), the sequential assignment procedure resulted in only 85% correlation of the spin systems with corresponding residues, *assuming the published sequences to be correct*, the regions of greatest difficulty being residues 8–12 and 66–70. Examination of the left-hand portion of Figure 3 illustrates the problems encountered.

Assuming the published sequence to be correct, no NOEs between the C_α proton of the *i*th residue and the amide proton of residue $i + 1$ could be identified (i.e., the $d_{\alpha N}$ cross-peaks) in the sequence 8–12 and only one of four possible cross-peaks between residues 66 and 70. A survey of distances between various types of protons at distances of less than about 8 Å has been made (Billeter et al., 1982; Wüthrich et al., 1984; Wüthrich, 1986). Assuming a trans peptide bond, C_α proton–amide distances ($d_{\alpha N}$) were shown to be less than or equal to 3.6 Å, regardless of the Ψ angle—the only independent variable. Therefore, given the mixing time of 160 ms, any C_α proton–amide proton cross-relaxation should result in an ob-

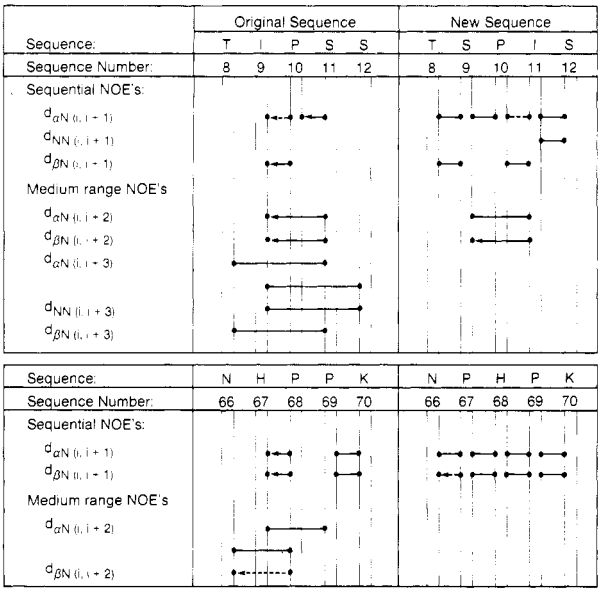

FIGURE 3: Summary of the NOE assignments for the two disputed sequences in α-bungarotoxin. (A) Residues 8–12. (B) Residues 66–70. The left-hand portion of (A) and (B) shows the assignments of the NOEs based on the published sequence and the right-hand side those based on the revised sequences. The nomenclature used is described in the first paper in this series (Basus et al., 1988) as suggested by Wüthrich et al. (1984), except as follows: (−−) an NOE that could also be assigned to a second spin system; (arrow) a reverse NOE, i.e., j to i instead of i to j. For the "amide proton" of proline read the δ-methylene protons instead.

servable NOESY cross-peak. Further, a cross-peak of reverse directionality, i.e., the distance $d_{N\alpha}$ shown as an arrow in Figure 3, is not expected unless the intervening peptide bond is cis (Arseniev et al., 1983; Wüthrich et al., 1984). While such cross-peaks are indicated in Figure 3, there is no strong supporting evidence, neither crystallographic nor spectral, for cis peptide bonds in bungarotoxin. [However, the computer graphics model of the unrefined 2.8-Å structure of α-cobratoxin reveals a cis peptide bond between residues 6 and 7, which are homologous to residues 8 and 9 of α-bungarotoxin (Walkinshaw et al., 1980).]

Two of the simpler explanations for the lack of identifiable sequential NOEs involving the C_α proton of the ith residue and the amide proton of residue $i + 1$ are (1) the presence of cis peptide bonds (then $d_{\alpha N}$ would be greater than 4 Å) and (2) the relevant cross-peaks occurring in crowded regions of the spectrum with such severe overlap that identification was impossible. For the first explanation, we have no corroborating evidence. The latter explanation was also considered unlikely; the expected cross-peaks were not in regions of significant overlap (Basus et al., 1988).

If the two sequences in question were ignored and the cross-peaks used instead to dictate sequence, then the most logical sets of sequences and cross-peak assignments based on the theoretical distance limitations and no cis peptide bonds are shown in the right-hand side of Figure 3. The attractive features of the new sequences are (1) the presence of the sequential $d_{\alpha N}$ cross-peaks, (2) the presence of at least five dipeptides (and probably six) with two different types of sequential NOEs, raising the probability, that these residues are sequential, to 95%, and (3) the larger number of sequential NOEs at the expense of medium-range NOEs. However, as emphasized by Wüthrich (1986), a protein sequence must be determined independently of the NMR assignments. Therefore, the relevant regions of Mono-S-purified α-bungarotoxin were resequenced.

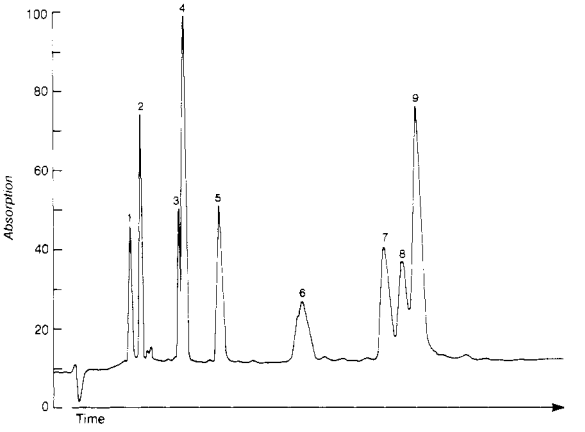

FIGURE 4: Separation of α-bungarotoxin tryptic peptides on reverse-phase chromatography. Details of the separation are given in the text. Assigned sequences based on amino acid analysis: fraction 1, residues 71–74; fraction 2, residues 65–70; fraction 4, residues 53–64; fraction 5, residues 39–51; fraction 7, residues 26–36; fraction 8, residues 27–36; fraction 9, residues 1–25. Fraction 3 was assumed to be residues 52–64 on the basis of its absorption at 280 nm and its relationship to fraction 4. The dipeptide, residues 37–38, was not identified.

Intact S-carboxymethylated α-bungarotoxin was sequenced from residue 1 to residue 20. The sequence found was I-V-C-H-T-T-A-T-S-P-I-S-A-V-T-C-P-P-G-E. This disagrees with the published sequence only at positions 9 and 11. At positions 9–11, the proposed sequence based on the NMR studies was confirmed, being Ser-Pro-Ile.

In order to sequence the region containing residues 67–69, S-carboxymethylated α-bungarotoxin was first digested with trypsin. Separation of the tryptic peptides was achieved by reverse-phase chromatography on the PEP RPC 5/5 system (Figure 4). Each fraction was analyzed for its amino acid composition; fractions 1–5 and 7–9 compose almost the entire expected group of tryptic peptides. (The identities of the peptides are given in the legend of Figure 4.) Fraction 6 may contain partial digestion products as it could not be unambiguously identified as a unique tryptic peptide and as its appearance varied in two separate digests. The peptide of interest, residues 65–70, was identified by its composition as fraction 2. Upon sequencing, it was shown to be C-N-P-H-P-K. Again the sequence suggested by the NOE data was correct.

Crystal Structure of α-Bungarotoxin. The X-ray structure of α-bungarotoxin (Love & Stroud, 1986) was corrected for the changes in sequence by model building (Jones, 1982) residues 9–11 and 67–69 into the $2F_o - F_c$ electron density map calculated with these residues omitted from the structure and then refining the new model by a least-squares procedure (Konnert & Hendrickson, 1981). The final residual was 0.25 on 2540 reflections in the range of 7.0–2.5 Å with intensities greater than 2σ. The rms deviation from ideal bond lengths was 0.026 Å and from ideal bond angles was 4.9°.

Residues 9–11 lie at the end of the first of the three loops in the bungarotoxin structure. These residues are not part of the β-sheet and, in the refined structure of Love and Stroud (1986), had higher than average temperature factors. The sequence corrections for these residues were relatively small, i.e., two-atom difference between the two side chains, and thus could be implemented in each monomer without major changes to the backbone structure. The average change in α-carbon position for the three residues was 1 Å. The final conformations of residues 9–11 were similar in the two monomers that comprise the crystallographic asymmetric unit, even though during refinement they were not constrained to be

Table I: Amino Acid Compositions of α-Bungarotoxin and α-Bungarotoxin$^{(Val-31)}$

amino acid	α-bungarotoxin	α-bungarotoxin$^{(Val-31)}$
aspartic acid	3.76 (94)[a]	3.86 (97)
threonine	6.39 (91)	6.48 (93)
serine	4.61 (77)	4.76 (79)
glutamic acid	5.04 (101)	5.19 (104)
proline	7.84 (98)	8.04 (101)
glycine	3.62 (91)	3.68 (92)
alanine	4.78 (96)	4.03 (81)
valine	4.04 (81)	5.01 (100)
methionine	0.80 (80)	0.85 (85)
isoleucine	1.56 (78)	1.58 (79)
leucine	2.02 (101)	2.05 (103)
tyrosine	1.99 (100)	1.99 (100)
phenylalanine	1.00 (100)	1.00 (100)
lysine	5.43 (90)	5.35 (89)
histidine	1.80 (90)	1.68 (84)
arginine	3.04 (101)	3.10 (103)

[a] Normalized so that phenylalanine equaled 1 mol/mol of protein. The numbers in parentheses are the percentage recovery based on the amino acid composition of α-bungarotoxin. (Carboxymethyl)cysteine content was not quantitated.

identical. Revision of the structure resulted in lower temperature factors for the backbone atoms of residues 9–11 and a better fit for the side chains of residues 9 and 11 to the electron density map.

Residues 67 and 68 are near the carboxy terminus of bungarotoxin. In order to accommodate the new sequence to the electron density map in this region, movements of up to 4 Å had to be made in the backbone structure. Residues 67–69 were finally built into the electron density map with different conformations for the two monomers. Residues 70–74 were also found in different conformations, but this was true also for the original crystal structure.

The new coordinates for α-bungarotoxin will be deposited in the Protein Data Bank.

Characterization of α-Bungarotoxin$^{(Val-31)}$. After purification by Mono-S chromatography, α-bungarotoxin$^{(Val-31)}$ was initially characterized by its amino acid composition. α-Bungarotoxin and α-bungarotoxin$^{(Val-31)}$ differed significantly only in a decrease in the number of alanines and a corresponding increase in the valine content of the latter's composition (Table I).

Attempts to characterize α-bungarotoxin$^{(Val-31)}$ by NMR spectroscopy were limited by the amount of protein available. The high sensitivity of the homonuclear Hartmann–Hahn experiment made it possible to obtain a HOHAHA spectrum at a protein concentration of about 1.5 mM. Figure 5 compares the region containing the α–β cross-peak of α-bungarotoxin's Ala-31 with that of α-bungarotoxin$^{(Val-31)}$. The relevant cross-peak is clearly absent in the latter's spectrum. All other alanine α–β cross-peaks could be identified in the HOHAHA spectrum of α-bungarotoxin$^{(Val-31)}$ (not shown). Unfortunately, some of the other regions of the HOHAHA spectrum did not contain sufficiently intense cross-peaks to allow us to assign proton resonances that might have been affected by the mutation. But overall, the two proteins' spectra are nearly identical when cross-peak positions could be compared.

To prove conclusively that α-bungarotoxin$^{(Val-31)}$ was a mutant of α-bungarotoxin with only a single residue change, the S-carboxymethylated protein was digested with trypsin, and the peptides were separated under the same conditions as in Figure 4. The peptide map of α-bungarotoxin$^{(Val-31)}$ is shown in Figure 6. Peptides 7 and 8 of Figure 4 have essentially disappeared, and two new peptides, 7a and 8a, migrating after peptide 9 have appeared. In other respects, the major peptides of α-bungarotoxin and α-bungarotoxin$^{(Val-31)}$ were identical. Analysis of the first seven residues of peptides 7a and 8a gave the sequences K-M-W-C-D-V-F and M-W-C-D-V-F-C, respectively.

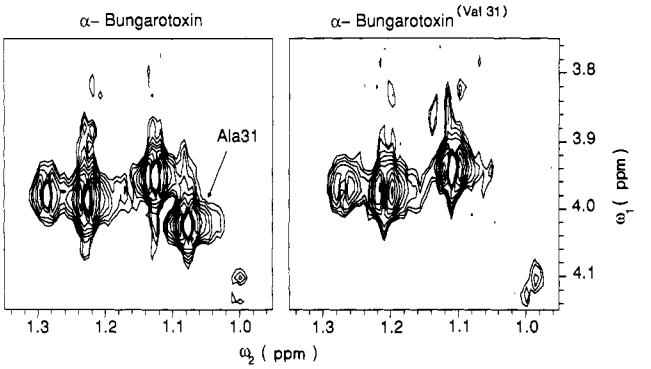

FIGURE 5: A small portion of the HOHAHA spectrum of α-bungarotoxin (left) and α-bungarotoxin$^{(Val-31)}$ (right), showing the presence and absence, respectively, of the Ala-31 α–β cross-peak. Contour levels are plotted with a factor of 1.4 between consecutive levels.

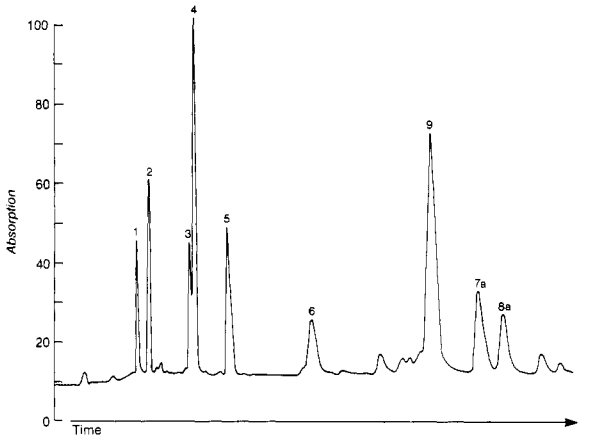

FIGURE 6: Reverse-phase chromatography of the tryptic peptides of α-bungarotoxin$^{(Val-31)}$. Conditions are the same as those in Figure 4. The starting material contained ~5% α-bungarotoxin.

We also characterized α-bungarotoxin and α-bungarotoxin$^{(Val-31)}$ by two additional chromatography techniques: nondenaturing polyacrylamide gel electrophoresis and reverse-phase chromatography. Upon electrophoresis, α-bungarotoxin$^{(Val-31)}$ migrated with a mobility 99% that of α-bungarotoxin and upon reverse-phase chromatography with a relative mobility of 101%. Clearly in the first case, it would be nearly impossible to identify a mixture of the two toxins as such and difficult in the second case, even though the resolution by reverse-phase chromatography tends to be better than that of gel electrophoresis.

Finally, the binding affinity of α-bungarotoxin$^{(Val-31)}$ for the acetylcholine receptor was characterized. As shown in Figure 7, α-bungarotoxin$^{(Val-31)}$ binds to the receptor with the same relative affinity as that of α-bungarotoxin.

Discussion

A number of procedures have been developed for α-bungarotoxin purification [e.g., Clark et al. (1972), Lee et al. (1972), Mebs et al. (1972), Hanley et al. (1977), Ravdin and Berg (1979), Quik and Lamarca (1982), and Ferragut et al. (1984)]. Typically, fractionation of venom on a CM-type resin (either Sephadex or cellulose) is the first step and is followed either by rechromatography on the same resin or chromatography on the alternative resin. In only one case (Ferragut

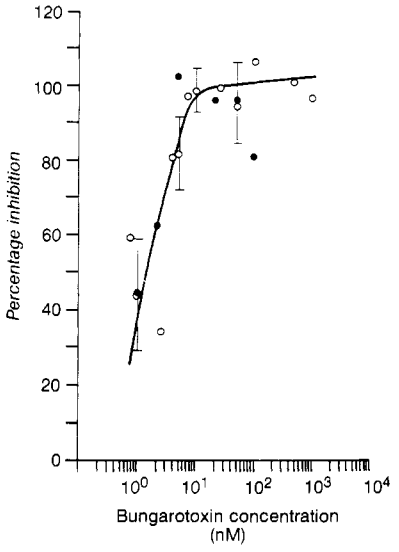

FIGURE 7: Percentage inhibition of binding to the acetylcholine receptor by ^{125}I-labeled bungarotoxin after a 30-min preincubation with α-bungarotoxin$^{(Val\text{-}31)}$ (unfilled circles) or α-bungarotoxin (filled circles). The error bars show the deviations between two or three sets of measurements. Other details are given in the text.

et al., 1984) prior to this paper have the newer resins, which operate under greater than ambient pressure, been exploited for purification purposes.

The usual goal of bungarotoxin purification procedures is removal of contaminating enzymes, e.g., acetylcholinesterase and phospholipase A, and other toxins which would interfere with functional studies on α-bungarotoxin. Our aim was somewhat different. The criterion sought was an NMR spectrum transparent to all macromolecules except α-bungarotoxin. This would not preclude contaminating species at a level of a few percent, but the 10% contaminating level of α-bungarotoxin$^{(Val\text{-}31)}$ was readily apparent. (Removal of proteases capable of degrading α-bungarotoxin was also an obvious concern. Since the bungarotoxin preparations have not deteriorated over a period of months, significant protease activity directed against α-bungarotoxin is assumed not to be present.) Consequently, we have not assayed for enzymatic activities nor tested for other types of toxins, but by the criteria of reverse-phase chromatography and nondenaturing polyacrylamide gel electrophoresis, purification of both α-bungarotoxin and α-bungarotoxin$^{(Val\text{-}31)}$ can be achieved to at least 99% with respect to any other one macromolecule after Mono-S chromatography.

In this paper we have provided the chemical evidence necessary to substantiate the inferred errors in the α-bungarotoxin sequence and the inferred presence of the second toxin provided by NMR spectroscopy. This study represents the fifth case known to us where ambiguities in NMR data have lead to either sequence corrections or the identification of a homologous species. Wüthrich and colleagues were the first to report an inconsistency between a publish protein sequence, that of the protease inhibitor IIA from bull seminal plasma, and its deduced NMR sequence assignments, resulting in the reordering of the carboxy-terminal dipeptide (Štrop & Wüthrich, 1983; Frank, 1983). They also noted the deterioration of the protein sample with time and attributed, but did not prove, the deterioration to hydrolysis of asparagine and glutamine side chains. Wüthrich's group has also reported discrepancies in the rabbit liver metallothionein 2 sequence and the presence of a chemically unique, but as yet incompletely characterized, metallothionein (Wagner et al., 1986). Wemmer and colleagues found it necessary to resequence toxin II from *Radianthus paumotensis* (a sea anemone) before being able to successfully analyze the NMR spectra of that protein. We have also identified and characterized a substantial contaminant in commercial preparations of bovine pancreatic trypsin inhibitor that was first noticed in the course of NMR spectroscopy. Here, the sulfur of the inhibitor's single methionine is oxidized to the level of the sulfoxide (P. A. Kosen, unpublished results). Undoubtedly NMR spectroscopy will continue to provide a sensitive check on both purity and sequence errors as the solution structures of additional proteins are begun.

The binding of neurotoxins, in general, and α-bungarotoxin, in particular, to the acetylcholine receptor depends on multifocal interactions. No single-site mutation nor chemical modification completely abolishes activity without also destroying structure, although significant decreases in activity have been noted upon chemical modification [summarized by Karlsson (1979), Dufton and Hider (1983), and Love and Stroud (1986)]. An exact description of the toxin–receptor interface remains a controversial area and requires techniques of higher resolution than are currently available. Even so, some simple statements relating the sequence errors and the new isotoxin, reported in this paper, to the structure–function relationship of α-bungarotoxin are appropriate, especially if α-bungarotoxin is considered to be one of the more primitive of the long neurotoxins (Strydom, 1979).

Neither residues 9–11 nor residues 67–69 have unambiguously identified functions. Neither is part of the triple-strand β-sheet common to all neurotoxins. Residues 9–11 are part of the first loop in the three-loop structure characteristic of snake neurotoxins. Residues 66–69 are found on the convex side of the long neurotoxins—the face that is not involved in receptor binding—and contact the second loop whose concave face is important to binding. The sequence surrounding residues 9–11 contains primarily aliphatic residues with little sequence conservation among the long neurotoxins, except at Thr-8. In the absence of genetically cloned long neurotoxins and site-specific mutagenesis, the functional or structural significance of residues 9–11 remains speculative at best. However, the sequence between residues 59 and 69 could be described by a consensus sequence: Cys-Cys-Ser-Thr-Asp-Asp/Asn-Cys-Asp/Asn-Pro-His/Phe-Pro. For α-bungarotoxin the residue at position 64 is a lysine; but the corrected sequence at positions 67–69 now conforms to the sequence Pro-X-Pro, with X an aromatic residue, in 80% of the long neurotoxins known (Dufton & Hider, 1983). Because His-68 is not protonated at neutral pH (Inagaki et al., 1985), the histidine may be structurally or functionally interchangeable with the phenylalanine at physiological pH.

Ours is the first report of a valine at position 31 of a long neurotoxin. Alanine, glycine, and asparagine have been found at this position. The simplest explanation of valine at this position is the presence of a second allele in an unknown fraction of the *Bungarus multicinctus* population. Only a single nucleotide change is needed to specify valine instead of alanine. We have found this isotoxin in two different preparations of venom and the valine methyl resonances of α-bungarotoxin$^{(Val\text{-}31)}$ can also be seen in the 1D ^{1}H NMR spectrum recorded by Inagaki et al. (1985).

Strydom (1979) has calculated a phylogenic tree of toxins from proteroglyphae venoms, including the then known 19 long neurotoxins. Examination of the tree shows that glycine at position 31 probably appeared independently at least 3 times and asparagine at least once from glycine. Position 31 is near residues that have been implicated in function, i.e., Trp-28, Asp-30, Phe-32, and Arg-36, or in structure, i.e., Cys-29 and

-33 and Gly-37. But, at least in the crystal structure of the homologous long neurotoxin α-cobratoxin, the corresponding side chain, Ala-28, is shielded from the toxin–receptor binding face by other residues and is at the very periphery of the protein. This is consistent with our finding that valine at position 31 does not alter toxin binding. That a valine has not been found at this position before could be the consequence of either a statistically insignificant population of sequenced neurotoxins or a marginally detrimental stability effect due to the increased hydrophobicity of the valine side chain coupled with its solvent-accessible position. We have not tested for the latter possibility, although the extreme stability of α-bungarotoxin makes this an unlikely possibility unless substitution of the valine increases the susceptibility to proteolysis.

Added in Proof

It has come to our attention that, recently, α-bungarotoxin was completely resequenced (Ohta et al., 1987). In addition to the two sequence errors that we have reported, a correction is also made at positions 71–72 with Gln-Arg being the new sequence. The crystal structure has been refined to account for this additional correction.

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Registry No. α-Bungarotoxin, 11032-79-4; α-bungarotoxin$^{(Val-31)}$, 113321-68-9.

References

Albuquerque, E. X., Eldefrawi, A. T., & Eldefrawi, M. E. (1979) in *Snake Venoms* (Lee, C.-Y., Ed.) p 377, Springer-Verlag, Berlin.

Arseniev, A. S., Kondakov, V. I., Maiorov, V. N., Volova, T. M., Grishin, E. V., Bystrov, V. F., & Ovchinnikov, Yu. A. (1983) *Bioorg. Khim. 9*, 768.

Basus, V. J., Billeter, M., Love, R. A., Stroud, R. M., & Kuntz, I. D. (1988) *Biochemistry* (first paper of three in this issue).

Bax, A., & Davis, D. G., (1985) *J. Magn. Reson. 65*, 355.

Billeter, M., Braun, W., & Wüthrich, K. (1982) *J. Mol. Biol. 155*, 321.

Bystrov, V. F., Tsetlin, V. I., Karlsson, E., Pashkov, V. S., Utkin, Yu., Kondakov, V. I., Pluzhnikov, K. A., Arseniev, A. S., Ivanov, V. T., & Ovchinnikov, Yu. A. (1983) in *Toxins as Tools in Neurochemistry* (Hucho, F., & Ovchinnikov, Yu. A., Eds.) p 194, de Gruyter, Berlin.

Changeux, J.-P., Kasai, M., & Lee, C.-Y. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1241.

Clark, D. G., Macmurchie, D. D., Elliott, E., Wolcott, R. G., Landel, A. M., & Raftery, M. A. (1972) *Biochemistry 11*, 1663.

Dufton, M. J., & Hider, R. C. (1983) *CRC Crit. Rev. Biochem. 14*, 113.

Endo, T., Inagaki, F., Hayashi, K., & Miyazawa, T. (1981) *Eur. J. Biochem. 120*, 117.

Ferragut, J. A., Gonzalez-Ros, J. M., Peterson, D. L., Weir, D. L., Franson, R. C., & Martinez-Carrion, M. (1984) *Arch. Biochem. Biophys. 235*, 628.

Frank, G. (1983) *J. Mol. Biol. 166*, 665.

Hanley, M. R., Eterovič, V. A., Hawkes, S. P., Hebert, A. J., & Bennett, E. L. (1977) *Biochemistry 16*, 5840.

Hendrickson, W. A., & Konnert, J. H. (1981) in *Biomolecular Structure, Function, Conformation, and Evolution* (Srinivasen, R., Ed.) Vol 1, p 43, Pergamon, Oxford.

Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J., & Hood, L. E. (1983) *Methods Enzymol. 91*, 399.

Inagaki, F., Clayden, N. J., Tamiya, N., & Williams, R. J. P. (1982) *Eur. J. Biochem. 123*, 99.

Inagaki, F., Hider, R. C., Hodges, S. J., & Drake, A. F. (1985) *J. Mol. Biol. 183*, 575.

Jones, T. A. (1982) in *Computational Crystallography* (Sayre, D., Ed.) p 303, Oxford University Press, Oxford.

Karlsson, E. (1979) in *Snake Venoms* (Lee, C.-Y., Ed.) p 159, Springer-Verlag, Berlin.

Lee, C.-Y., Chang, S. L., Kau, S. T., & Luh, S.-H. (1972) *J. Chromatogr. 72*, 71.

Love, R. A., & Stroud, R. M. (1986) *Protein Eng. 1*, 37.

Low, B. W. (1979) in *Snake Venoms* (Lee, C.-Y., Ed.) p 213, Springer-Verlag, Berlin.

Mebs, D., Narita, K., Iwanaga, S., Samejima, Y., & Lee, C.-Y. (1971) *Biochem. Biophys. Res. Commun. 44*, 711.

Mebs, D., Narita, K., Iwanaga, S., Samejima, Y., & Lee, C.-Y. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 353*, 243.

Ohta, M., Ohta, K., Nishitani, H., & Hayaski, K. (1987) *FEBS Lett. 222*, 79.

Quik, M., & Lamarca, M. V. (1982) *Brain Res. 238*, 385.

Ravdin, P. M., & Berg, D. K. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2072.

Saenger, W., Walkinshaw, M. D., & Maelicke, A. (1983) in *Toxins as Tools in Neurochemistry* (Hucho, F., & Ovchinnikov, Yu. A., Eds.) p 151, de Gruyter, Berlin.

Schmidt, J., & Raftery, M. A. (1973) *Anal. Biochem. 52*, 349.

Štrop, P., Wider, G., & Wüthrich, K. (1983) *J. Mol. Biol. 166*, 641.

Strydom, D. J. (1979) in *Snake Venoms* (Lee, C.-Y., Ed.) p 213, Springer-Verlag, Berlin.

Surin, A., Utkin, Y., Pluzhnikov, K., & Tsetlin, V. (1983) in *Toxins as Tools in Neurochemistry* (Hucho, F., & Ovchinnikov, Yu. A., Eds.) p 171, de Gruyter, Berlin.

Wagner, G., & Wüthrich, K. (1982) *J. Mol. Biol. 155*, 347.

Wagner, G., Neuhaus, D., Wörgötter, Ed., Vasák, M., Kägi, J. H. R., & Wüthrich, K. (1986) *Eur. J. Biochem. 157*, 275.

Walkinshaw, M. D., Saenger, W., & Maelicke, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2400.

Wemmer, D. E., Kumar, N. V., Metrione, R. M., Lazdunski, M., Drobny, G., & Kallenbach, N. R. (1986) *Biochemistry 25*, 6842.

Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, New York.

Wüthrich, K., Wider, G., Wagner, G., & Braun, W. (1982) *J. Mol. Biol. 155*, 311.

Wüthrich, K., Billeter, M., & Braun, W. (1984) *J. Mol. Biol. 180*, 715.