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Proton Nuclear Magnetic Resonance Studies on the Variant-3 Neurotoxin from *Centruroides sculpturatus* Ewing: Sequential Assignment of Resonances†

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ABSTRACT: We report the sequential assignment of resonances to specific residues in the proton nuclear magnetic resonance spectrum of the variant-3 neurotoxin from the scorpion *Centruroides sculpturatus* Ewing (range southwestern U.S.A.). A combination of two-dimensional NMR experiments such as 2D-COSY, 2D-NOESY, and single- and double-RELAY coherence transfer spectroscopy has been employed on samples of the protein dissolved in D_2O and in H_2O for assignment purposes. These studies provide a basis for the determination of the solution-phase conformation of this protein and for undertaking detailed structure–function studies of these neurotoxins that modulate the flow of sodium current by binding to the sodium channels of excitable membranes.

The venom from the scorpion *Centruroides sculpturatus* Ewing (range southwestern U.S.A.) is a complex mixture that contains a large number of small basic proteins (MW ~ 7000) which are responsible for the neurotoxic effects (Watt et al., 1978; Watt & Simard, 1984). This venom, like that from other species of scorpions, is unique in that the various toxins present in the venom can differ markedly in their toxicity to a given species, despite their closely related chemical properties and similar modes of action. In addition, the toxins display a degree of target specificity: e.g., some may be more toxic to mammals than to insects while the others are more toxic to insects than to mammals (Watt et al., 1978; Watt & Simard, 1984; Zlotkin et al., 1972a,b). Toxicity of the scorpion venoms and the isolated toxins is a consequence of the depolarizing action of the toxins on membranes of excitable cells. A broad range of receptive excitable cells, e.g., neurons (central and peripheral) and muscle (skeletal and smooth), are depolarized and, in the case of nerve endings, depolarized with the release of neurotransmitters (Rochat et al., 1979; Zlotkin et al., 1978). Results of electrophysiological studies and pharmacological assays suggest that the scorpion toxins could be divided into two different groups, the scorpion α- and β-toxins, that bind to two distinct receptor sites on the sodium channels of excitable membranes (El Ayeb et al., 1986; Wheeler et al., 1983; Meves et al., 1982). The α-toxins bind to the sodium channels in a voltage-dependent manner and prolong the inactivation of the sodium current, an activity that is also shared by the sea anemone toxins (Romez et al., 1976). The binding of the β-toxins, which enhance the flow of sodium inward current, is voltage independent. Among the various toxic proteins isolated and characterized from the venom of *C. sculpturatus* Ewing, CsE-v1 to CsE-v6 (also referred to as variants 1–6) and CsE-V prolong inactivation of the sodium current, whereas CsE toxins I, III, IV, VI, and VII affect sodium activation (Meves et al., 1982; Simard et al., 1986).

The above results suggest the possibility of intriguing conformation–function relationships that contribute to the ob-

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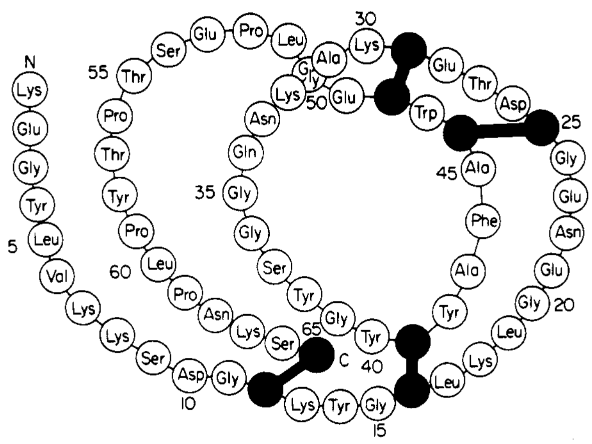

FIGURE 1: Amino acid sequence for the CsE-v3 toxin from *Centruroides sculpturatus* Ewing.

served diversity in toxicities, pharmacological specificities, and sodium channel binding properties for the different scorpion toxins in general. Considerable information on the structure–antigenicity relationships and on the receptor binding sites for the scorpion toxins on the sodium channel is also now available [e.g., Angelides and Nutter (1983a,b), Ayeb et al. (1986), Bahraoui et al. (1986), and Novotny and Haber (1986)]. The CD studies by Dufton et al. (1986) on toxins from five species of scorpions indicate that they exhibit variations in their CD-detectable structures. The studies on *Tityus* and *Centruroides* toxins by Possani et al. (1981) using CD/NMR spectroscopic methods indicate differences in the conformational flexibilities of the different toxins.

The venom from *C. sculpturatus* has about 25–30 different toxins, and the amino acid sequences for six of these proteins are known (Babin et al., 1974; Fontecilla-Camps et al., 1981; Mole and Watt, unpublished data). The crystallographic structure for one of these toxins, viz., the variant-3 toxin (also referred to as CsE-v3), has been characterized in considerable detail (Almassy et al., 1983; Fontecilla-Camps et al., 1982, 1981). Like other scorpion toxins the amino acid sequence for this toxin, shown in Figure 1, is characterized by four disulfide bridges. This is one of the least toxic proteins present in the *Centruroides* venom and prolongs the inactivation of the sodium current. The overall structure of CsE-v3 appears to be unrelated to that of erabutoxin (Tsernoglou & Petsko, 1976; Low et al., 1976) and α-cobratoxin (Walkenshaw et al., 1980), two snake toxins that act at the postsynaptic level by binding to the acetylcholine receptor. Recent NMR studies (Gooley & Norton 1986; Wemmer et al., 1986) on the sea anemone toxins, which possess very little sequence homology with the scorpion toxins, suggest that their overall conformations are different from that of toxin variant-3. On the other hand, some common structural domains appear to be present between the variant-3 toxin and two short toxins, a scorpion insectotoxin (Bystrov et al., 1983) and the bee venom toxin apamin (Wemmer & Kallenbach, 1983; Zell et al., 1985).

As a prelude to a detailed definition of the structure–function relationships of the various toxins from *C. sculpturatus*, we have initiated a detailed solution-phase NMR spectroscopic characterization of the CsE-v3 toxin. A preliminary characterization of this protein has been reported previously (Krishna et al., 1983). In the present investigation, we report the sequence-specific assignment of the backbone NH and C$^{\alpha}$H and the side-chain CH proton resonances of almost all the residues using the 2D NMR spectroscopic methodology outlined by the laboratory of Wuthrich and his co-workers (Billeter et al., 1982; Wuthrich et al., 1982).

Materials and Methods

Sample Preparation. The isolation and purification of the variant-3 toxin has been described elsewhere (Watt & Simard, 1984; Babin et al., 1974). All solutions of the protein were unbuffered and contained in 5-mm NMR sample tubes. For solutions in H_2O, 10% (v/v) D_2O was added to provide an internal lock. Solutions in D_2O were first adjusted to the appropriate pH in 99.8% D_2O, followed by centrifugation and lyophilization. The dry residue was then redissolved in 0.3 mL of 99.996% D_2O (Stohler Isotopes Chemicals). The protein concentrations were 8 mM in H_2O and 2 mM in D_2O solutions. In general, the solution pH was maintained at 2.7 for all 2D NMR experiments.

NMR Spectroscopy. All of the ^{1}H NMR experiments with the D_2O solutions of the protein were performed at 400 MHz on a Bruker WH-400 NMR spectrometer, equipped with an Aspect 2000 computer and two Diablo 31 disk drives connected in series. Most of the experiments with the H_2O solutions were performed at 500 MHz on a WM-500 spectrometer equipped with an Aspect 2000A computer and a 32-MB disk drive. Additional data also were obtained with the WH-400 spectrometer. Quadrature detection was employed with the carrier frequency always maintained at the solvent resonance. Two-dimensional NOESY experiments (Jeener et al., 1979; Macura & Ernst 1980) were performed in the phase-sensitive mode (States et al., 1982) whereas the 2D-COSY spectra (Aue et al., 1976; Bax & Freeman, 1981; Nagayama et al., 1980) were obtained in the absolute-value mode. The COSY and NOESY spectra at 500 MHz were collected as 400 and 512 t_1 experiments, respectively, each with 2K data points. The 2D NMR spectra on the WH-400 were collected as 256 t_1 experiments with 1K data points and processed with the Bruker software on the Aspect 2000 computer. A mixing time of 200 ms was used for all the NOESY experiments. The strong proton signal from the solvent (HDO or H_2O) was suppressed by presaturation for 2 s prior to the experimental pulse sequence, and the decoupler was gated off during the t_1 and t_2 periods. In the case of NOESY experiments, the decoupler was turned on during the mixing time. To minimize the transfer of saturation from the H_2O resonance to the amide proton resonances upon presaturation, the sample pH was adjusted to 2.8. Since the presaturation of the H_2O resonance invariably resulted in the suppression (bleaching) of cross-peaks from protons resonating very close to water, the 2D NMR experiments were performed at two different temperatures (303 and 313 K) in order to shift the water resonance and thus recover some of the suppressed cross-peaks. The data obtained on the WM-500 were transferred to a Vax 11/780 computer and subsequently processed with the software developed by Dr. Dennis Hare. After 2D transformation, the residual t_1 ridges were suppressed by the subtraction method (Klevit, 1985). The chemical shifts were all referenced to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). The RELAY experiments at 400 MHz were performed with a mixing time of 42 ms, while those at 500 MHz were performed with a 52-ms mixing time (Eich et al., 1982). The double-RELAY experiments at 400 MHz were performed with a mixing time of 30 ms and the pulse and phase cycling sequences described in the literature (Bax & Drobny, 1985).

Results

The assignment of the proton resonances in the CsE-v3 toxin was performed in two distinct steps: (i) identification of

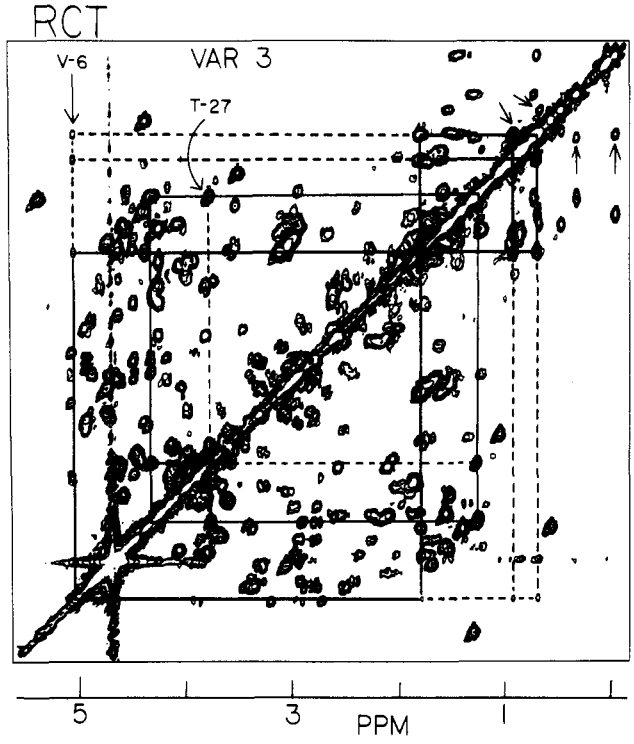

FIGURE 2: A 400-MHz RCT–COSY NMR spectrum of CsE-v3/D_2O (pH 2.5). The solid lines are the normal COSY connectivities while the dashed lines show the coherence transfer connectivities involving the $C^{\alpha}H$ proton and the CH_3 protons of Val-6 and Thr-27. The arrows indicate CH_3–CH_3 coherence transfer peaks.

resonances according to the amino acid type and (ii) sequential assignment of resonances. To accomplish the first step, considerable effort was devoted to a study of the protein in D_2O solvent with 2D-COSY, RELAY and double-RELAY, and 2D-NOESY techniques. Three amino acids, viz., Val, Phe, and Trp, are unique to CsE-v3 in that they appear only once in the amino acid sequence and occupy positions 6, 44, and 47, respectively (see Figure 1). Hence, identification of the resonances from these residues will provide some of the suitable starting points for sequential assignment of resonances of the adjacent stretches of residues. Identification of the valine residue from the 2D-COSY spectrum of the protein in D_2O proved to be somewhat difficult due to the crowding of the cross-peaks; however, this difficulty was easily overcome with a RELAY–COSY experiment which gave an unequivocal identification of the proton resonances from the valine residue. Typical results are shown in Figure 2. The 2D-COSY identification of the aromatic CH proton resonances and the 2D-NOESY identification of the aliphatic CH proton resonances from the single Phe and Trp residues and from the six tyrosines are discussed elsewhere (Krishna et al., 1989) together with a discussion of the microenvironment of all the aromatic residues. In addition to the valine residue which was already identified by the RELAY–COSY method, the other methyl-group-containing residues in the CsE-v3 toxin are three alanines (at positions 31, 43, and 45), three threonines (at positions 27, 55, and 57), and five leucines (at positions 5, 17, 19, 51, and 60). Of these, the methyl group resonances from the leucines could easily be identified from the 2D-COSY spectrum since they give rise to two cross-peaks each corresponding to the connectivity between the two methyl groups and the $C^{\gamma}H$ proton for each of the leucines. A further confirmation of the methyl resonances from the valine and leucine residues is provided by the presence of CH_3–CH_3 RELAY coherence transfer peaks, also shown in Figure 2.

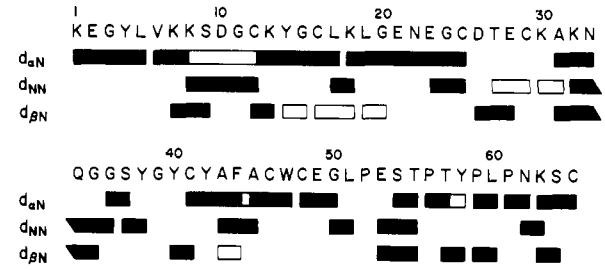

FIGURE 3: Sequential connectivities established in the CsE-v3 toxin. The solid and open bars are a qualitative representation of strong and weak connectivities, respectively, observed in the spectra. the connectivities shown are based on data obtained at two different temperatures, 313 and 303 K. Not shown are the connectivities involving the $C^{\delta}H_2$ protons of prolines-56 and -61 with the $C^{\alpha}H$ protons of residues 55 and 60, respectively.

Such peaks have also been observed by others [e.g., Holak and Prestegard (1987)]. In this manner the methyl resonances for four of the five leucines in the CsE-v3 toxin have been easily identified. The identification of resonances from the remaining protons of leucines proved to be nontrivial, and a combination of techniques including double-RELAY–COSY (Bax & Drobny, 1985) that gives CH_3–$C^{\alpha}H$ coherence transfer peaks, RELAY–COSY, COSY, and 2D-NOESY was used to study the protein in D_2O. The CH_3–$C^{\alpha}H$ double-RELAY coherence transfer peaks were in general very weak (presumably from a loss of signal intensity due to tranverse relaxation during the delay periods), thus permitting the identification of $C^{\alpha}H$ resonances for only two of the five leucines. The alanines and the threonines give rise to only one cross-peak for the methyl groups in the 2D-COSY spectrum. Further separation into alanines and threonines was accomplished by a combined use of COSY and RELAY–COSY experiments (not shown). The resonances for four out of the nine glycine residues have been readily identified by noting the twin cross-peaks between the NH and the two $C^{\alpha}H$ protons in a 2D-COSY spectrum of the protein in H_2O. Although the twin cross-peaks from four additional glycines were also located, their assignment remained tentative in the early stages due to the spectral overlap of the cross-peaks. Their assignment was however confirmed by a subsequent sequential assignment procedure. Identification of some of the resonances, as described above, according to the amino acid type facilitated the sequence-specific assignment of practically all of the resonances in the CsE-v3 toxin.

Sequence-Specific Assignments. The sequential assignment procedure (Billeter et al., 1982; Wuthrich et al., 1982) exploits the existence of a high statistical frequency with which a given amide proton can find itself in close spatial proximity to one of the following protons of the preceding residue: the $C^{\alpha}H$, $C^{\beta}H$, and NH. The spatial proximities of these protons manifest themselves as cross-peaks [corresponding to connectivities $d_{\alpha N}$, $d_{\beta N}$, and d_{NN}, respectively, according to the notation of Stassinopoulou et al. (1984)], between the appropriate protons in the 2D-NOESY spectrum of the protein in H_2O solutions. Then, starting from a known assignment for a given amino acid, the assignments for the adjacent residues could be deduced by following the sequential connectivities in the 2D-NOESY spectrum. This procedure is facilitated by first identifying, through sequential connectivities, as many short (di-, tri-, etc.) peptide segments as possible involving some of the amino acids identified above (e.g., GY, YAFA, etc.). A summary of such sequential connectivities established in the CsE-v3 toxin is shown in Figure 3 and is discussed below. The fingerprint identifying the various NH–$C^{\alpha}H$ COSY cross-peaks for the CsE-V3 toxin is shown

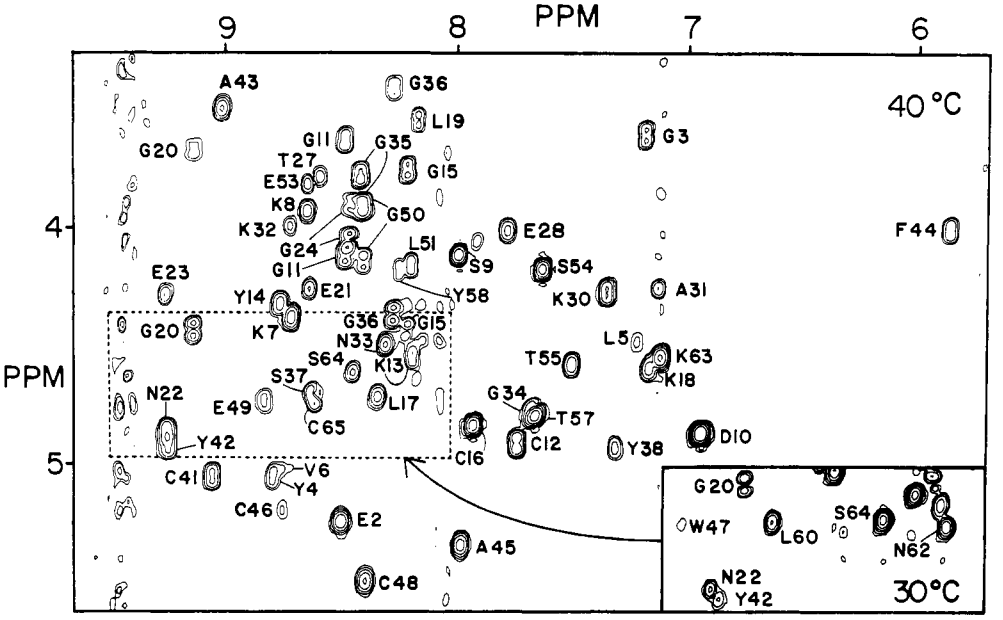

FIGURE 4: Fingerprint for the CsE-v3 toxin at 500 MHz, identifying the N*H*–C$^{\alpha}$*H* COSY cross-peaks at 313 K. The inset shows part of the region at 303 K.

in Figure 4. Many of the sequence-specific assignments relied upon the proper identification of many short as well as some long stretches of $d_{\alpha N}$ connectivities (Figure 3). The long $d_{\alpha N}$ stretches (with four or more amino acids) cover residues 1–5, 6–17, 18–25, and 41–47 in the amino acid sequence. The sequence-specific assignments in these long stretches, as well as in the other stretches of the sequence, will be discussed below.

Residues 1–5. The assignment for this stretch became possible after first identifying a $d_{\alpha N}$ connectivity for the dipeptide GY. Since this dipeptide segment occurs at two different locations, viz., at 3, 4 and 39, 40 in the sequence, the final choice of the first pair was made after noting that the amide proton of the glycine residue shows a $d_{\alpha N}$ connectivity to a nonaromatic residue (the C$^{\alpha}$H positions of all the tyrosines having been identified earlier, before the start of the sequence-specific assignments). This latter residue was assigned to be residue 2. These connectivities were further extended on either side to residues 1 and 5 by following the $d_{\alpha N}$ connectivities.

Residues 6–17. The sequential assignment in this stretch was facilitated by the prior unequivocal assignment of the resonances from the Val-6 residue (Figure 2) and the identification of dipeptide segment YG, corresponding to residues 14 and 15 within the sequence. This dipeptide also appears at another location in the sequence (as residues 38 and 39); however, the final assignment to residues 14 and 15 was made after noting that the α proton of the glycine residue shows a $d_{\alpha N}$ contact to a nonaromatic residue, which was assigned to Cys-16. Starting from these assignments, the assignments were extended to the other residues within the stretch by following $d_{\alpha N}$ connectivity cross-peaks. The $d_{\alpha N}$ contacts between K-8 and S-9 and between G-11 and C-12 show up as weak cross-peaks at 310 K. Figure 5 shows the sequential $d_{\alpha N}$ connectivities established among the first 17 residues. There was no contact of any kind between residues 5 and 6. In addition to the $d_{\alpha N}$ contacts, residues 8–12 also exhibit d_{NN} connectivities (Figure 6). The $d_{\beta N}$ connectivities have been observed for the following stretches: 7–9, 12 and 13, 14 and 15, and 16–18. Some of these $d_{\beta N}$ connectivities served as additional checks on the assignments.

Residues 18–29. The assignments from the previous stretch were extended to the amide proton of residue 18 by the observation of d_{NN} and $d_{\beta N}$ contacts between residues 17 and 18. The assignments were further facilitated by the prior identification of the unique (appearing only once in the sequence) dipeptide segment LG, corresponding to residues 19 and 20. L-19 was identified on the basis of 2D-COSY, and double-RELAY–COSY experiments. Observation of $d_{\alpha N}$ and $d_{\beta N}$ contacts between L-19 and a glycine residue helped the identification of the dipeptide LG. Residues 21–25 were easily assigned by following the $d_{\alpha N}$ connectivities. Weak d_{NN} contacts were exhibited by residues 23–25 and 27–29. Some of these contacts were very weak and could be observed only after the appropriate window functions were selected to enhance the weak cross-peaks (Figure 7). The d_{NN} cross-peak between 28 and 29 is very close to the main diagonal, and to the extent we could not rule out the possibility of this being an artifact, the assignment for the amide proton of residue 29 remains tentative. No COSY cross-peak between the NH and C$^{\alpha}$H protons for this residue could be observed in the fingerprint. The amide proton of residue 26 failed to show any NOESY contacts to the two neighboring residues. It was, however, assigned by noting a $d_{\beta N}$ connectivity from the amide proton of a threonine to the β-hydrogens of an AMX spin system. These residues were assigned as Thr-27 and Asp-26, respectively. The positions for the C$^{\alpha}$*H* and the N*H* resonances of Asp-26 were obtained from the COSY spectra. Residues 26–28 are also connected by $d_{\beta N}$ cross-peaks.

Residues 30–38. The sequence-specific assignments in this stretch were facilitated by the identification of the resonances from Ala-31 (which, in turn, was done after the identification of the other two alanines in the stretch 40–51 discussed below) and from the unique tripeptide GSY corresponding to residues 36–38. The tripeptide was identified by noting that a glycine shows a $d_{\alpha N}$ connectivity to the amide proton of another residue which, in turn, shows a d_{NN} contact to a tyrosine. The amide proton of lysine-30 was identified from its d_{NN} contact with Ala-31. Residues 32 and 33 were easily located from the $d_{\alpha N}$ contacts.

We have also observed d_{NN} contacts starting from residue 32 up to 36 (Figure 6). The observation of $d_{\beta N}$ contacts was also helpful in the sequence-specific assignment of residues 31–35. A d_{NN} contact completed the connectivity for the

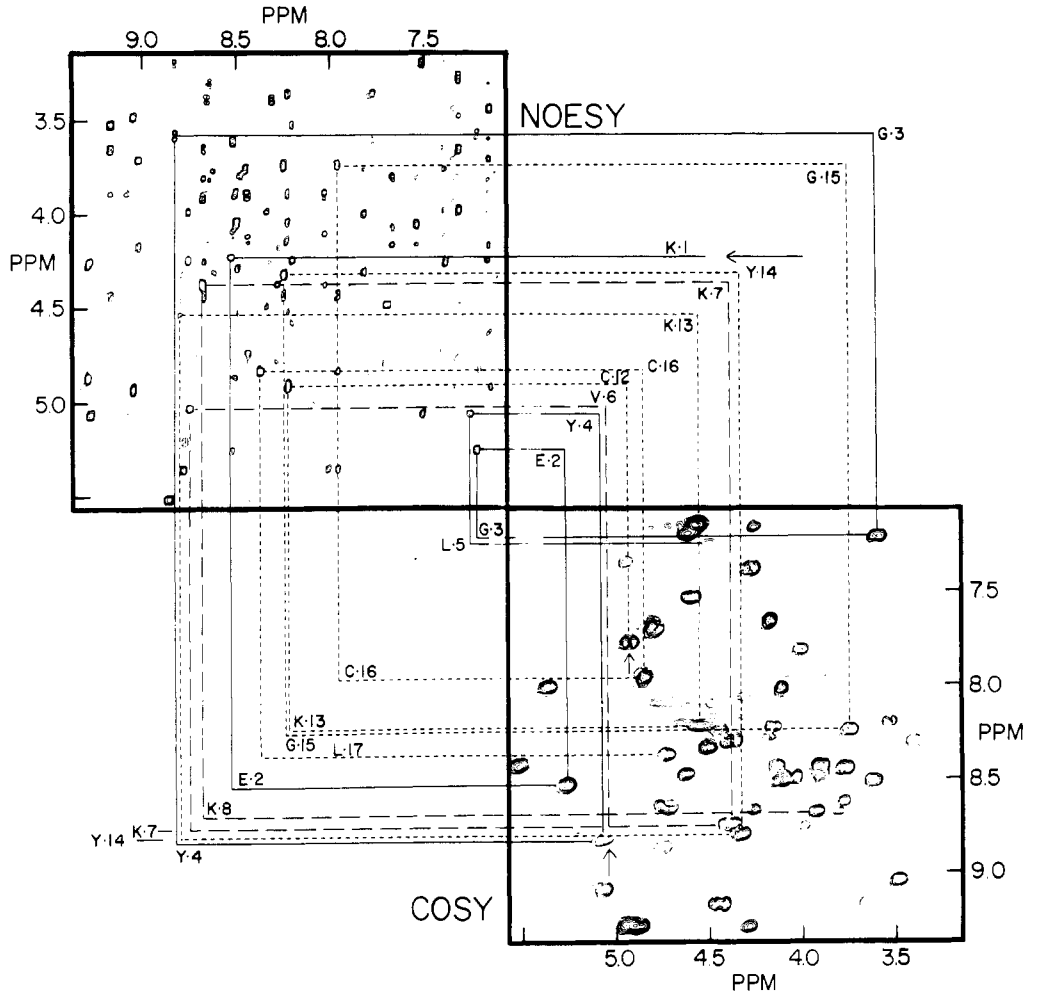

FIGURE 5: The 500-MHz 2D-COSY (absolute-value mode) and 2D-NOESY (phase-sensitive mode) NMR spectra of the CsE-v3 toxin in H_2O (pH 2.7, 313 K) showing the sequential $d_{\alpha N}$ connectivities established for the stretches of residues 1–5, 6–8, and 12–17. The NOESY connectivities for residues 8–12 are weak and have not been plotted in this figure. The connectivity between residues 9 and 10 was observed only at 303 K.

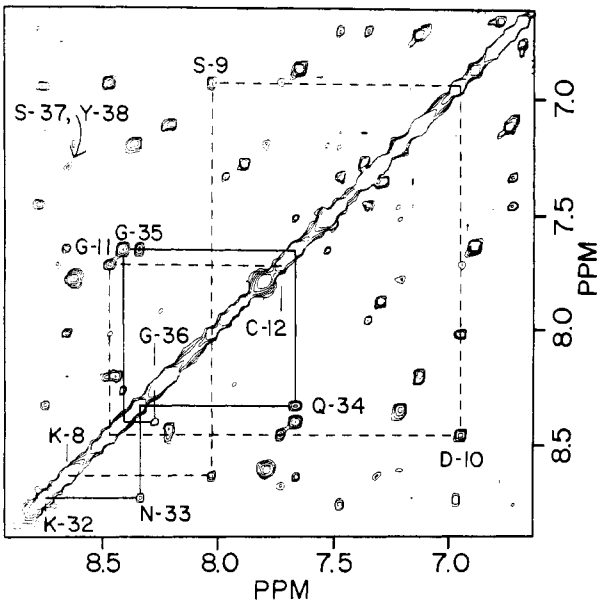

FIGURE 6: d_{NN} connectivities established for residues 8–12 and 32–36 in the 500-MHz 2D-NOESY spectrum of the CsE-v3 toxins (90% H_2O, pH 2.7, 303 K). Shifted sine bell (by 35°) function was used in both the t_1 and t_2 dimensions before the Fourier transformation.

dipeptide GG at positions 35 and 36. There was a break in the connectivity between residues 29 and 30 and between residues 38 and 39. At this point, residue 39 remains unassigned.

Residues 40–51. In this stretch, the tetrapeptide segment YAFA corresponding to residues 42–45 was first unambiguously identified from the prior identification of the amino acid types and from the observed $d_{\alpha N}$ contacts. These assignments were then extended on either side to 41 and 47 following the $d_{\alpha N}$ connectivities. Observation of a $d_{\beta N}$ cross-peak between the amide proton of Cys-41 and the $C_\beta H$ protons of a tyrosine (identified earlier) complete the assignment of Tyr-40. Sequence-specific assignment of residues 48–51 (CEGL) became possible only after finding that the amide hydrogen of a glycine showed a d_{NN} contact to another amide hydrogen. This latter amide proton was identified to be from a leucine on the basis of the intra NOESY contacts between this amide proton and the two CH_3 groups. The only location where a GL dipeptide occurs in the sequence is at positions 50 and 51. Following $d_{\alpha N}$ contacts with Gly-50, residue 49 and subsequently 48 could be identified. No connectivity could be observed between 47 and 48. The $C^\alpha H$ and the $C^\beta H$ protons of Leu-51 did not show any connectivity peaks to the amide hydrogen of the next residue up in the sequence. This is compatible with Leu-51 being followed by a proline (residue 52). Residue 52 remains unassigned.

Residues 53–65. Assignment of this stretch was done in sections. First, residues 56–58 (PTY) were assigned by locating the $d_{\alpha N}$ and $d_{\beta N}$ cross-peaks from the unique dipeptide

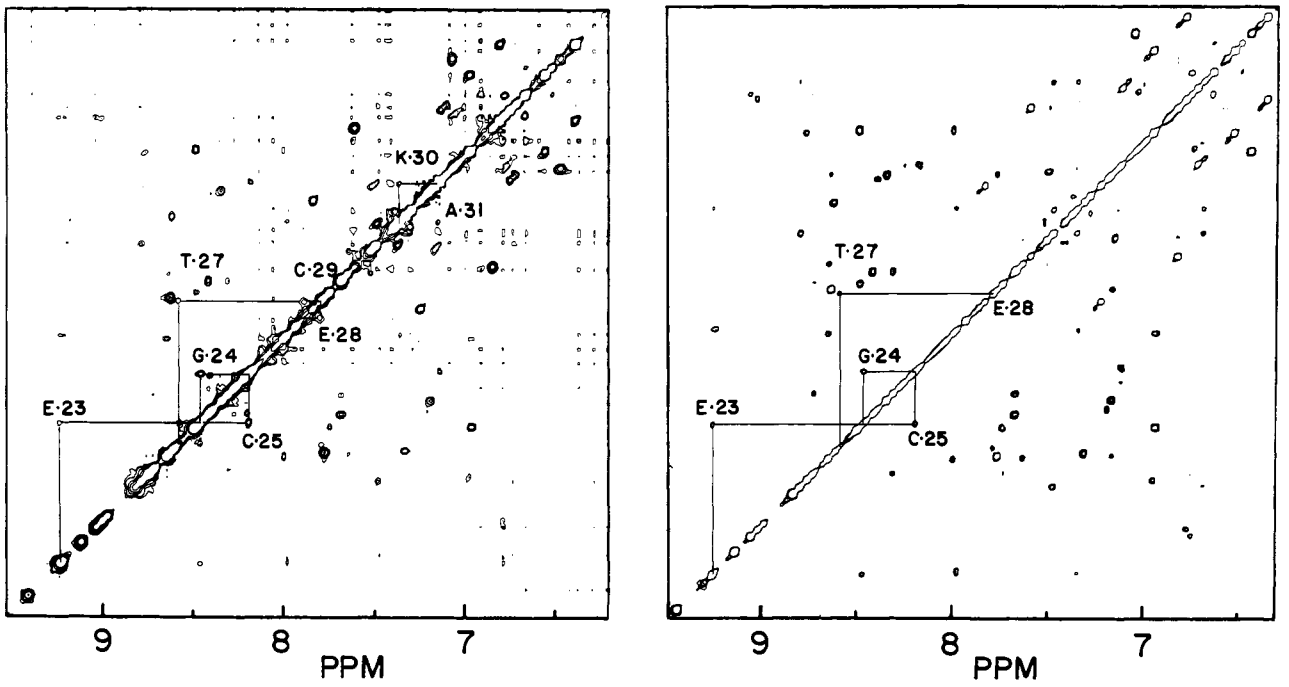

FIGURE 7: Identification of d_{NN} sequential connectivities for the residues 23–30 in the CsE-v3 neurotoxin at 313 K. The spectrum on the left was obtained with the use of shifted sine bell (by 35°) in both the t_1 and t_2 dimensions prior to Fourier transformation. The spectrum on the right was obtained with a 45° shifted sine bell in the t_1 direction and an unshifted sine bell in the t_2 direction.

TY and extending the assignment to Pro-56 from a $d_{\alpha N}$ cross-peak. Having assigned two (Thr-27, Thr-57) of the three threonines as described above, the remaining threonine was assigned to be residue 55 by elimination. The $C^{\alpha}H$ proton of this residue showed a NOESY connectivity to two methylene protons of a long side chain residue (in D_2O solution). This connectivity was interpreted to be $d_{\alpha N}$ type involving the $C^{\delta}H_2$ of Pro-56. Following the $d_{\alpha N}$ and the $d_{\beta N}$ cross-peaks from Thr-55, the preceding residues 54 and 53 were assigned. The next segment assigned involved residues 59–65, and this was less straightforward. One starting point was provided by the identification of a NH–$C^{\alpha}H$ COSY cross-peak at 313 K from an AMX spin system (identified later as residue 65). No 2D-NOESY cross-peaks involving this amide proton could be observed at 313 K. Interestingly, at the lower temperature of 303 K, this COSY cross-peak essentially disappears with the concomitant appearance of a $d_{\alpha N}$ cross-peak involving this amide proton and the $C^{\alpha}H$ proton of another amino acid (residue 64). The amide proton of this latter residue (identified from the 2D-COSY spectra at both 313 and 303 K) shows both $d_{\alpha N}$ and $d_{\beta N}$ contacts to a long side chain residue. Following a d_{NN} contact from this residue and a subsequent $d_{\alpha N}$ contact, the sequence terminated with what appeared to be a proline residue, as evidenced by the absence of any kind of peaks involving an amide proton from this residue. Hence, the $C^{\alpha}H$ proton of this residue was assigned to Pro-61, and the residues 62–65 were assigned from the NOESY contacts mentioned earlier. The assignments for residues 59 and 60 were made from the $d_{\alpha N}$ and the $d_{\beta N}$ contacts between an unassigned amide hydrogen and the $C^{\alpha}H$ and the $C^{\beta}H$ protons of a proline. The $C^{\alpha}H$ proton of residue 60 showed a NOESY contact with the methylene protons ($C^{\delta}H_2$) of a long side chain residue (Pro-61).

Assignment of Side-Chain Resonances. After the backbone NH and $C^{\alpha}H$ proton resonances were assigned for practically all of the residues in CsE-v3 toxin, the chemical shifts for the side-chain protons of the individual residues were obtained from the 2D-COSY spectra and from the intraresidue connectivities in the 2D-NOESY spectra. In regions where there is an extensive crowding of peaks, the RELAY–COSY experiment was useful in tracking down the proper COSY connectives.

Discussion

The principal objective of the current investigation is to provide sequence-specific assignments for the CsE-v3 toxin and to provide a basis for the determination of the solution-phase three-dimensional conformation for this protein. Table I provides the chemical shifts determined for the backbone and side-chain protons of the individual residues in the CsE-v3 toxin with the sequence-specific assignment procedure. A detailed analysis of the three-dimensional structure of the CsE-v3 toxin using the 2D-NOESY data sets obtained at 500 and 400 MHz in conjunction with the distance geometry calculations (Braun, 1987; Havel et al., 1983) will be reported elsewhere. However, it is instructive to compare, in a qualitative manner, some of the secondary structural features of this toxin deduced from the solution-phase NMR studies with the detailed crystallographic structure published in the literature (Almassy et al., 1983; Fontecilla-Camps et al., 1981, 1982).

The crystal structure is characterized by two and a half turns of α-helix involving residues 23–32 and a short three-strand stretch of β-sheet formed by residues 1–4, 37–41, and 46–50 (middle strand). We see evidence from the NMR studies for the presence of two of these β-strands (as indicated by $d_{\alpha N}$ connectivities) formed by residues 1–4 and 46–49. Our observation of cross-strand α–α proton NOEs between residues 2 and 49 and between residues 4 and 47 (data not shown) indicates that these two β-strands are in antiparallel arrangement. Because the Gly-39 residue did not contribute any observable resonances (presumably due to the overlap of its CH resonances with the solvent at both temperatures), we were not able to ascertain with certainty the $d_{\alpha N}$ connectivities corresponding to a β-strand for residues 37–41.

Figure 7 shows the d_{NN} contacts observed for residues 23–25, 27–29, and 30–31. No d_{NN} connectivity cross-peaks could be found connecting residues 29 and 30 at either of the

Table I: Proton Resonance Assignments of the Individual Residues in the Variant-3 Neurotoxin (pH 2.8, 313 K)

residue	NH	$C^{\alpha}H$	$C^{\beta}H$	other
Lys-1		4.14		
Glu-2	8.49	5.23	2.29, 2.05	
Gly-3	7.19	3.54, 1.85		
Tyr-4	8.80	5.04	3.19, 3.09	m.H 7.49; o.H 7.37
Leu-5	7.22	4.44	1.65, 1.40	$C^{\gamma}H$ 1.55; CH_3 0.66, 0.47
Val-6	8.75	4.99	1.79	CH_3 0.91, 0.69
Lys-7	8.71	4.35	1.82	
Lys-8	8.64	3.90	1.87	
Ser-9	8.00	4.08	3.87	
Asp-10	6.93	4.85	3.26, 2.76	
Gly-11	8.48	4.08, 3.59		
Cys-12	7.74	4.90	3.34, 2.81	
Lys-13	8.19	4.53	1.80, 1.27	
Tyr-14	8.77	4.30	2.90, 2.78	m.H 6.96; o.H 6.52
Gly-15	8.21	4.43, 3.71		
Cys-16	7.93	4.80	3.36, 2.93	
Leu-17	8.33	4.45	1.69	$C^{\gamma}H$ 1.61; CH_3 0.91, 0.86
Lys-18	7.16	4.57		
Leu-19	8.18	3.49	1.71, 1.35	$C^{\gamma}H$ 1.44; CH_3 0.70, −0.03
Gly-20	9.14	4.41, 3.64		
Glu-21	8.63	4.24	2.09	
Asn-22	9.24	4.85	2.90, 2.43	
Glu-23	9.25	4.26	2.21	
Gly-24	8.47	4.01, 3.87		
Cys-25	8.15			
Asp-26	7.92	4.02	3.08, 3.03	
Thr-27	8.55	3.75	4.29	CH_3 1.28
Glu-28	7.79	3.98	1.94, 1.59	$C^{\gamma}H$ 2.16
Cys-29	7.90			
Lys-30	7.35	4.25	1.50	
Ala-31	7.14	4.21	1.56	
Lys-32	8.72	3.98	1.92, 1.85	
Asn-33	8.30	4.47	2.93, 2.78	
Gln-34	7.68	4.79	2.33, 2.12	
Gly-35	8.41	3.86, 3.72		
Gly-36	8.27	4.33, 3.37		
Ser-37	8.63	4.68	3.88	
Tyr-38	7.32	4.90	3.44, 2.52	m.H 7.12; o.H 6.73
Gly-39				
Tyr-40		4.66	3.10, 2.88	m.H 6.81; o.H 6.63
Cys-41	9.05	5.04	3.86, 2.71	
Tyr-42	9.24	4.90	2.86, 2.53	m.H 6.78; o.H 6.34
Ala-43	9.02	3.45	1.05	
Phe-44	5.87	3.96	3.63, 3.24	m.H 7.28; o.H 7.39; p.H 7.33
Ala-45	7.97	5.33	1.30	
Cys-46	8.74	5.18	2.85, 2.66	
Trp-47	9.30	4.32+	2.81	ring protons (NH 9.6, C2H 6.53, C4H 5.79, C5H 6.68, C6H 7.06, C7H 7.16)
Cys-48	8.39	5.49	2.48	
Glu-49	8.82	4.69		
Gly-50	8.41	4.14, 3.91		
Leu-51	8.20	4.12	1.38, 1.18	$C^{\gamma}H$ 1.30; CH_3 0.74, 0.34
Pro-52				
Glu-53	8.65	3.79	2.56, 2.10	
Ser-54	7.63	4.14	4.05, 3.79	
Thr-55	7.50	4.55	4.03	CH_3 1.52
Pro-56		4.47	2.25, 1.75	$C^{\gamma}H$ 1.9; $C^{\delta}H$ 3.83; $C^{\delta}H$ 4.06
Thr-57	7.66	4.73	4.35	CH_3 0.56
Tyr-58	8.26	4.14	2.98, 2.77	m.H and o.H 7.13
Pro-59		3.69	1.68, 1.11	$C^{\gamma}H$ 1.75; $C^{\delta}H$ 3.59; $C^{\delta}H$ 3.44
Leu-60[a]	8.98	4.56	1.85	$C^{\gamma}H$ 1.68; CH_3 0.96, 0.93
Pro-61		4.13	2.3, 1.8	$C^{\gamma}H$ 1.97; $C^{\delta}H$ 3.62; $C^{\delta}H'$ 3.85
Asn-62	8.17	4.61	2.96	
Lys-63	7.12	4.53	1.57	
Ser-64	8.44	4.60	3.78	
Cys-65	8.61	4.70	3.25, 3.0	

[a] At 30 °C.

temperatures. Similarly, residue 26 failed to show d_{NN} cross-peak to its two neighboring residues at both these temperatures. In general, the intensities of the d_{NN} cross-peaks in the stretch 23–31 tended to be very weak. We have however found $d_{\alpha(i)\beta(i+3)}$ cross-peaks (data not shown) between residues 23 and 26 and between residues 27 and 30. These data suggest an α-helical conformation for residues 23–31. The generally weak intensities observed for the α-helical region may be a consequence of a rather dynamic conformation for the helix or of a partial destabilization of the helix under the experimental conditions (pH, temperature, etc.) employed in this study. On the basis of the predictions of the helix dipole model for the charged group effect (Blagdon & Goodman, 1975; Shoemaker et al., 1985), protonation of the acidic groups near the N-terminus (and/or the deprotonation of basic groups near the C-terminus) of an α-helix would tend to destabilize the helix. At the pH employed in the present study on the CsE-V3 toxin, both Glu-23 and Asp-26 are protonated, and thus, the protonation of these residues could contribute to the helix destabilization.

It is a common practice to perform 2D-NOESY experiments on proteins in H_2O at acidic pH to minimize the transfer of solvent saturation to the amide protons. Our discussion above suggests that under these conditions, on the basis of the validity of helix dipole model with charged group effect, α-helices with acidic groups near the N-terminus might become destabilized and hence might contribute weak NOESY cross-peaks, or might escape detection altogether. Thus, it is advisable to supplement the 2D-NOESY data obtained at acidic pH with additional NOESY data obtained at a pH where the acidic groups are deprotonated, and yet the amide protons are not substantially exchange broadened. To avoid the transfer of saturation by chemical exchange from water protons to the amide protons at these pH values, water suppression techniques that avoid saturation of the solvent resonance [e.g., Hore (1983)] could be gainfully employed.

Our data (Figure 6) also indicate turns at residues 8–12 and 32–35, in general agreement with crystallographic structure. The cis conformation observed for Pro-59 in the crystal structure is also preserved in the solution phase, as indicated by the presence of a NOESY contact between the α-protons of Tyr-58 and Pro-59 (data not shown). We have not discussed the aromatic residues in this paper; a detailed discussion of the microenvironment of the aromatic residues on the basis of NOESY experiments and from an analysis of the pH dependence of their proton chemical shifts will be presented elsewhere (Krishna et al., 1989).

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