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Structure of P401 (mast cell degranulating peptide) in solution

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P401 (also known as mast cell degranulating protein, MCD) is a minor component of honeybee venom. Its primary structure is related to that of apamin. We have studied the structure of P401 in solution by high-resolution two-dimensional ^{1}H-NMR spectroscopy. Almost all the backbone proton resonances have been assigned by sequential assignment strategy. Analysis of NOEs shows that P401 has a conformation very similar to that of apamin. N-terminal residues Ile-1–Cys-5 are in an extended conformation and residues His-13–Asn-22 on the C-terminus are in an α-helical structure. These two secondary structural elements are connected by two tight turns.

1. Introduction

There is today growing interest in the potential for forming secondary structure in small peptides. Both the nature and extent of structure in fragments of protein are of concern in understanding how these molecules elicit anti-peptide antibodies, and the basis for cross-reactivity of these antibodies with those raised against native proteins [1]. Small structured peptides have provided fundamental tools for analysing the determinants of α-helix stability [2]. Both stabilizing interactions, such as appropriate distribution of charged groups within a helix, and stop signals that abruptly terminate a helix, have been identified in the short S-peptide fragment of RNase [3–5]. We also believe that aspects of protein folding mechanisms can be investigated in greater detail using ordered small peptides than larger, more complex proteins.

The venom of the honeybee, *Apis mellifera*, contains three major protein components (melittin, phospholipase A_2 and hyaluronidase) together with a set of small, remarkably stable, highly structured peptides including the neurotoxin apamin, the mast cell degranulating peptide designated as MCD or P401, secapin and tertiapin [6–8]. These minor venom components share a common pattern of disulfide connectivity as well as some sequence similarity [9] (fig. 1) suggestive of a common gene origin [10]. Based on CD spectroscopic analysis, apamin and MCD were shown to have a common α-helical content indicated by the presence of strongly negative molar ellipticity at 222 nm [11]. Functionally, each of these molecules is distinct. Apamin is a neurotoxin

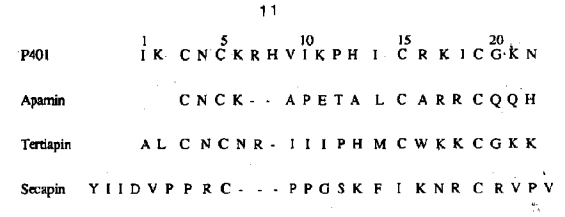

Fig. 1. Amino acid sequence comparison of proteins of the apamin family. The sequences are aligned according to Hider and Ragnarsson [9].

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acting on Ca^{2+}-dependent K^+ channels, and capable of crossing the blood-brain barrier to act on the central nervous system (CNS) [6]. P401 exhibits toxicity only on injection into the CNS, and is associated with two independent pharmacological activities which are paradoxical [6,12]. It degranulates rat peritoneal mast cells and produces an inflammatory response due to release of histamine and 5-hydroxytryptamine. On the other hand, it acts as an anti-inflammatory agent. Secapin is found in high concentration only in the venom of queen bees [13], and its function, like that of tertiapin, remains unknown. However, a comparative structural investigation by Hider and Ragnarsson [9] using Levitt's parameters for secondary structural propensities [14] predicts that all these molecules share a common conformational pattern.

A two-dimensional ^{1}H-NMR analysis by Wemmer and Kallenbach [15] showed that apamin folds to form a β-turn–turn–α-helix motif, as was in fact predicted by Hider and Ragnarsson [9]. Sequential assignments in apamin were carried out spanning two segments, the first encompassing two turns comprising residues Cys-1 and Ala-5, the second from Glu-7 to His-18. We report the sequential assignments for P401 and show that qualitatively the structures of P401 and apamin are very similar. Both these proteins have an N-terminal β-turn and a C-terminal helix. We observe major and minor conformers in this protein as reported by Walde et al. [16] but do not know the structural differences between these two forms. Our NMR data support the argument that apamin, P401 and tertiapin have very similar folded structures.

2. Materials and methods

P401 was prepared from lyophilized bee venom (purchased from Bulgar Coop, Sofia, Bulgaria) according to the procedure of Gauldie et al. [7,8]. Purification steps include forced dialysis of the venom to segregate high molecular weight species, gel filtration, and ion exchange on SP Sephadex followed by extensive desalting on Sephadex G-15. All NMR experiments were carried out on Bruker WM500 and AM500 spectrometers equipped with Aspect computer systems. Standard pulse sequences and phase cycles were used in COSY [17], DQFCOSY [18], NOESY [19], RELAY [20,21] and TOCSY [22,23] experiments. Quadrature detection in the ω_1 dimension in the DQFCOSY experiment was achieved by time-proportional, phase incrementation, TPPI [24–26] while hyper-complex Fourier transformation [27] was used in phase-sensitive NOESY experiments. The MLEV-17 type of composite pulse sequence [23] was used for isotropic mixing in TOCSY experiments in which spins evolve collectively under an effective Hamiltonian which does not have any Zeeman terms [22]. Mixing times in the NOESY, RELAY and TOCSY experiments were 200–300, 25–40 and 100 ms, respectively. In general, 400–512 free induction decays (t_1 dimension) with 1024 complex data points in the t_2 dimension were acquired and appropriately zero-filled to give final 1024×1024 real point matrix in the frequency domain. Skewed sine bell apodization was employed in both dimensions. The FTNMR program kindly provided by Dr. Dennis Hare was used for off-line data processing of all the spectra on a VAX 11/750 or 11/780 computer.

3. Results and discussion

We used the sequential assignment strategy developed by Wüthrich et al. [28–30] to assign the two-dimensional NMR spectra and elucidate the structure of P401 peptide. In this procedure, coupling networks of the spin systems corresponding to individual amino acids (or classes of amino acids) are identified and the NOEs between the next-neighbor residues are used to obtain the sequence-specific resonance assignments. Despite its relatively small size (22 amino acids), unusual amino acid composition (4 isoleucines, 4 lysines, 2-arginines, 1 valine, 4 cysteines, etc.), degeneracies in chemical shifts (spectral overlap) and particularly the presence of minor species [16] make the assignment of the MCD spectrum a nontrivial problem [31]. We identified the AMX spin systems corresponding to the residues Cys, Asn and His and these residues will be collectively referred

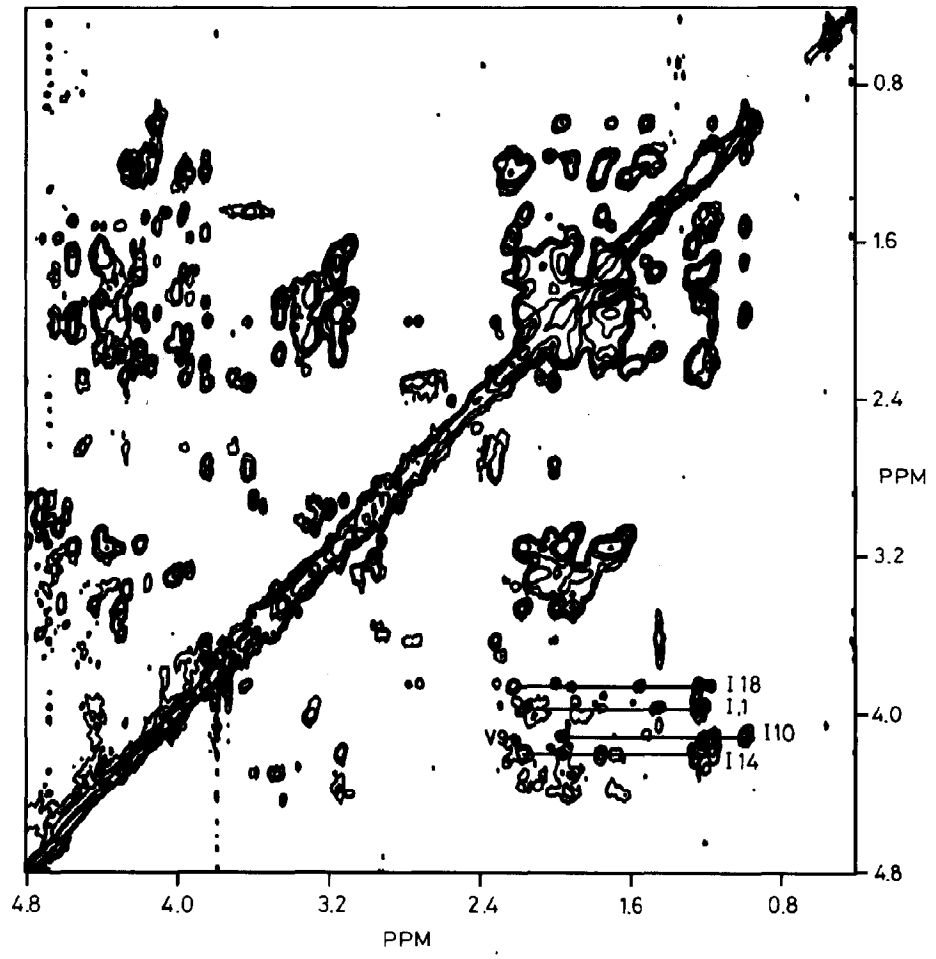

Fig. 2. Identification of Ile and Val spin systems in the TOCSY spectrum of P401 in ^2H_2O. Horizontal lines show the remote connectivities from the αCH resonances to γ protons.

to as 'class X' in the later discussions. Analysis of TOCSY (fig. 2); RELAY and NOESY spectra in ^2H_2O made it possible to identify unique spin systems for all four isoleucines, one valine and one proline residue. Isotropic mixing in TOCSY experiments causes all the spins of any coupled network to evolve collectively, providing efficient coherence transfer among them. For example, in isoleucine cross-peaks are observed between αCH protons, and β, γ and δ protons (fig. 2). Although the RELAY experiment in principle can give similar information, βCH_2 protons act as an efficient sink and make the transfer between α and γ protons rather weak in practice. Complete identification of spin systems of Lys and Arg residues was not possible because of the overlap. However, complex multiplet structure of the α-β cross-peak in the DQFCOSY spectrum, the high-field chemical shifts and identification of Ile and Val residues give us likely candidates for these residues. These residues are collectively labeled as 'class R' in sequential assignments. NH-$^{\alpha}$CH cross-peaks in the fingerprint region (fig. 3) corresponding to these spin systems were identified from the RELAY experiment in H_2O which connects the amino proton shifts with the β proton

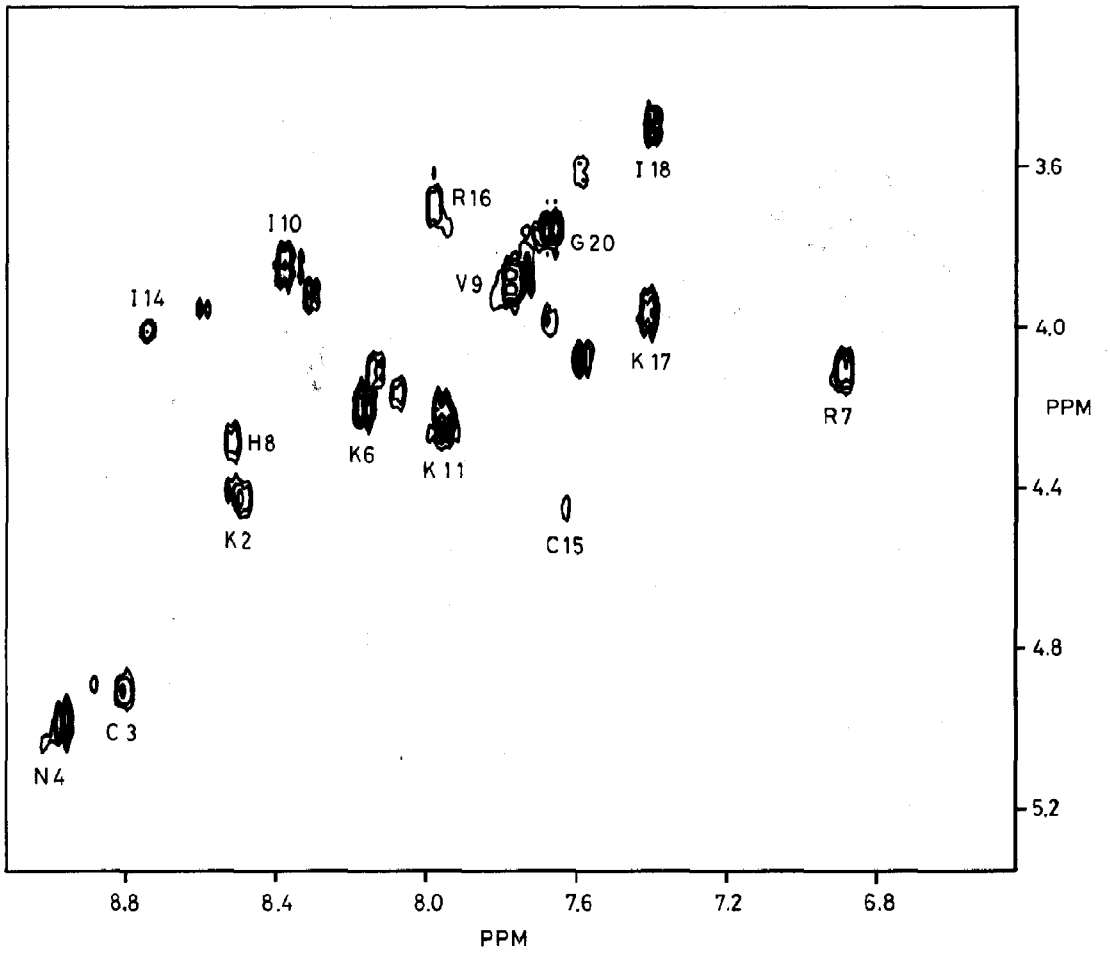

Fig. 3. Fingerprint region of COSY spectrum of P401 in H_2O. Amide NH-αCH cross-peaks are labeled with corresponding sequence-specific assignments. Resonances of C5 and H13 are very weak in this spectrum and are observed under different conditions.

shifts of a particular residue, thereby lifting the degeneracy in α chemical shifts.

Sequence-specific assignments were obtained from the results of COSY and NOESY experiments in H_2O. As shown by Wüthrich et al. [28–30], NOEs between NH_i and αCH_{i-1} as well as the NOEs between the amide protons of the neighboring residues are of central importance for this purpose. The NOEs observed in the P401 spectrum are summarized in fig. 4. A relatively small number of NOEs are observed in the NH-αCH region of the P401 spectrum. These NOEs, denoted by $d_{\alpha N}$, link the NH proton of residue i with the αCH of residue $i-1$. We observe four cross peaks of this kind corresponding to a sequence of IRXXX, where R is a Lys or Arg, and X is any residue with an AMX spin system. This pentapeptide sequence uniquely identifies the N-terminal segment IKCNC. The d_{NN}-type NOE cross-peak between Cys-5 and its neighbor of class R assigns Lys-6. The $d_{\alpha N}$-type NOEs along the chain give specific assignments for the residues Arg-7–Lys-11. Lys-6 and Arg-7 are also connected by a d_{NN}-type NOE between their NH protons. Assignments for the residues His-8 and Val-9 are further corroborated by the presence of weak d_{NN}

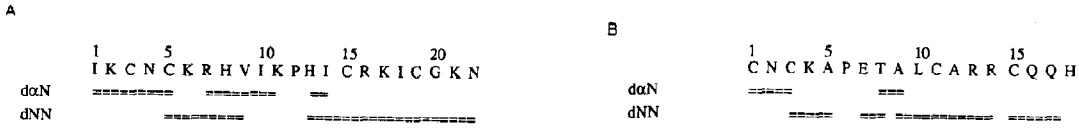

Fig. 4. Summary of $d_{\alpha N}$ and d_{NN}-type NOEs in (A) P401 and (B) apamin [15].

NOEs (fig. 5). Proline at position 12 interrupts the sequential connectivities beyond Lys-11, and the assignments for the C-terminal half of the protein are derived mostly from the d_{NN}-type NOEs. As shown in fig. 5, each residue from His-13 to Asn-22 is connected via these NOEs to the NH proton of its neighboring amino acid residue. It should be noted that the amide proton chemical shifts of Lys-17 and Ile-18 are almost degenerate and the corresponding NOE cross-peak falls es-

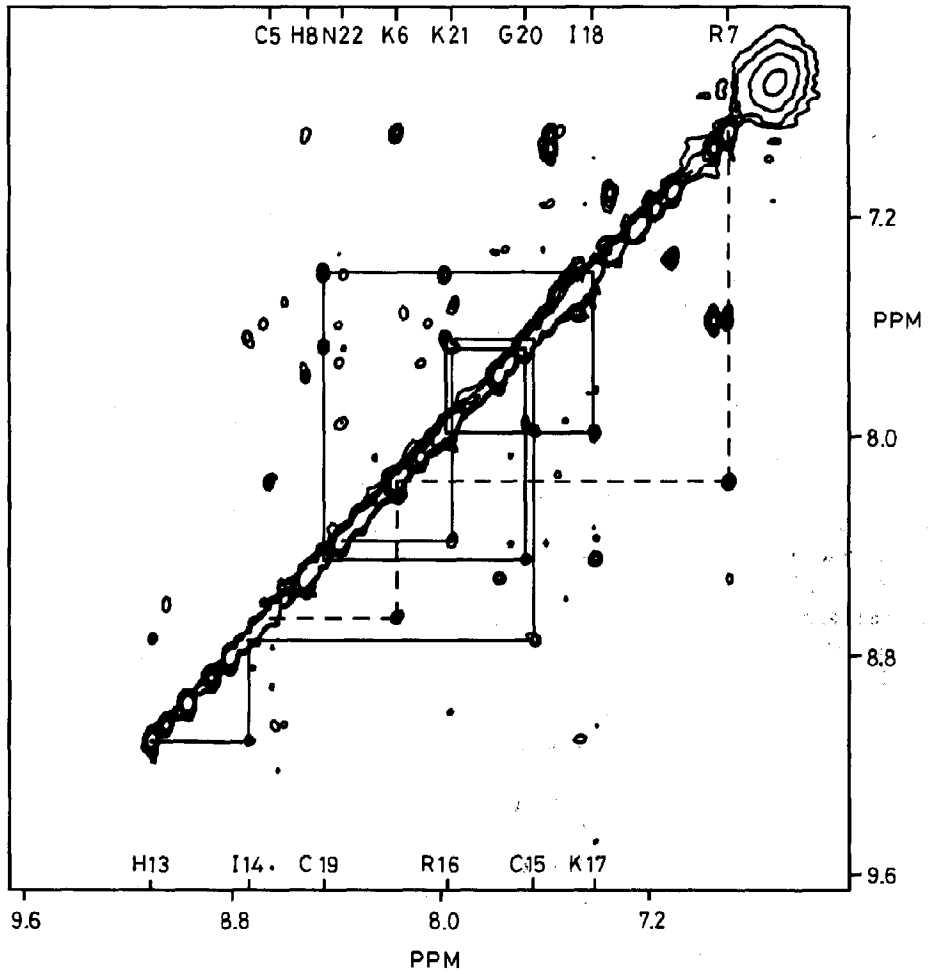

Fig. 5. d_{NN}-type NOEs observed in the spectrum of P401 in H_2O. Sequence-specific assignments are shown at the amide proton positions. Dashed lines show connectivities in the Cys-5–Arg-7 segment.

sentially on the diagonal, and hence could not be clearly observed.

4. Structure of MCD peptide

While individual NOEs are useful for the sequential assignments, the patterns of these NOEs reveal the characteristic secondary structure of the protein segment concerned. It is well established statistically by now that contiguous stretches of d_{NN}-type NOEs represent a signature of α-helix while the stretches of $d_{\alpha N}$-type NOEs are indicative of extended β-structures [30]. Fig. 4 shows the summary of NOEs observed in P401 and in apamin by Wemmer and Kallenbach [15]. The $d_{\alpha N}$-type NOEs show that the residues Ile-1–Cys-5 are in the extended conformation. The discontinuity in these NOEs and the presence of NOE between the amide protons of Cys-5 and Lys-6, as well as Lys-6 and Arg-7, indicate that these residues are in the characteristic type-I turn [32]. The residues His-13–Asn-22 are in α-helical conformation as shown by the long stretch of NOEs between the neighboring amide protons. However, it is difficult to determine the exact beginning of the helix. Proline at position 12 probably causes a tight turn in the backbone. A weak $d_{\alpha N}$ NOE cross-peak between His-13 and Ile-14 suggests a deviation from the regular α-helical conformation and is consistent with the β-turn predicted by Hider and Ragnarsson [9] and is essential for the observed disulfide bonds (3–15 and 5–19). Weak NOEs in the C-terminus (fig. 5) indicate fraying of the helix which was also observed in apamin [15]. Overall similarity in the NOE patterns in P401 and apamin (fig. 4) reflects their common secondary structures. The major differences in their structures are in the second turn in which there are two additional residues in P401. We have observed cross-peaks corresponding to the minor forms of P401 [16] in the fingerprint region of COSY and NOESY spectra. However, no details of the structure of this minor form are known.

In conclusion, we have sequentially assigned the two-dimensional NMR spectrum of P401 and shown that its secondary structure is very similar to that of apamin. Further investigations on the tertiary structure of these proteins are in progress.

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