Structure of Apamin in Solution: A Two-Dimensional Nuclear Magnetic Resonance Study†

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ABSTRACT: A two-dimensional (2-D) Fourier-transform nuclear magnetic resonance (NMR) study of the 18 amino acid neurotoxin apamin isolated from honeybee venom is reported. Combining 2-D *J*-correlated spectra with 2-D nuclear Overhauser spectra in H_2O solution permits essentially complete assignment of the ^1H NMR spectrum of apamin at a fixed pH, including a number of spin systems that are reported for the first time. The structural model previously derived by Bystrov et al. [Bystrov, V. F., Okhanov, V. V., Miroshnikov, A. I., & Ovchinnikov, Yu. A. (1980) *FEBS Lett. 119*, 113–116] from NMR data is shown to be largely correct. The 2-D nuclear Overhauser effect (NOE) spectrum in particular reveals a series of amide–amide NOE's consistent with an α-helical core (residues 9–15) in the molecule. NOE's between amide and C^α protons, followed by amide to amide NOE's, indicate a β-turn involving residues 3–5 and a nonstandard turn including residues 6–8. We find no evidence for the β-type structure postulated at the C terminus, however. Instead, it appears that the α-helix continues with increasing fraying from residues 16–18.

The neurotoxin apamin from honeybee venom is among the smallest known peptide toxins and possesses unusual functional as well as structural properties (Habermann, 1972). Apamin is remarkable among peptides in its ability to cross the blood–brain barrier and act on the central nervous system. Its primary structure consists of the 18 amino acids shown in Figure 1, with two disulfide bridges connecting the four cystine residues in an overlapping pattern that recurs in at least one other venom peptide component (Gauldie et al., 1978). The secondary or tertiary structure of apamin is exceptionally stable with respect to pH, temperature, and denaturants (Miroshnikov et al., 1978). Its size and stability make apamin an excellent molecule for NMR[1] investigations. Bystrov and his co-workers have reported extensive ^1H and ^{13}C NMR studies on apamin (Bystrov et al., 1978; Okhanov et al., 1980). Relying primarily on information derived from hydrogen exchange rates and vicinal coupling constants, Bystrov et al. (1980) have presented a structural model of apamin consisting of an α-helical core flanked by two regions with β-type turns. The model is generally consistent with predictions of theoretical conformational analysis by Hider & Ragnarsson (1981), as well as with CD spectral data (Miroshnikov et al., 1978).

We show here that application of 2-D FT NMR [reviewed by Freeman & Morris (1978)] makes it possible to complete and correct the assignments of the ^1H spectrum of apamin. In particular, the 2-D nuclear Overhauser experiment not only provides information crucial to assigning a number of resonances but also identifies both the α-helix and β-turn structures in this molecule. Our revised structure lacks the β-turns proposed by Bystrov et al. (1980) to exist at the C terminus of the molecule. We believe the α-helix continues to the C terminus as suggested by Hider & Ragnarsson (1981) but with progressively increasing fraying from residues 16–18.

Materials and Methods

Apamin was prepared from lyophilized venom of *Apis mellifera* (purchased from Sigma as grade IV) following the procedure of Gauldie et al. (1976). Two forms of apamin occur in the venom, one with the N-terminal amino group free (apamin 1) and one with this group formylated (apamin 2). The N-blocked species is the major contaminant of the purified material we have prepared (see Figure 2); it is present at 10% of the concentration of apamin 1, the major species present, and does not contribute to the 2-D analysis. The extinction coefficient of apamin was determined by ninhydrin assay (Crestfield et al., 1963). Two-dimensional NMR spectra were recorded on solutions of 0.05 M apamin, in a solvent containing 0.0 or 0.13 M NaCl, pH 2, and 10% D_2O.

NMR Spectroscopy. Two-dimensional *J*-correlated (Aue et al., 1976; Bax et al., 1981) and NOE (Jeener et al., 1979; Kumar et al., 1980a,b) spectra were recorded on the modified Bruker HXS 360 spectrometer equipped with a Nicolet 1180 data system at the Stanford Magnetic Resonance Laboratory. Each 2-D experiment required 512 measurements with delays of $0 < t_1 < 75$ ms for both pulse sequences. Data blocks were zero filled to give a 512×512 point matrix in the frequency domain. Correlated spectra designed to enhance weak couplings were carried out with an additional 50-ms delay following each of the 90° pulses (Bax & Freeman, 1981). Spectra were symmetrized by the method of Baumann et al. (1981). The solvent H_2O signal was suppressed by continuous low-power irradiation during all time periods except t_2. For NOE spectra, a mixing time of 300 ms was used, and for some spectra, additional solvent suppression was obtained by audio-frequency notch filtering (Marshall et al., 1979). All spectra reported here were taken at 25 °C.

Results and Discussion

The most difficult problem in ^1H NMR spectroscopy of proteins is generally that of assigning resonances to individual proton sites in the molecule. Many assignments in apamin have been made previously, with classical homo- and heteronuclear spin-decoupling techniques, pH dependence of chemical shifts, and $^3J_{^{13}CNC^\alpha H}$ couplings in certain cases (Okhanov

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[1] Abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; FT, Fourier transform; CD, circular dichroism.

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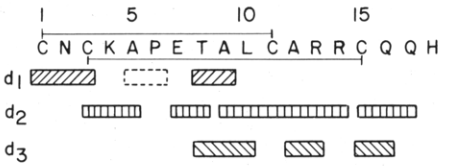

FIGURE 1: Primary sequence of neurotoxin apamin, with a summary of observations at each residue position used for sequential resonance assignments. The distances are $d_1 = d(C^{\alpha} H_i, N H_{i+1})$, $d_2 = d(N H_i, N H_{i+1})$, and $d_3 = d(C^{\beta} H_i, N H_{i+1})$ (Billeter et al., 1982). Bars indicate residues for which an NOE between designated protons was observed, indicating a distance <3 Å approximately. The dashed box in d_1 represents an NOE from the proline δ-H to the C^{α} H of residue 5.

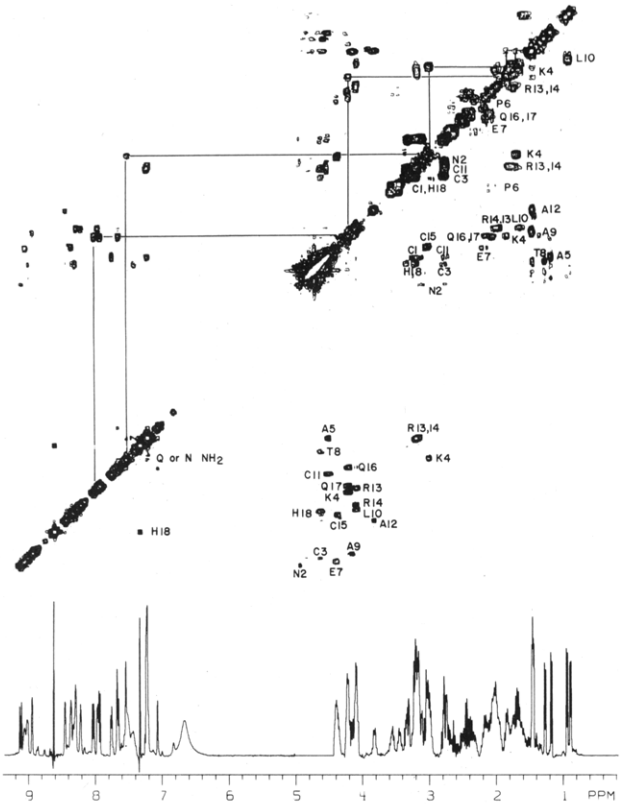

FIGURE 2: 360-MHz two-dimensional autocorrelated spectrum of apamin (50 mM, pH 2, in 90% H_2O–10% D_2O). $T = 25$ °C. The spectrum contains 512 points in each dimension and has been symmetrized. The connectivity of the resonances of lysine-4 are indicated with a solid line; cross peaks are labeled according to residue.

et al., 1980). Even in a small molecule that exhibits very highly resolved ^1H spectra, this approach is limited by its low sensitivity and inability to discriminate among residues for which the α-protons have the same chemical shifts. Instead, we have applied the approach of sequential assignments on the basis of NOE measurements between adjacent residues in the chain. The method and its application to the pancreatic trypsin inhibitor and glucagon have been described in detail by Wuthrich and his co-workers (Wuthrich et al., 1982; Billeter et al., 1982; Wagner & Wuthrich, 1982; Wider et al., 1982). Briefly, the procedure is as follows. The first step is to identify resonances belonging to the same residue; this is done primarily by using the *J*-correlated spectrum (Figure 2; see also Figure 3). The number of protons in a spin system and their chemical shifts relative to calibrations on free amino acids (McDonald & Phillips, 1969) and small peptides (Bundi & Wuthrich, 1979a,b) make it possible to determine the type of amino acid, in a number of cases, normally including the amino acids A, R, I, L, K, P, T, and V. If the type of actual amino acid cannot

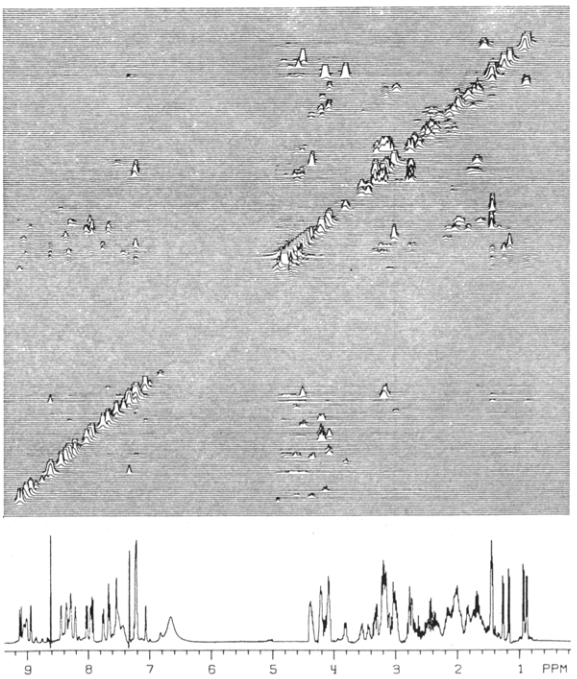

FIGURE 3: A stacked plot representation of the 2-D autocorrelated spectrum in Figure 2, showing magnitudes of cross peaks.

be specified, at least one can classify spin systems as belonging to a group of possible amino acids; AX_2 patterns, for example, correspond to N, D, C, H, F, S, W, or Y; see Wider et al. (1982) for further examples. The usual difficulty arises when more than one resonance occurs at a particular chemical shift, as results when several residues of the same amino acid occur in similar environments. Tracings for one complete system in apamin and identification of cross peaks are indicated in Figure 2. Certain side-chain resonances lack sufficiently strong spin connectivities to identify them; these include most aromatic groups as well as most exchangeable hydrogens. For these, the through-space connectivities determined by NOE have to be used.

In apamin, it is possible to identify the spin systems of all nonexchangeable C^{α} H and side-chain hydrogens. Ambiguities arise for several backbone N H's, however, since the C^{α} H positions are degenerate. Complete assignment of the spin system in H-18 is made possible by observation of four-bond couplings C2 H–C4 H and C4 H–C^{β} H; in larger molecules with broader resonances, this might not be possible. Coupling between two pairs of side-chain amides from Q and N was observed, but not to the rest of the side chain. These weak peaks were confirmed by their greatly increased intensity when extra delays were included following the 90° pulses in the correlation spectral sequence (Bax & Freeman, 1981). Reasonably strong peaks were observed for the ϵ-CH_2–NH_3 coupling in K and the δ-CH_2–ϵ-NH coupling in both R side chains.

The next step in sequential assignment requires the through-space connectivities provided by the NOE experiment. These both complete the assignments in apamin and furnish fundamental structural information. The critical observation is that in proteins of known structure NOE's between amide and non-nearest-neighbor protons are rare (Billeter et al., 1982). With high probability, the amide of a particular residue will be close enough to either the α-proton, amide proton, or β-proton of its adjacent residue to exhibit a significant NOE. Guided by the primary sequence, sequential networks of NOE's can be identified that assign residues with subsections or segments of the protein chain. These stretches are usually interrupted at proline residues. Billeter et al. (1982) have

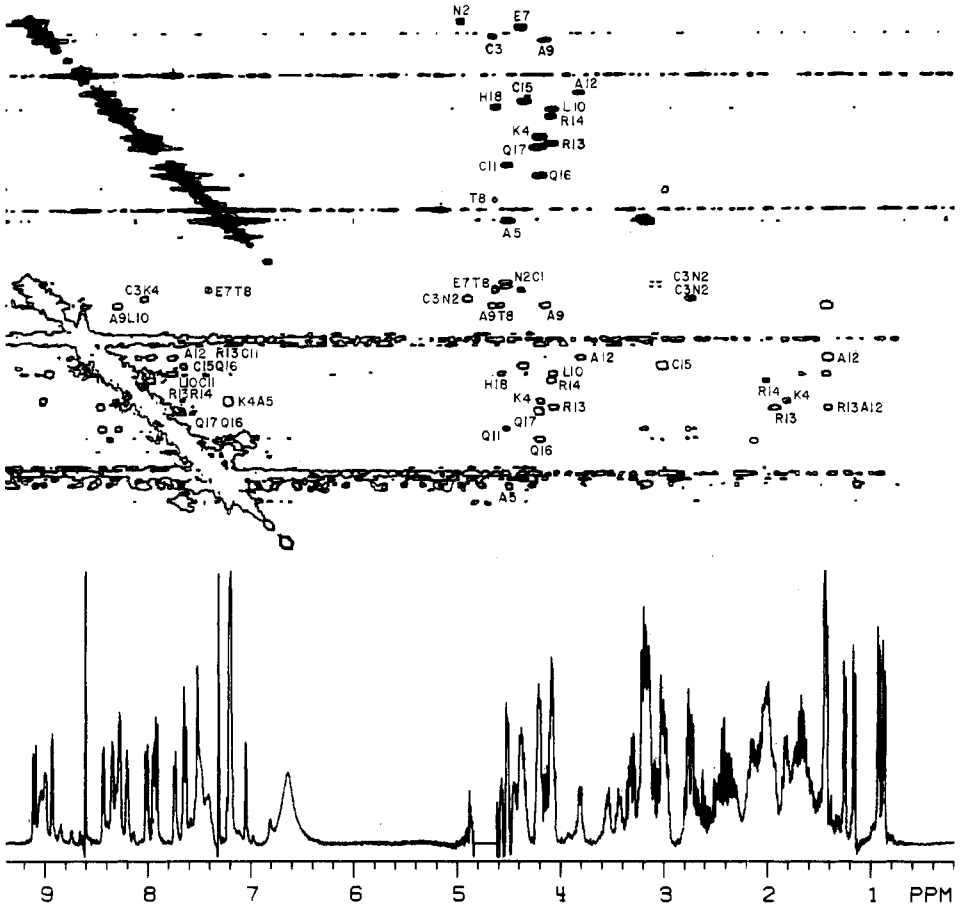

FIGURE 4: Expansions of two-dimensional correlated (top) and NOE (middle) spectra, with a normal spectrum (bottom), taken on the same sample as that in Figure 1. A mixing time of 300 ms was used in the NOE experiment.

introduced a convenient notation for the distances, d, between the three protons that tend to exhibit NOE's from one residue to the next in the chain:

$$d_1 = d(C^{\alpha} H_i, N H_{i+1}) \qquad d_2 = d(N H_i, N H_{i+1})$$
$$d_3 = d(C^{\beta} H_i, N H_{i+1})$$

Figure 1 summarizes the basis of assignments in apamin from the NOE experiment, the terms of these NOE distance measurements. The amide regions of the correlated and NOE spectra are shown together in Figure 4. Since apamin contains only one proline, the assignments of two sequential segments, residues 1–5 (I) and 7–18 (II), broken at P-6 can be considered separately.

Segment I. Assignments in segment I rely on d_1 and d_2 NOE's. Residue K-4 is unique; the NOE between its amide and that of an alanine (d_2) identifies A-5. A second NOE connects the amide of K-4 to a cystine amide, which must be C-3. An NOE from the C-3 amide to the furthest downfield amide identifies N-2, which in turn exhibits an NOE to a C^{α} H, which has no corresponding amide, corresponding to C-1. We see no signal attributable to the terminal NH_3^+, as expected because of its rapid exchange with water.

Segment II. Many assignments in this segment also depend on d_2 NOE's. The amide region of Figure 4 is shown expanded in Figure 5. Much of the H-18 spin system can be identified by spin–spin couplings. The C^{α} H position of H-18 coincides with that of T-8, however. Identification of the T-8 amide by NOE (described below) assigns H-18 by elimination.

The spin systems of glutamines (Q-16 and Q-17) and glutamate (E-7) can be identified by their coupling patterns; an NOE between two of the amides directly indicates Q-16 and

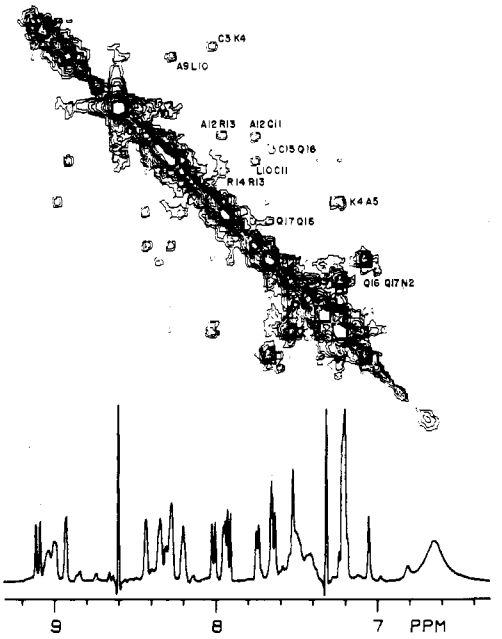

FIGURE 5: Expansion of amide region of NOE experiment in Figure 3 showing amide to amide NOE's. The very strong upfield peaks are from NH_2 protons in the side chains of asparagine and glutamine.

Q-17. The amide of Q-16 shows NOE's to both the amide proton and β-proton of a cystine; this must be residue C-15. No NOE's are observed for the amide of C-15, which can be attributed to residue 14. By means of spin couplings, however, the C^{α} H protons in R-13, R-14, and L-10 are all found to have the same chemical shift. An NOE is observed between

Table I: Summary of Resonance Assignments in ^{1}H NMR Spectrum of 0.05 M Apamin 1[a]

apamin residue	N H	C^α H	C^β H	C^γ H	C^δ H	C^ϵ H	other H
Cys-1		4.55	3.23, 3.10				
Asn-2	9.11	4.84	3.05, 2.74				
Cys-3	9.00	4.63	3.30, 2.75				
Lys-4	8.02	4.20	1.85	1.45	1.68	2.96	7.50 (NH_3)
Ala-5	7.24	4.52	1.16				
Pro-6		4.40	2.31	2.14, 1.98	3.44, 3.55		
Glu-7	9.05	4.39	2.16	2.31			
Thr-8	7.43	4.60	4.66	1.25			
Ala-9	8.93	4.14	1.44				
Leu-10	8.28	4.07	1.62	1.57	0.88, 0.92		
Cys-11	7.75	4.52	3.16, 2.74				
Ala-12	8.44	3.81	1.43				
Arg-13	8.21	4.08	1.92	1.70	3.15		7.20 (NH)
Arg-14	7.96	4.08	1.98	1.74	3.16		7.22 (NH)
Cys-15	8.35	4.38	2.99				
Gln-16	7.65	4.20	2.15	2.44			
Gln-17	7.92	4.22	2.04	2.36			7.32 (C2 H)
His-18	8.31	4.63	3.23, 3.13				8.61 (C4 H)
side chain							
NH_2	(7.66, 7.06), (7.53, 7.22) (7.50, 6.82) unassigned						
Gua NH_2	6.65 (6.92, 6.52, low temperature)						

[a] Conditions: pH 2, 298 K, no salt. Shifts are in ppm from external sodium 4,4-dimethyl-4-silapentanesulfonate.

one pair of the corresponding amide protons, identifying arginine-13 and -14. Since the frequencies of the amide resonances of residues R-14 and C-15 are very close, it is possible that an NOE between them could have been obscured by the diagonal peaks. The amide of R-13 shows a further NOE to the amide of an alanine, identifying residue A-12. This amide in turn shows an NOE to the amide of a cystine, identifying residue C-11. Again, a sequential NOE is observed to the amide of leucine-10, previously identified by its chemical shift following elimination of two arginines, R-13 and R-14. The leucine amide shows NOE's to both the amide proton and β-proton of alanine-9; the former connects to the α- and β-proton of threonine-8. A weak NOE between the amides of E-7 and T-8 serves to complete assignments of all amides. The coupling pattern assigns the resonances of the unique proline, P-6.

Side Chains. Most of the side chains can be immediately assigned from the backbone assignments. However, very small chemical shift differences in the C^α H positions of arginine-13 and R-14 and glutamine-16 and -17 make the side-chain–side-chain identification through couplings impossible. But in both cases, an NOE is observed from the amide to the C^β H of the same residue. Using these, we assign the side chain of R-13 to be slightly upfield of R-14, except for the N1 H. The N2 H protons of these side chains cannot be distinguished as they form an exchange-broadened line at 6.65 ppm. This exchange broadening was confirmed by its temperature dependence: two broad lines are observed at 5 °C that coalesce at about 15 °C and continue to sharpen with increasing temperature. Two pairs of coupled side-chain amide protons are observed in Figure 2 arising from either N or Q. It is probable that the line at 6.8 ppm and one near 7.5 ppm form the third pair of side-chain protons, although the coupling between them was not directly observed. None of these could be assigned to particular residues. The very strong cross peak observed for two pairs of amide protons may arise from chemical exchange via rotation about the amide bond in addition to "spin exchange". The line at 6.82 ppm broadens with increasing temperature, supporting this argument.

While most assignments we have made independently confirm those by Okhanov et al. (1980), there are differences. The most serious is that of L-10, since interpretation of its exchange rate is important to their model. Under a wide variety of conditions, the C^α H's of L-10, R-13, and R-14 are extremely close, and assignment via ^{13}C couplings is difficult at best. Our NOE measurements permit resolution of C-11 from C-15, R-13 from R-14, and Q-16 from Q-17. The chemical shifts of resonances in apamin (pH 2, 90% H_2O–10% D_2O, 25 °C) are summarized in Table I.

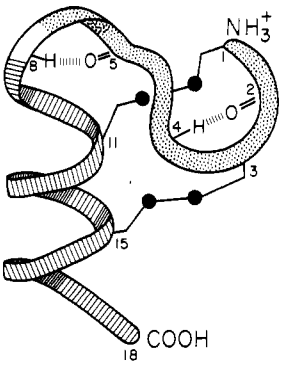

FIGURE 6: A schematic drawing showing the features of the structure of apamin on the basis of NMR measurements in this work. These data provide a "low-resolution" picture of these structural features.

Structural Conclusions. The NOE data observed and applied to assigning residues in apamin evidently furnish structural information in terms of the short-range distances d_1, d_2, and d_3. Without attempting to quantitate the values of these distances, we can view them as providing a set of constraints on the structure (Braun et al., 1981; Wuthrich et al., 1982; Wider et al., 1982). In principle, a sufficiently large number of distance constraints, in appropriate ranges, can delineate the overall structure with surprising precision (Crippen, 1979; Havel et al., 1979). Application of the distance-geometry algorithm to constraints provided by NOE data is under way (Braun et al., 1981; Crippen et al., 1981). However, without resorting to this level of analysis, distinctive and identifiable patterns of NOE's can be associated with regular secondary structural conformations (Dubs et al., 1979; Billeter et al., 1982; Wagner & Wuthrich, 1982). When these data are considered together with other information, notably CD spectroscopy, amide proton exchange rates, and the ϕ-angle-dependent $^3J_{HN-C^\alpha H}$ coupling constants, a reasonable approximation to a structure can be generated.

In the case of apamin, the CD spectrum (Miroshnikov et al., 1978) indicates substantial α-helical content. The theoretical conformational analysis by Hider & Ragnarsson (1981) also proves to be particularly useful in confirming conclusions from NMR studies. The structural features that we can identify with some certainty and the supporting arguments are the following (see Figure 6 for an illustration and Figure 1 for a summary):

(1) Residues C-1, N-2, and C-3 are in an extended conformation. This segment exhibits two NOE's, one from the C^{α} H of C-1 to the amide of N-2 and one from the C_{α} H of N-2 to the amide of C-3, that are common in the linear regions of β-sheets [e.g., Wagner & Wuthrich (1982)]. The $^3J_{HN-C^{\alpha}H}$ coupling constant for N-2 has a large value (9.5 Hz), consistent with this structure (Bystrov et al., 1978).

(2) Residues C-3, K-4, and A-5 form a turn, possibly a type-1 β-turn (Chou & Fasman, 1978). This is indicated by the presence of d_2 NOE's between the amides of C-3 and K-4 and K-4 and A-5, the latter being particularly strong. This indicates a turning of the chain. The fact that the ^3J coupling constants for residues K-4 and A-5 are fairly large suggests the bending does not produce an α-helical configuration. In addition, the hydrogen exchange rates of amides K-4 and A-5 are slow, with A-5 being slower than that of K-4 (D. Wemmer and N. R. Kallenbach, unpublished results; Bystrov et al., 1980). These observations are consistent with residue A-5 lying in the middle of a turn, and a stereochemically reasonable hydrogen bond between the amide of K-4 and the carbonyl in the side chain of N-2 can also be formed.

(3) Residues E-7, T-8, and A-9 probably form another turn, starting at proline-6. Thus the amides of E-7 and T-8 are connected via an NOE, and the hydrogen exchange rate of the T-8 amide is significantly retarded. A reasonable H bond to the C=O of A-5 could explain this. On the other hand, the NOE between the A-9 amide and both C^{α} H and C^{β} H of T-8, together with a large ^3J coupling constant for T-8, suggest an extended conformation between residues T-8 and A-9. The chain thus bends between residues P-6, E-7, and T-8 but appears more extended between T-8 and A-9; this is a nonstandard turn.

(4) Residues A-9, L-10, C-11, A-12, R-13, R-14, and C-15 form an α-helix. This is consistent with several observations and/or predictions for apamin: (a) A series of consecutive amide–amide NOE's connects A-9 through R-14 (and beyond, see below); (b) NOE's connecting the C^{β} H to its neighboring amides (d_3) are observed between A-9 and L-10 and A-12 and R-13; (c) All these residues have small values of $^3J_{HN-C^{\alpha}H}$ coupling constants consistent with a right-handed α-helix (Bystrov et al., 1978); (d) Slow exchange rates are seen for the N–H of Al-12, R-13, R-14, and C-15; (e) The conformational analysis of apamin and two related sequences from bee venom indicates a likely common folding, including two β-turns (residues 1–4 and 5–8) with an α-helix from T-8 to H-18 (Hider & Ragnarsson, 1981).

(5) Residues C-15, Q-16, and Q-17 are probably α-helical but with increasing disorder toward residue H-18, which is disordered. Amide–amide NOE's connect C-15–Q-16 and Q-16–Q-17, while the coupling constants $^3J_{HN-C^{\alpha}H}$ increase progressively from residues R-14 to H-18. Exchange of amide Q-16 is fairly slow, and the rates observed follow the order H-18 > Q-17 > Q-16 >> C-15.

Finally, we note that we repeatedly observe one NOE that is not consistent with any of the observations just presented. This is an NOE apparently connecting the amide of E-7 to the C^{α} H of C-3, a constraint incompatible with any model of the chain we have been able to make that also incorporates all the other NOE's. We believe this may reflect an intermolecular phenomenon, and this possibility is under investigation.

In conclusion, on the basis of recent 2-D NMR techniques, we can assign essentially all the resonances in the spectrum of apamin. Combination of the NOE data with information including coupling constants, hydrogen exchange, and CD permits us to construct a structural model with features resembling those of Bystrov et al. (1980) and Hider & Ragnarsson (1981), but with distinctive differences from each. Lacking an X-ray structure for apamin, the model is fairly detailed. Refinement should be possible by quantifying the NOE's and by application of the distance-geometry algorithm. Comparison of the assignments presented here with those obtained by one-dimensional methods makes it clear that the latter gives rise to ambiguities whenever several protons resonate at a common frequency. This situation naturally becomes more frequent in larger molecules.

Acknowledgments

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Registry No. Apamin, 24345-16-2.

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Proton Nuclear Magnetic Resonance Assignments and Surface Accessibility of Tryptophan Residues in Lysozyme Using Photochemically Induced Dynamic Nuclear Polarization Spectroscopy†

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ABSTRACT: Tryptophan resonances in the 360-MHz ^1H photochemically induced dynamic nuclear polarization spectrum of hen egg white lysozyme are investigated in detail. All resonances of one tryptophan and six of another are identified and assigned to their respective protons. The methods employed, all involving nuclear spin polarization, include the study of cross-relaxation effects and the use of selective radio-frequency irradiation, Gd^{3+} as a paramagnetic probe, and riboflavin as the chemically induced dynamic nuclear polarization generating dye. From a comparison of the experimental results with the known X-ray structure of lysozyme, second-stage assignments of the two tryptophan residues (Trp-62 and Trp-123) are proposed. A number of other resonances are characterized, among them Trp-63 C(2)H and four indirectly polarized methyl groups.

An essential prerequisite for a nuclear magnetic resonance (NMR)[1] investigation of the structure and properties of a molecule is the assignment of its NMR spectrum, that is, the identification of each spectral line with the nucleus responsible for it. In large molecules such as proteins, this task can be quite formidable, and one has often to settle for a partial assignment of the total spectrum. Among the many methods developed to tackle this problem (Campbell et al., 1975; Dobson, 1977; Jardetzky & Roberts, 1981; Wüthrich et al., 1982; Wagner & Wüthrich, 1982; Wider et al., 1982), an approach which is specific for certain aromatic amino acid residues on the surface of a protein is that of photochemically induced dynamic nuclear polarization (photo-CIDNP) (Kaptein, 1978, 1982). The method depends on reversible photochemical reactions of the protein with a dye to generate nuclear spin polarization, and hence NMR intensity enhancements, in the side chains of reactive residues. If accessible to the photoexcited dye (usually a flavin), tyrosine, histidine, and tryptophan can be polarized in this way. Subtraction of "light" and "dark" protein spectra gives a vastly simplified spectrum containing only polarized resonances (Kaptein, 1978).

The three residues have characteristic photo-CIDNP spectra which are easily distinguished. First-stage assignment (to a specific type of proton) of His and Tyr resonances is straightforward, owing to the simplicity of the spin systems, while Trp residues, in which all five aromatic protons become polarized, can be more awkward especially when, as in lysozyme, more than one Trp is accessible to the dye.

In a previous photo-CIDNP investigation of hen egg white lysozyme (Kaptein, 1978), two polarized Trp residues were found, and some tentative assignments were made, which are now shown to be partially incorrect. In what follows, we describe new experimental work designed to arrive at a more convincing set of assignments.

Materials and Methods

Photo-CIDNP Spectra. All experiments were performed at 360 MHz on a Bruker HX-360 NMR spectrometer controlled by a BNC 12 or an Aspect 2000 computer. The photo-CIDNP technique has been described previously (Kaptein, 1978; Kaptein et al., 1978), the only modification being the use of an optical fiber in some of the measurements to couple the argon laser beam into the NMR probe. As before, light pulses are controlled from the computer by means of a mechanical shutter.

"Light" and "dark" spectra are recorded alternately and subsequently subtracted to give a photo-CIDNP difference spectrum in which unpolarized resonances do not appear. Typically, five scans were necessary to obtain an acceptable signal to noise ratio; a CIDNP spectrum of 1 mM protein could thus be acquired in less than 2 min.

All spectra, except those required for chemical shift measurements, were recorded at 25 °C to ensure reasonable

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[1] Abbreviations: NMR, nuclear magnetic resonance; CIDNP, chemically induced dynamic nuclear polarization; NOE, nuclear Overhauser effect; rf, radio frequency; ppm, parts per million; flavin I, 7,8,10-trimethyl-3-carboxymethylisoalloxazine; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

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