Proton Nuclear Magnetic Resonance Characterization of the Aromatic Residues in the Variant-3 Neurotoxin from *Centruroides sculpturatus* Ewing†

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ABSTRACT: The amino acid sequence for the variant-3 (CsE-v3) toxin from the venom of the scorpion *Centruroides sculpturatus* Ewing contains eight aromatic residues. By use of 2D NMR spectroscopic methods, the resonances from the individual protons (NH, $C^{\alpha}H$, $C^{\beta}H',H''$, and the ring) for each of the individual aromatic residues have been completely assigned. The spatial arrangement of the aromatic ring systems with respect to each other has been qualitatively analyzed by 2D-NOESY techniques. The results show that Trp-47, Tyr-4, and Tyr-42 are in close spatial proximity to each other. The NOESY contacts and the ring current induced shifts in the resonances of the individual protons of Tyr-4 and Trp-47 suggest that the aromatic ring planes of these residues are in an orthogonal arrangement. In addition, the spatial proximity of the rings in the pairs Tyr-4, Tyr-58; Tyr-42, Tyr-40; and Tyr-40, Tyr-38 has also been established. A comparison with the published crystal structure suggests that there is a minor rearrangement of the aromatic rings in the solution phase. No 2D-NOESY contacts involving Phe-44 and Tyr-14 to any other aromatic ring protons have been observed. The pH dependence of the aromatic ring proton chemical shifts has also been studied. These results suggest that the Tyr-58 phenolic group is experiencing a hydrogen-bonding interaction with a positively charged group, while Tyr-4, -14, -38, and -40 are experiencing through-space interactions with proximal negatively charged groups. The Trp-47 indole NH is interacting with the carboxylate groups of two proximal acidic residues. These studies define the microenvironment of the aromatic residues in the variant-3 neurotoxin in aqueous solution.

The variant-3 (CsE-v3) neurotoxin from the venom of the scorpion *Centruroides sculpturatus* Ewing (range southwestern U.S.A.) is one of the least toxic of the different proteins present in the venom. Like other α-toxins, this toxin binds in a voltage-dependent manner to the sodium channels of excitable membranes and prolongs the inactivation of the sodium current (Meves et al., 1984). This activity is also shared by other α-toxins isolated thus far not only from *Centruroides* venom, viz., CsE-v1, CsE-v2, CsE-v4–v6, and CsE-V, but also from venoms of various species of scorpions, worldwide. On the other hand, the β-toxins such as CsE-I, -III, and -IV isolated from the *Centruroides* venom enhance activation of the inward sodium current through voltage-independent binding on the membrane (Meves et al., 1982). The various toxins also exhibit target specificity (Watt et al., 1978; Watt & Simard, 1984; Zlotkin et al., 1972). As part of a general program on defining the structure–function relationships among these *Centruroides* toxins, our laboratories have been investigating the three-dimensional structures of the individual toxins. The crystallographic structure of the CsE-v3 toxin has been reported in great detail (Almassy et al., 1983; Fontecilla-Camps et al., 1982, 1981). The crystallographic conformation is characterized by a continuous stretch of hydrophobic residues forming a hydrophobic surface on one side of the molecule. Such a hydrophobic surface might facilitate the interaction of the toxin with the nerve cell membrane at sodium channel sites. Some of the highly conserved amino acid residues in the various scorpion toxin sequences are contained in this hydrophobic surface (Fontecilla-Camps et al., 1982).

We have initiated the high-field NMR spectroscopic characterization of the CsE-v3 toxin to determine the solution-phase conformation of this protein. In an accompanying paper, the sequence-specific assignment of resonances in the proton NMR spectrum of the CsE-v3 has been reported (Nettesheim et al., 1989). In the current investigation we will discuss, in detail, the characterization of the individual aromatic residues and their microenvironment. This is of particular interest since of the eight aromatic residues in the CsE-v3 sequence (see Figure 1 in the accompanying paper) seven are contained in the hydrophobic patch shown in the crystal structure (Fontecilla-Camps et al., 1982). Of these, Tyr-4 and to a lesser extent Tyr-40 and Trp-47 are conserved. In the current investigation we will also describe briefly the assignment of the individual aromatic residues using 2D NMR spectroscopic methods. Because the ring protons from the aromatic residues usually resonate in a spectral window from 6 to 8 ppm without complications due to spectral overlap from the aliphatic protons, and further could be studied in D_2O solvent, a characterization of the aromatic residues serves as an important step toward the complete sequence-specific assignment of proton resonances from a protein and the subsequent determination of its three-dimensional conformation. A preliminary characterization of this toxin has been reported

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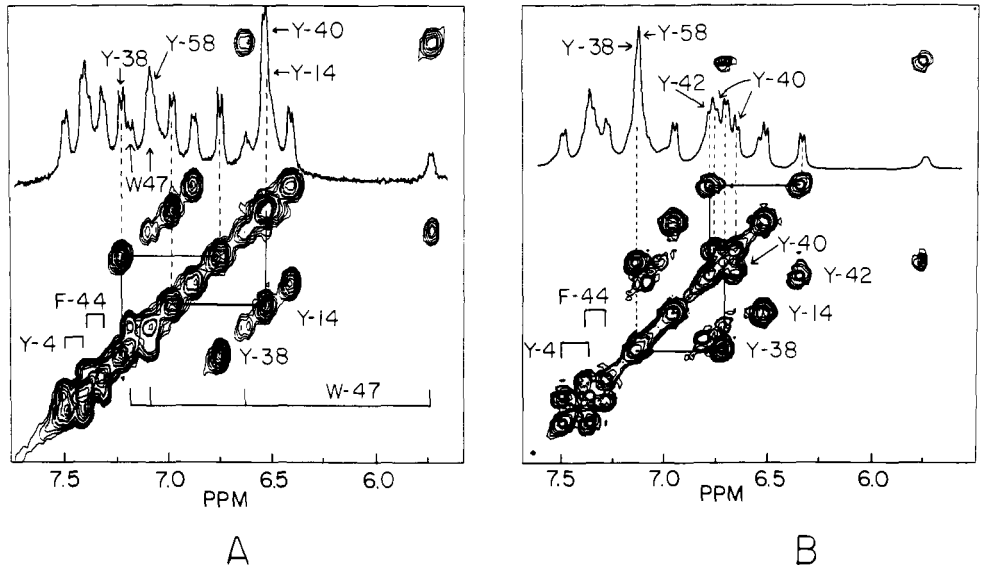

FIGURE 1: 400-MHz 2D-COSY NMR spectra of the aromatic C*H* resonances in the CsE-v3 toxin in D_2O at pH 2.8 (A, left) and at pH 8.5 (B, right). The 1D NMR spectra as well as the assignments and line positions for some of the residues are also shown.

earlier (Krishna et al., 1983).

Materials and Methods

The isolation and purification of the CsE-v3 toxin, the sample preparation, and the details of the 2D NMR spectroscopic measurements on this protein at 400 and 500 MHz are described in the accompanying paper (Netteshiem et al., 1989).

Results

There are eight aromatic residues within the amino acid sequence of the CsE-v3 toxin. These consist of six tyrosines (at positions 4, 14, 38, 40, 42, and 58), a single phenylalanine (at 44), and a single tryptophan (at 47). Figure 1 shows the 2D-COSY NMR spectra for the aromatic CH proton resonance region of the CsE-v3 toxin at two different pH values. In the low-pH spectrum, five of the six tyrosines are easily recognized from the cross-peaks between the ortho and meta proton resonances. The sixth tyrosine (identified as Tyr-58 during sequential assignment) failed to exhibit COSY cross-peaks presumably due to a partial degeneracy of its ortho and meta proton resonance chemical shifts in the pH range studied. The COSY cross-peaks connecting the ring protons C4H, C5H, C6H, and C7H for the lone trytophan are easily recognized in the spectrum at pH 8.5. The indole C2H peak (a singlet in D_2O) shows up as a shoulder to a tyrosine doublet at 6.5 ppm in the spectrum at low pH. The indole NH proton resonance was identified from its characteristically low-field chemical shift in a spectrum of the protein in H_2O and from its COSY cross-peak connectivity to the indole CH proton in a 2D-COSY experiment in H_2O. The resonances from the lone phenylalanine were identified from the characteristic AA'XX'M type of spectra for its ring protons in resolution-enhanced 1D NMR spectra and from the COSY cross-peaks between its C (3,5)H and C (2,6)H protons. The COSY cross-peaks involving its C4H proton are too close to the main diagonal to be discerned.

Having identified the ring proton chemical shifts for the individual aromatic residues in the CsE-v3 toxin, the next step toward the characterization of these residues involved the identification of the chemical shifts for the aliphatic protons ($C^{\alpha}H$, $C^{\beta}H',H''$) associated with each of these aromatic residues. This was most conveniently accomplished by a 2D-NOESY experiment on the protein in D_2O. Typical results of the NOESY spectra at pH 8.5, identifying the NOESY peaks that arise due to interaction between the ring C (2,6)H protons and the $C^{\alpha}H$ and $C\beta H',H''$ protons for all the six tyrosines and the lone phenylalanine, are shown in Figure 2. The tryptophan ring protons did not exhibit any NOESY cross-peaks to the aliphatic protons of this residue. They were identified by the sequence-specific assignment method at a later stage. The positions for the amide hydrogens of the six tyrosines and the phenylalanine were identified from the NH–$C^{\alpha}H$ COSY cross-peaks in the 2D-COSY spectrum of this protein in H_2O and were further confirmed from a RELAY–COSY spectrum in H_2O (not shown).

While the tryptophan and the phenylalanine proton resonances are easily assigned to positions 47 and 44, respectively, in the sequence because of their uniqueness (present only once in the amino acid sequence), the assignment of the individual tyrosines required the use of a sequence-specific assignment procedure by 2D-NOESY spectroscopy (Billeter et al., 1982; Wuthrich et al., 1982). Elsewhere, we have reported the detailed sequence-specific assignments for the backbone NH and $C^{\alpha}H$ and the side-chain CH proton resonances for CsE-v3 toxin (Nettesheim et al., 1989). A number of 2D NMR spectroscopic measurements such as 2D-COSY (Aue et al., 1976; Bax & Freeman, 1981; Nagayama et al., 1980), phase-sensitive 2D-NOESY (States et al., 1982), and RELAY- and double-RELAY–COSY (Eich et al., 1982; Bax & Drobny, 1985) were performed on the protein in H_2O and D_2O to extract the sequence-specific assignments for the individual residues in the toxin. The 2D-NOESY spectra of the protein in H_2O permitted us to identify the sequential connectivities [$d_{\alpha N}$, d_{NN}, and $d_{\beta N}$ as defined by Stassinopoulou et al. (1984)] between the backbone NH and $C^{\alpha}H$ and the side-chain $C^{\beta}H$ protons. Here, for the sake of completeness, we will briefly discuss the assignment procedure for a small stretch of residues, viz., residues 40–47, that contains four of the eight aromatic amino acids in the sequence. Figure 3 shows the sequential connectivities established in this stretch. A prior identification of some of the COSY cross-peaks according to the amino acid type (e.g., alanines, tyrosines, and Phe-44) facilitated the sequential assignment in this stretch. A convenient starting point is provided by the identification of the $C^{\alpha}H$–NH COSY cross-peak for the Phe-44 with the data in Figure 2 for the NOESY contacts between its ring protons and the $C^{\alpha}H$ and $C^{\beta}H',H''$ protons (also shown in

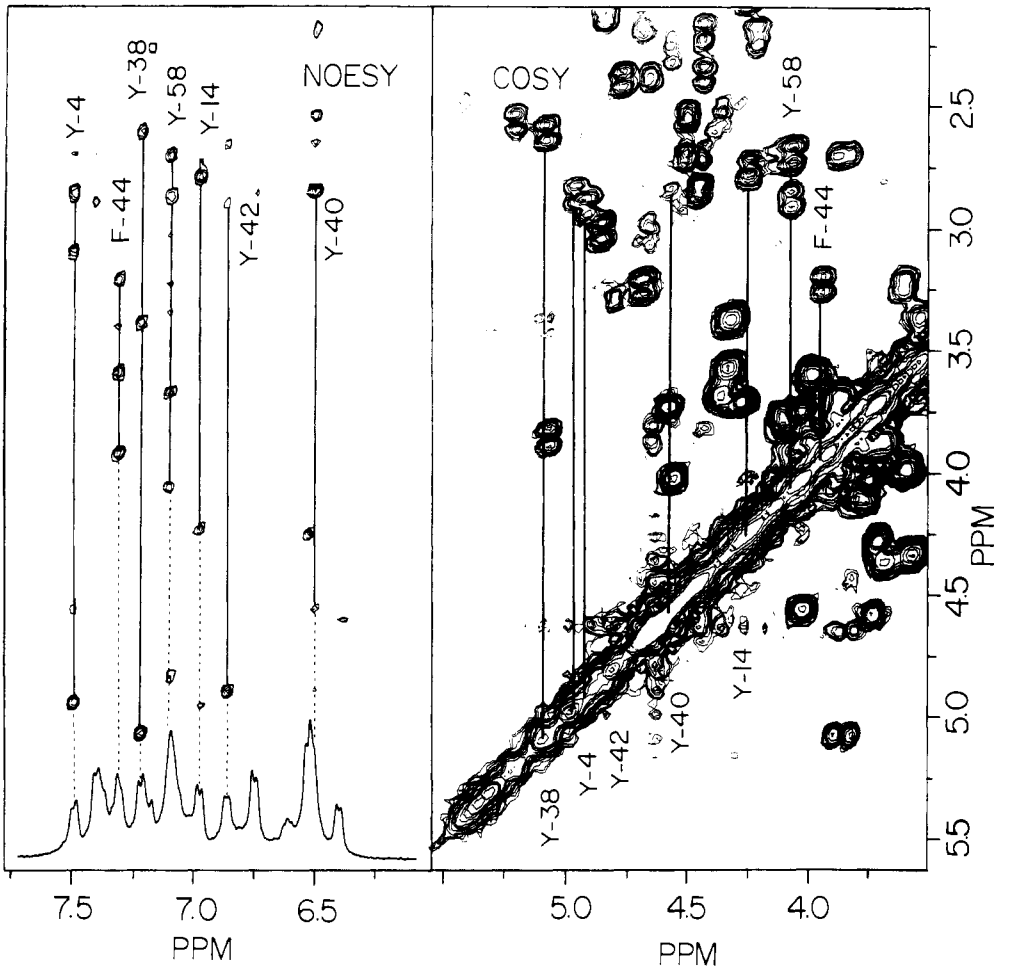

FIGURE 2: 2D-NOESY/COSY NMR spectra of CsE-v3/D_2O (pH 8.5, 400 MHz). The left panel shows the 1D NMR spectrum for the aromatic proton resonances along with the NOESY cross-peaks involving the ring protons and the $C^{\alpha}H$ and $C^{\beta}H',H''$ protons. The right panel shows the 2D-COSY spectrum for the aliphatic protons. The assignments for the protons of interest are also indicated in both the panels.

Figure 3) and the RELAY–COSY spectrum of the protein in H_2O. The $C^{\alpha}H$ proton of Phe-44 shows a weak $d_{\alpha N}$ connectivity (not shown in Figure 3) to the NH of an alanine which was assigned to residue 45. The NH of Phe-44 exhibited a $d_{\alpha N}$ connectivity to the $C^{\alpha}H$ of another alanine which was assigned to be Ala-43. d_{NN} connectivities have also been observed between residues 43–45. From Ala-43, the assignments for the preceding two residues were obtained by following the $d_{\alpha N}$ connectivities. The appearance of $d_{\beta N}$ connectivities between the NH of Cys-41 and the $C^{\beta}H',H''$ protons of a tyrosine completed the assignment for Tyr-40. Residues 46 and 47 were assigned by following the $d_{\alpha N}$ connectivities from Ala-45. The sequence-specific assignments for the remaining aromatic residues (Tyr-4, -14, -38, and -58) were completed in a similar manner.

The assignments for the individual aromatic residues in the CsE-v3 toxin, together with the chemical shifts for the NH, the $C^{\alpha}H$, the $C^{\beta}H',H''$, and the ring protons, are listed in Table I. Having completed the assignments, the microenvironment around each aromatic residue was probed by 2D-NOESY experiments and from the pH dependence of the ring proton chemical shifts.

2D-NOESY Measurements. Figure 4 shows the 2D-NOESY spectrum (200-ms mixing time) for the aromatic CH protons of the CsE-v3 toxin in D_2O. The presence of a significant number of cross-peaks indicative of ring–ring proton interactions is noteworthy in the spectrum. The ortho and meta protons of Tyr-4 show NOESY connectivities with the C4H and the C5H protons of Trp-47. The four cross-peaks

Table I: Proton Chemical Shifts for the Aromatic Residues in the Variant-3 Neurotoxin (pH 2.8, 310 K)

residue	NH	$C^{\alpha}H$	$C^{\beta}H$	ring protons
Tyr-4	8.80	5.04	3.19, 3.09	(2,6)H 7.49; (3,5)H 7.37
Tyr-14	8.77	4.30	2.90, 2.78	(2,6)H 6.96; (3,5)H 6.52
Tyr-38	7.32	4.90	3.44, 2.52	(2,6)H 7.12; (3,5)H 6.73
Tyr-40		4.66	3.10, 2.88	(2,6)H 6.81; (3,5)H 6.63
Tyr-42	9.24	4.90	2.86, 2.53	(2,6)H 6.78; (3,5)H 6.34
Phe-44	5.87	3.96	3.63, 3.24	(2,6)H 7.28; (3,5)H 7.39; (4)H 7.33
Trp-47	9.30	4.32	2.81	ring protons (NHC2H 6.53, C4H 5.79, C5H 6.68, C6H 7.06, C7H 7.16)
Tyr-58	8.26	4.14	2.98, 2.77	(2,6)H and (3,5)H 7.13

corresponding to these connectivities are equally intense. No other cross-peaks between the Tyr-4 ortho and meta protons and the remaining Trp-47 protons (e.g., C6H, C7H, C2H) are observed in the spectrum. Other interesting NOESY contacts that are established between the ring systems are Y-42–W-47, Y-4–Y-58, Y-4–Y-42, and Y-38–Y-40. These results are summarized in Table II. The 2D-NOESY spectrum also contains intra-NOESY cross-peaks between the C4H and C6H protons and between C5H and C7H protons of Trp-47 (distance 4.3 Å). The intensities of these weak cross-peaks serve as a convenient qualitative measure of the distances for the other aromatic ring–ring proton NOESY contacts. The weak intensity of these intra-NOESY cross-peaks, as well as our observation that the inter-NOESY contact between Trp-47 and Tyr-42 is confined to only one type of proton on each ring system, suggests that spin diffusion related effects are negli-

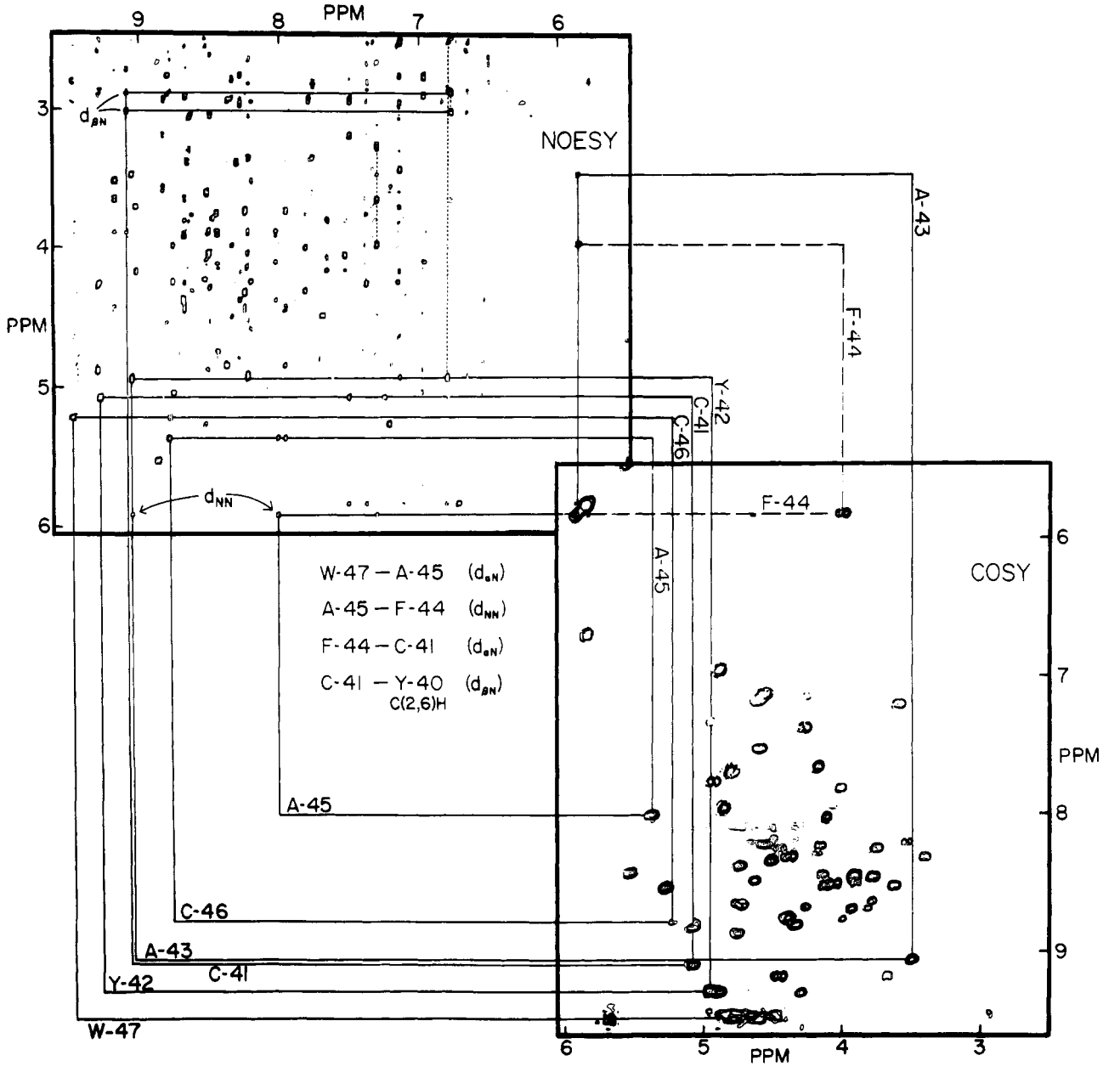

FIGURE 3: Sequential connectivities established for residues 41–47 in the CsE-v3 toxin (90% H_2O, pH 2.7, 310 K) on the basis of 2D-NOESY and COSY spectra obtained at 500 MHz. The details of the connectivities are described in the text.

Table II: Inter Aromatic Ring Proton 2D-NOESY Contacts in the Variant-3 Toxin

NOESY contacts	distance in the crystal structure (Å)[a]
Y-4 C(3,5)H/Y-42 C(2,6)H	3.93
Y-4 C(3,5)H/W-47 C4H	3.33
Y-4 C(3,5)H/W-47 C5H	2.78
Y-4 C(2,6)H/W-47 C4H	3.44
Y-4 C(2,6)H/W-47 C5H	3.06
Y-38 C(2,6)H/Y-40 C(2,6)H	2.6
Y-42 C(2,6)H/W-47 C4H	5.15[b]
Y-4 C(2,6)H/Y-58 (C2,6)H	3.93

[a] From 1.5-Å resolution data. Only the shortest distance is shown. [b] From 1.2-Å resolution data (unpublished).

gible at the mixing time (200 ms) employed in the studies. Tyr-14 and Phe-44 do not exhibit NOESY contacts to other ring protons.

pH Dependence of Aromatic Proton Chemical Shifts. We have monitored the pH dependence of the chemical shifts of the aromatic ring CH protons in D_2O and the Trp-47 indole NH in H_2O for the CsE-v3 toxin. The results are shown in Figures 5 and 6. The chemical shifts of these protons are sensitive to the spatial proximity of ionizable groups. The tyrosines also exhibit, in addition, the pH dependence due to the ionization of the phenolic groups. Only those ring CH protons whose chemical shifts could be followed unambiguously in the pH range studied are included in Figure 6. The pK_a values estimated from a least-squares fitting (Marquardt, 1963) of the data in Figures 5 and 6 to standard equations for multiple ionization (Dwek, 1975) are listed in Table III.

Discussion

An examination of the NMR spectrum for the aromatic protons in the CsE-v3 toxin shows that with the exception of Tyr-58 all other tyrosines exhibit characteristic AA'BB' type of spectra for the ortho and meta protons suggesting that these tyrosines are rapidly flipping about the C^{β}–C^{γ} axes at a rate that is fast compared to the NMR time scale. That two of the tyrosines, viz., Tyr-4 and Tyr-42, experience somewhat

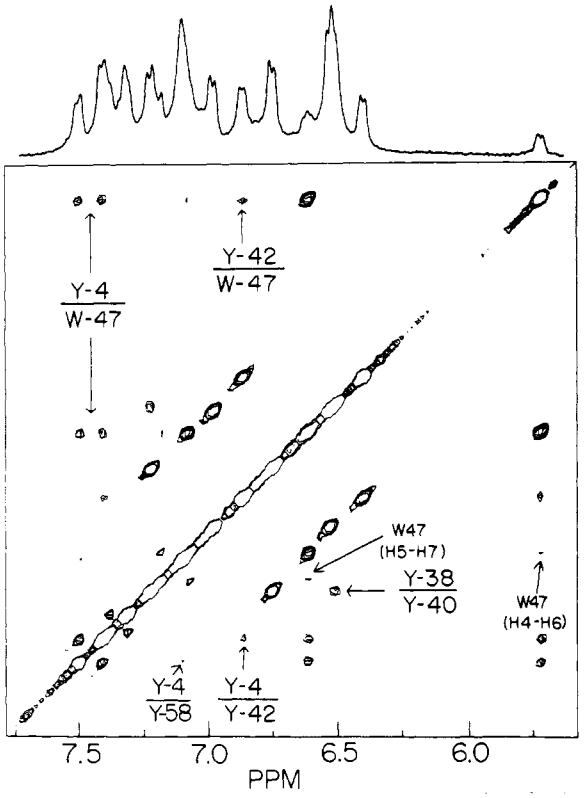

FIGURE 4: 2D-NOESY pure absorption mode NMR spectrum of CsE-v3/D_2O (pH 8.5, 310 K, 400 MHz) showing the aromatic ring–ring NOESY contacts. A mixing time of 200 ms was used. Also shown are intra-NOESY cross-peaks between the C4*H* and C6*H* protons and between the C5*H* and C7*H* protons (4.3 Å) of Trp-47.

restricted mobility compared to the others (e.g., Tyr-14, Tyr-38) is indicated by their line widths which are large enough to partially obscure the ortho–meta proton coupling (see Figures 1 and 2). The ortho and meta proton resonances of Tyr-58 are either partially or completely degenerate and could not be resolved in the pH range employed for the NMR measurements. It is also possible that one of the resonances is exchange broadened due to intermediate rate of rotation about the C^{β}–C^{γ} bond (Campbell & Dobson, 1979). This observation suggests an unusual environment for Tyr-58, which is further discussed below.

An examination of the chemical shifts listed in Table III for the various aromatic protons in the CsE-v3 toxin indicates that many of these protons experience strong ring current induced perturbations (Perkins, 1982) from proximal aromatic ring systems. For example, the ortho and meta protons of tyrosines exhibit substantial deviations from the random coil chemical shift values of 6.86 and 7.15, respectively (Bundi & Wuthrich, 1979). These deviations are also reflected in the chemical shift differences for the ortho and meta protons of the individual tyrosines (0.1 ppm for Y-40, 0.14 ppm for Y-4, 0.42 ppm for Y-38, 0.44 ppm for Y-14, and 0.45 ppm for Y-42) compared to the random coil value of 0.292 ppm (Bundi & Wuthrich, 1979). Similarly, the C4, C5, and C6 protons of Trp-47 exhibit substantial deviations from the random coil values of 7.65, 7.17, and 7.24, respectively, with the C4H proton exhibiting an upfield shift of as much as 1.87 ppm from its random coil value.

That these deviations in the chemical shifts from the random coil values are to a large extent due to ring current interactions is supported by the observation of several ring–ring proton NOESY contacts listed in Table II on the basis of the 2D-NOESY spectrum in Figure 4. The ortho and meta protons

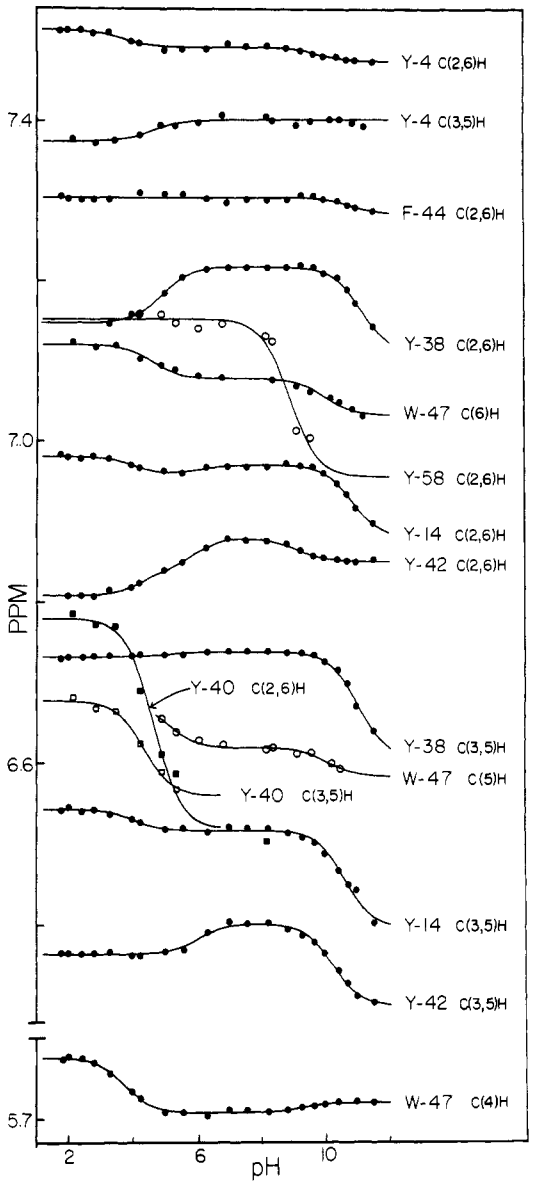

FIGURE 5: pH dependence of the aromatic CH proton resonance chemical shifts in the CsE-v3 toxin in D_2O. The solid curves represent the least-squares fitting of the data according to standard equations for multiple ionization.

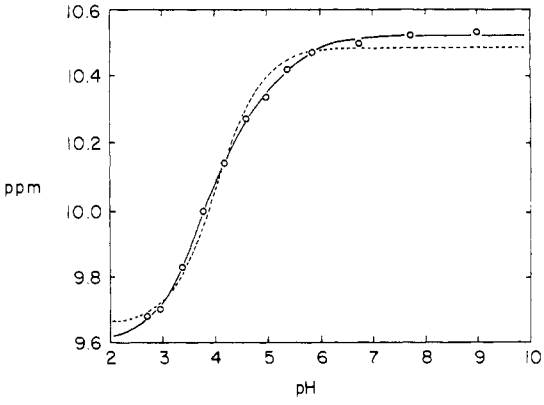

FIGURE 6: pH dependence of the Trp-47 indole N*H* resonance in CsE-v3/H_2O. The solid curve is the least-squares fitting obtained with pK_a values of 3.7 and 5.25. The dashed curve is the least-squares fit obtained with a pK_a of 4.04.

of Tyr-4 show strong NOESY contacts with the C4H and the C5H protons of Trp-47, and no contacts with the C6H and the C7H protons. These data suggest an orthogonal type of

Table III: Summary of pH Titration Results for the Aromatic Ring Protons in CsE-v3 Toxin[a]

	ppm_I	δ ppm	pK_a	δ ppm	pK_a	δ ppm	pK_a
Tyr-4							
(2,6)H	7.514	−0.0209	3.69			0.0179	9.78
(3,5)H	7.375						
Tyr-14							
(2,6)H	6.981	−0.0233	4.03	0.0121	6.06	−0.0890	10.80
(3,5)H	6.543	−0.0268	4.02			−0.1201	10.54
Tyr-38							
(2,6)H	7.148	0.0690	4.91			−0.1047	11.08
(3,5)H	6.732	0.0058	5.18			−0.1309	10.96
Tyr-40							
(2,6)H	6.781	−0.263	4.63				
(3,5)H	6.679	−0.1185	4.32				
Tyr-42							
(2,6)H	6.809	0.0291	4.25	0.0410	5.89	−0.0293	9.12
(3,5)H	6.364			0.0377	6.02	−0.1029	10.18
Tyr-58 (2,6)H	7.153					−0.198	8.89
Phe-44 (2,6)H	7.304					−0.0215	10.79
Trp-47 NH	9.605	0.697	3.70	0.217	5.25		
C(4)H	5.777	−0.0690	3.71			0.0116	9.22
C(5)H	6.677	−0.0575	5.09			−0.0363	10.01
C(6)H	7.121	−0.0428	4.61			−0.0456	9.96

[a] ppm_I, limiting value of chemical shift at low pH. δ ppm, change in the chemical shift due to ionization (for the pK_a value as shown).

arrangement of Trp-47 and Tyr-4 ring systems, with the C4H and C5H protons pointing toward the ring plane of Tyr-4. This also explains the unusual high-field shift observed for the C4H of Trp-47. The ortho protons of Tyr-4 also show a NOESY contact to the meta protons of Tyr-42, which in turn show a NOESY contact to the C4H of Trp-47. A very weak contact between the meta protons of Tyr-4 and the ring protons of Tyr-58 is also observed in the 2D-NOESY spectrum. The other ring–ring NOESY contact observed is the one between Y-38 and Y-40. These observations define a unique spatial arrangement of the aromatic rings from Y-4, W-47, Y-42, Y-40, Y-38, and Y-58 with respect to one another. Presumably, aromatic interactions of the type observed in the CsE-v3 toxin may play a crucial role in protein structure stabilization (Burley & Petsko, 1985). Even though no NOESY contacts to other aromatic ring protons were observed from Y-14 and F-44, these two could be close enough to other ring systems to experience ring current interactions, but far enough (distance greater than 4 Å) not to exhibit strong NOESY contacts at the mixing time employed in these studies. A comparison of the observed ring–ring NOESY contacts with the crystallographic data on the CsE-v3 toxin (Almassy et al., 1983; Fontecilla-Camps et al., 1982) shows that the side chains for some of the aromatic residues undergo some minor conformational rearrangement in the solution phase. In interpreting the experimental NOESY data, the effect of the side-chain motional dynamics should also be considered in principle, though here we use the NOESY cross-peaks as a qualitative measure of spatial proximity of a given pair of protons, so that one could envision the approximate spatial arrangement of some of the residues. For example, the shortest distance between the Trp-47 C4H and one of the meta protons (2,6H) of Tyr-42 in the crystallographic structure is 5.15 Å, a distance for which no significant NOESY cross-peak is expected for the mixing time used for the experiment in Figure 4 (as a qualitative measure, the intra-NOESY cross-peak between the C4H and the C6H protons, separated by 4.3 Å, shows up as a small period in this spectrum). Nevertheless, the 2D-NOESY spectrum exhibits a healthy cross-peak between these protons, indicating that these protons are closer than in the crystal structure. The possibility that this may be due to spin diffusion has been considered and ruled out by us (vide supra). This is supported by the failure of Y-42 (3,5) protons to show similar NOESY contacts. We have also considered the possibility that an aliphatic proton located between Trp-47 and Tyr-42 might mediate cross-relaxation between the two ring protons. Such an aliphatic proton would have exhibited strong NOESY cross-peaks to both Trp-47 and Tyr-42 ring protons; none were found. Further, the published crystal structure shows no such aliphatic proton (Fontecilla-Camps et al., 1982). In a similar fashion, the shortest distance between the Y-4 (3,5) and the Y-42 (2,6) protons, as well as between the pair of Y-4 (2,6) H and Y-58 (2,6) H, is 3.93 Å in the crystal structure. Thus, NOESY cross-peaks of similar intensity would be expected for these pairs of protons, a prediction that is at variance with the experimental NOESY data in Figure 4. The reason for these discrepancies becomes clear when one examines the crystal structure in greater detail—it shows that during the process of crystallization one of the solvent molecules used as a precipitating agent, viz., methylpentanediol (MPD), is situated with its hydrophobic side packed slightly into the apolar pocked formed by the aromatic rings of Trp-47, Tyr-4, Tyr-42, and Tyr-40 [see Figure 11 in Almassy et al. (1983)]. Thus, in the absence of a bound MPD molecule in the solution phase, the aromatic ring systems corresponding to these four residues assume their natural positions in the three-dimensional structure whereas in the crystal structure they undergo a minor conformational rearrangement to accommodate an MPD molecule. The observed discrepancies in the microenvironment of the aromatic ring systems between the solution and crystal structures thus appear to be the result of an artifact of crystallization. A detailed quantitative comparison of the three-dimensional structures deduced from the solution-phase NMR studies together with the distance geometry calculations (Braun, 1987; Havel et al., 1983) and the published crystal structure will appear elsewhere.

The pH dependence of the aromatic proton chemical shifts reflects the proximity of these protons to ionizable groups, as well as pH-dependent conformational changes. Least-squares fitting of the indole NH data (solid curve, Figure 6) suggests that this proton is in close proximity to two acidic groups with apparent pK_a values of 3.7 and 5.25. The dashed curve in the figure corresponds to a least-squares fitting of the same data for the ionization of a single acidic group (pK_a = 4.04). It is clear from Figure 6 that the curve with two pK_a values gives much better fit. A statistical analysis of the indole NH data using Hamilton's R-factor ratio criterion (Hamilton, 1965) showed that the fitting with a single pK_a could be rejected at

better than 99.5% confidence level. The crystal structure shows that the indole NH is in close contact with the carboxylate groups of Glu-2 and Glu-49. Even though we have not yet established the identity of these two acidic groups by NMR spectroscopy, our NMR data appear to suggest that this microenvironment for the indole NH is preserved in the solution phase as well, though a definitive conclusion to this effect must await model building efforts currently in progress. It is interesting that the two acidic groups exhibit substantial deviations in their pK_a values from the random coil value of 4.3 expected for Glu (Bundi & Wuthrich, 1979). The lower pK_a of 3.7 for one acidic residue might be the result of a hydrogen-bonding interaction between the carboxylate group and a proton from a donor group (e.g., W-47 indole NH or the hydroxyl proton of a proximal tyrosine). The elevated pK_a of 5.25 for the second acidic group might be the result of its spatial proximity to the first acidic group. The pH dependence of the chemical shifts for the remaining protons of Trp-47 reflects their proximity to an acidic group and to the phenolic group of a tyrosine. Five of the six tyrosines exhibit inflections in their pH vs chemical shift curves with pK_a values ranging from 3.7 for Tyr-4 to 5.2 for Tyr-38. These may be associated with through-space effects from proximal acidic groups such as aspartic and glutamic acids. The phenolic groups titrations observed for the different tyrosines show some variations from the random-coil value of 10.3 for a solvated tyrosine (Bundi & Wuthrich, 1979) and reflect local environments. Of particular note is the Tyr-58 for which a pK_a of 8.9 is observed. The lowering in the pK_a value (from 10.3) may be the result of a hydrogen-bonding interaction with the charged group of a basic amino acid such as lysine. Such an interaction is expected to lower the pK_a of the tyrosine and raise the pK_a of the basic group. Examination of the crystal structure reveals that the side-chain amino group of Lys-13 is located adjacent to the Y-58 ring system, though no hydrogen-bonding interaction is present. Additional inflections observed for Tyr-14 and Tyr-42 with pK_a values close to 6 may reflect pH-dependent conformational changes.

In summary, in the present investigation we have provided absolute asssignments for the individual proton resonances of all the aromatic residues in the CsE-v3 toxin by 2D NMR spectroscopy. An analysis of the 2D-NOESY data establishes the presence of a cluster of ring systems formed by the residues Y-4, Y-38, Y-40, Y-42, W-47, and Y-58 that exhibit aromatic–aromatic ring interactions in this small protein. Two of the remaining aromatic residues, viz., F-44 and Y-14, appear to be sufficiently far away not to show any significant NOESY contacts with the other atomatic protons. A qualitative comparison of the microenvironment for the aromatic residues based on the solution-phase NMR studies and the published crystallographic data for the CsE-v3 toxin suggests that these two are essentially similar in gross features, although they differ somewhat in terms of a minor rearrangement for some of the aromatic residues. This difference may be the result of an artifact of crystallization. Our studies on the characterization of the aromatic residues provide a basis for further studies on the function of these residues in terms of their role in stabilizing the tertiary structure of the scorpion neurotoxins, as well as their role in facilitating interactions with the sodium channel.

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