The interactions with solvent, heat stability, and $^{13}$C-labelling of alamethicin, an ion-channel-forming peptide

Adelinda A. YEE, Kirk MARAT and Joe D. J. O'NEIL

Department of Chemistry, University of Manitoba, Winnipeg, Canada

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The peptide alamethicin was labelled with $^{13}$C and $^{15}$N by growing the fungus *Trichoderma viride* in a medium containing [U-$^{13}$C]glucose and K$^{15}$NO$_3$. Spin-echo difference spectroscopy showed that $^{13}$C was incorporated to a level of about 50% and $^{15}$N to about 98%. Incorporation of $^{13}$C into the peptide provided residue-specific probes of the interactions with solvent and heat stability of this ion-channel-forming peptide. All of the carbonyl carbons and the $\alpha$-carbons of the $\alpha$-aminoisobutyric acid [Ala(Me)] residues of alamethicin in methanol were assigned using two-dimensional and three-dimensional heteronuclear correlation experiments. Measurements of $^{1}J_{CH}$ revealed hydrogen bonding with solvent at residues 1 and 19 at the ends of the peptide and at Gly11 in the middle. The data also support the thesis [see Juranic, N., Ilich, P. K. & Macara, S. (1995) *J. Am. Chem. Soc.* 117, 405–410] that intramolecular hydrogen bonds in proteins and peptides are weaker than hydrogen bonds to solvent. The sensitivity of alamethicin carbonyl and proton chemical shifts to perturbation by dimethyl sulfoxide correlates well with the calculated solvent accessibilities of the carbonyls in the crystal structures and reveals residues in the middle of the peptide and at the C-terminus which interact with solvent. Taken together with the $^{1}J_{CH}$ measurements, the data support a model in which hydrogen bonding to solvent at the Gly11/Leu12 amide could provide a site of hydration in the interior of the alamethicin channel structure. The temperature dependencies of the carbonyl chemical shifts support the suggestion that the peptide is flexible in the regions where solvent interacts with the backbone of the peptide. The linear temperature dependence of the carbonyl chemical shifts and molar ellipticity indicate that, due to steric constraints at the Ala(Me) residues, the peptide folding/unfolding transition is non-cooperative and that the peptide is remarkably heat stable.

*Keywords*: alamethicin; $^{13}$C labelling; dimethyl sulfoxide; ion channel; NMR.

Alamethicin is a 20-amino-acid antibiotic peptide secreted by the fungus *Trichoderma viride*. It forms a voltage-gated conductance in lipid bilayers (Eisenberg et al., 1973; Latore et al., 1981) and is one of the most extensively studied models for vertebrate ion channels [for reviews see Sansom (1993) and Cafiso (1994)]. The peptide has eight residues of $\alpha$-aminoisobutyric acid [Ala(Me)] which have $\alpha$-methyl in place of the usual $\alpha$-hydrogen found in the $\alpha$-amino acids. The mechanism by which alamethicin inserts into membranes, forms ion channels, and is regulated by an applied voltage is unclear. You et al. (1996) recently showed that covalent dimers of alamethicin form stabilized ion channels with a single predominant conductance state. They suggest that the channels consist of a bundle of six parallel monomers. Evidence for antiparallel interactions between monomers dissolved in methanol was obtained by North et al. (1994). Their measurements of the paramagnetic enhancements of nuclear relaxation also indicated that the peptide backbone can undergo large structural fluctuations which may be significant in the response of the peptide to an applied voltage. They suggested that the predominant conformation of the peptide in methanol is one which reduces the solvent accessibility of the polar residues in the peptide. However, experimental evidence of residue-specific conformational flexibility and interactions with solvent have been difficult to obtain in alamethicin (Spyracopoulos et al., 1996). We report that the $^{13}$C carbonyl spectrum and heteronuclear coupling constants yield residue-specific information about peptide interactions with solvent and conformation.

The chemical synthesis of alamethicin has been reported (Schmitt and Jung, 1985a; Gisin et al., 1981; Slomczynska et al., 1992). However, synthesis of $^{13}$C-labelled alamethicin would be expensive and there is the additional disadvantage that that Ala(Me) is not one of the commonly available amino acids. Alamethicin is enzymatically synthesized (Rindfleisch and Kleinkauf, 1976) and so it cannot be expressed in *Escherichia coli*, the most common method for labelling proteins. A systematic study of the fermentation conditions for *T. viride* by Brewer et al. (1987) showed that the fungus is able to grow on a variety of simple nitrogen sources and this allowed us to label alamethicin with $^{15}$N using inexpensive K$^{15}$NO$_3$ (Yee and O'Neil, 1992). Brewer et al. (1987) also reported that a complex carbon source (carboxymethyl-cellulose or dextrin) is required by *T. viride* for the production of alamethicin. However, *Trichoderma* spp., and other Deuteromycetes, Ascomycetes, and Basidiomycetes, are saprophytes. They digest complex carbohydrates by excreting...
cellulase complexes that hydrolyze cellulose extracellularly and then absorb the simpler sugars (Moore-Landecker, 1990). Since *T. viride* is able to grow on carboxymethyl-cellulose, we have explored growth conditions for the uptake of monomeric [U-\(^{13}\)C]glucose and the \(^{13}\)C-labeling of alamethicin.

There are several published reports on the usefulness of the \(^{13}\)C carbonyl resonance for providing information about peptide structure, dynamics, and interactions with solvent: (see Urry et al., 1974; Jameson and Mason, 1987; Tuchseng and Hansen, 1988; Ando et al., 1988; Hansen et al., 1992; Wishart et al., 1991; Wishart and Sykes, 1994). For example, helix-to-coil transitions in small alanine-based peptides were conveniently monitored via the carbonyl chemical shift (Shalongo et al., 1994a,b). Recently, intramolecular and intermolecular hydrogen bonds in proteins and model compounds were distinguished via the magnitude of the one-bond coupling between the carbonyl carbon and its directly attached nitrogen (\(J_{NC}\)) (Juranic et al., 1995).

The activities of enzymes in nonaqueous solvents are of growing interest in the field of biotechnology but an explanation of the effects of organic solvents on protein structure and dynamics is far from complete [see Zheng and Ornstein (1996) and references therein]. Proteins and peptides are highly soluble in dimethyl sulfoxide (MeSO), an aprotic solvent with a dielectric constant of 46, which denatures some proteins (Waterhouse and Johnson, 1994). Quantum mechanical calculations (Zheng and Ornstein, 1996) suggest that MeSO can strip away water of hydration from proteins owing to the strength of the MeSO-amide hydrogen bond compared to water-amide and amide-carbonyl interactions. Experimental evidence of significantly longer lifetimes for MeSO than for water molecules at the surfaces of proteins has also been obtained (Hills and Favret, 1994). Molecular dynamics simulations suggest that subtilisin has a higher number of intramolecular hydrogen bonds when dissolved in MeSO than in water (Zheng and Ornstein, 1996) which may explain why that solvent appears to stabilize some proteins. We report herein the interactions of the peptide backbone of alamethicin with methanol and MeSO using \(^{13}\)C-NMR and \(^{1}H\)-NMR and CD spectroscopy.

**MATERIALS AND METHODS**

**Materials.** *T. viride* NRRL 3199 was purchased from the American type culture collection. K\(^{15}\)NO\(_3\), (99%), D-[U-\(^{13}\)C]glucose (98%), and CD\(_2\)OH were from Cambridge Isotope Laboratories, Inc. CD\(_3\)OD was from Aldrich Chemical Co. Other chemicals, vitamins and minerals were from Fisher Scientific, Sigma Chemical Co. and Mallinckrodt. A Beckman Spherisorb 5-mm C-18 reverse-phase column was used for HPLC purification of alamethicin.

**\(^{13}\)C-labeling of alamethicin.** Initially, experiments were performed to test whether *T. viride* would grow in a medium containing only soluble glucose as a carbon source, and to determine if alamethicin is produced under these conditions. In addition, *T. viride* that was grown on \(^{15}\)N-containing minimal medium (Yee and O'Neil, 1992) was used to inoculate a modified malt/agar plate. The plate was incubated for two weeks at 25°C. A small piece of the agar with dense fungal growth was used to inoculate 50 ml of the \(^{15}\)N minimal medium described previously (Yee and O'Neil, 1992), and 50 ml of a glucose-containing medium which is the same minimal medium but with 0.5 g glucose substituted for 1 g dextrin. The cultures were incubated for 50 days with shaking at 80 rpm at 25°C. Visible growth of mycelium was observed and alamethicin was extracted and purified as described previously (Yee and O'Neil, 1992). To produce \([^{15}\text{N},^{13}\text{C}]\)alamethicin, the mycelium grown in the glucose-containing medium was used to inoculate 50 ml minimal medium containing K\(^{15}\)NO\(_3\), and [U-\(^{13}\text{C}\)]glucose and was grown under the same conditions as the test experiment. Alamethicin was extracted and purified as described previously (Yee and O’Neil, 1992).

**Circular dichroic spectroscopy.** CD spectra of HPLC-purified alamethicin in methanol were acquired using a Jasco J500A spectropolarimeter. The temperature of the sample in a 0.5-cm path-length cell was controlled by a Haake D1-G water bath. Mean residue ellipticities were calculated using a mean residue mass of 97.8 Da.

**NMR spectroscopy.** HPLC-purified alamethicin was dissolved in 0.6 ml CD\(_3\)OD or CD\(_2\)OH to a concentration of about 1 mM without adjustment of the pH; the measured apparent pH was 6.8 and the sample was placed in a 5-mm NMR tube (Wilmad 535 or 537). For the solvent perturbation study, Me\(_2\)SO was added to a CH\(_3\)OH/CD\(_3\)OH (1:1) to give 20% Me\(_2\)SO initially. After a spectrum was acquired, some of the solvent in the same sample was evaporated and the sample was filled to its original volume with Me\(_2\)SO; this increased the Me\(_2\)SO concentration to approximately 80%. The concentration of Me\(_2\)SO was determined using the ratio of the CH, signals from methanol and Me\(_2\)SO.

All NMR experiments were conducted on a Bruker AMX500 NMR spectrometer using a 5-mm triple-resonance probe head with the inner coil tuned to \(^{1}H\) and \(^{15}\)N, and the outer coil tuned to \(^{13}\text{C}\) and \(^{15}\text{N}\). One-dimensional \(^{1}C\) observe spectra were acquired with and without decoupling of \(^{1}H\) and \(^{15}\text{N}\). The carbonyl region was acquired with a spectral width of 1400 Hz and 4 K data points, and was zero filled to 32 K; the total number of scans was 1024. The \(^{13}\text{C},^{15}\text{N}\) correlation experiments were performed via heteronuclear zero and double-quantum coherence with \(^{1}H\) decoupling throughout the experiment and \(^{13}\text{C}\) decoupling during acquisition (Mooberry et al., 1989); 160 scans for each of the 64 \(t_i\) increments of 2 K data points each were acquired; the total experiment time was 14 h. For the variable-temperature experiments, the number of scans/increment was reduced to 32 and only 32 \(t_i\) increments were acquired; the total experiment time was 1 h. All two-dimensional (2D) data were zero-filled to 4 K and 128 points in the \(F_2\) and \(F_1\) dimensions, respectively. A \(\pi/2\)-shifted, sine-squared filter was applied to both dimensions before Fourier transformation. The spin echo difference experiment (Griffey et al., 1985) was performed with \(^{13}\text{C}\) decoupling and solvent presaturation. Proton decoupling was performed using WALTZ-16 composite pulse decoupling (Shaka et al., 1983), and \(^{13}\text{C}\) and \(^{15}\text{N}\) decoupling were performed using the GARP sequence (Shaka et al., 1985). The 3D \(1\text{H},^{15}\text{N},^{13}\text{C}\) correlation experiment was performed with inverse correlation for triple resonance using multiple INEPT transfer steps (Kay et al., 1990; Bax and Ikura, 1991). This was acquired with 16 scans/increment and 512 total data points were collected along the observed \(^{1}H\) dimension; the sweepwidth in the \(^{13}\text{C}\) dimension was set to 20 ppm centered in the \(^{13}\text{C}\) region and a total of 64 increments were acquired; the sweepwidth in the \(^{15}\text{N}\) dimension was 45 ppm and a total of 128 increments were acquired. All three dimensions were processed with a sine-squared window function shifted by \(\pi/2\). No zero filling was applied to the \(^{1}H\) and \(^{15}\text{N}\) dimensions and forward linear prediction was applied to the \(^{13}\text{C}\) dimension; 64 points were predicted and zero filled to 256 data points.

**RESULTS AND DISCUSSION**

**\(^{13}\text{C}\)-labeling of alamethicin.** In liquid culture, *T. viride* is usually grown in the presence of insoluble solids such as fish meal,
Fig. 1. $^{13}$C carbonyl spectra. $^{13}$C carbonyl spectra of alamethicin in CD,OD: (a) without decoupling, (b) with $^1$H decoupling and (c) with $^1$H and $^{15}$N decoupling.

dextrin (Reusser, 1967), and/or carboxymethyl-cellulose (Brewer et al., 1987). We have observed that when liquid culture medium containing soluble glucose as the sole carbon source is inoculated with spore dispersions, no visible growth of fungus occurs even after 50 days and no alamethicin is produced. The insoluble solids in the growth medium may be required to stimulate spore germination (Mandels and Darby, 1953) and/or for the fungus to anchor its rizhomorph. Thus, when a piece of agar containing growing fungus is used as inoculum, growth of the fungus and production of alamethicin are observed although the yield of peptide is only about 25% of that of the control medium. When [U-$^{13}$C]glucose is added to the medium the fungi incorporate $^{13}$C into alamethicin as indicated in the $^1$H-NMR spectra of the peptide (not shown) in which the methyl resonances of the Ala(Me) and Ala residues are split by 128 Hz ($J_{^1C-^1H}$). The extent of $^{13}$C incorporation was determined using the spin-echo difference experiment (Griffey et al., 1985) and indicates that the $^{13}$C-labelling in the peptide is between 51—57%. Incorporation of $^{15}$N was determined to be about 98%.

The fully coupled $^{13}$C spectrum of the carbonyl region of alamethicin dissolved in CD,OD is shown in Fig. 1a. The spectrum shows two groups of poorly resolved resonances at low field and some better resolved resonances to high field. When protons are decoupled, the resonances appear as doublets of doublets (Fig. 1b). The smaller separation of 15 Hz corresponds to the $J_{^1H-N}$ coupling. When both $^1$H and $^{15}$N are decoupled, the peaks collapse into doublets separated by 53 Hz and a smaller peak in the middle. This is clearly seen at the 174-ppm peak in Fig. 1c. The 53-Hz separation is the typical $J_{^1H-N}$ coupling and the small peak is due to the carbonyl carbons that do not have a neighboring $^{13}$Ca. The relative intensities of the $^{13}$C- and $^{13}$C-attached peaks in Fig. 1c show that the majority of $^{13}$C' atoms (85%) are directly bonded to $^{13}$Ca atoms whereas a smaller fraction are bonded to $^{13}$Ca (15%). This suggests that biosynthetic incorporation of $^{13}$C did not occur randomly and that most of the $^{13}$C is in highly enriched molecules. A possible explanation is that, after transfer to the $[^{13}$C]glucose medium, T. viride initially used up its stores of glucose and amino acids to produce predominantly unlabelled alamethicin and that, when these were depleted, $[^{13}$C]glucose was consumed and used to make predominantly $^{13}$C-labelled alamethicin. These observations suggest that, in addition to E. coli (Wang et al., 1992), several Bacillus strains (Teplyakov et al., 1992), algae and yeast (Sailer et al., 1993), fungi could be a useful vehicle for the production of $^{13}$C- and $^{15}$N-labelled proteins.

Assignment of the $^{13}$C' and Ala(Me) $^{13}$Ca resonances. The full 1D $^{13}$C spectrum of alamethicin in CD,OD is similar to that previously reported for the synthetic peptide with Glu in position 18 (Schmitt and Jung, 1985b). The a-carbons and the sidechains of all alamethicin non-Ala(Me) residues were assigned previously using natural abundance $^1$H-$^1$C correlation experiments (Kelsh et al., 1992; Yee et al., 1995). However, the carbonyl carbons and the quaternary a carbons of Ala(Me) could not be

Fig. 2. Carbonyl assignments. Proton-decoupled correlation of the $^{13}$C-carbonyls (residues $i$) with the $^{15}$N amides (residues $i+1$) (Mooberry et al., 1989) of alamethicin in CD,OD. The assignments given are for the $^{13}$CO($i$). The crosspeak splitting is due to couplings of the $^{13}$C' resonances to the $^{13}$Ca and $^{15}$N resonances as indicated.
The rest of the carbonyls resonate between 174.0-177.2 ppm. This pattern of chemical shifts is attributable to the p-effect on the chemical shift of the carbonyl carbon (Stothers, 1972). A similar effect was observed for the 13C resonances of alamethicin (Yee and O'Neil, 1991). Each panel in Fig. 3 is a 1H slice at the indicated chemical shift and shows the Ca to N correlation.

The carbonyls of Ala(Me)1 and Ala(Me)13 resonate at a higher field than those in a-helices (Wishart et al., 1991; Wishart and Sykes, 1994). The chemical shift index calculated for the carbonyl carbons in alamethicin confirms the predominantly helical conformation determined from other resonances such as the 13Ca, CaH, NH, and 15N resonances (see Yee et al., 1995).

The Ala(Me) a-carbon resonances, which severely overlap, were assigned using a 3D 1H-13C-15N correlation experiment (Kay et al., 1990; Bax and Ikura, 1991). This experiment has the advantage that the one-bond couplings to the a-carbons from the f- and carbonyl carbons can be eliminated by 13C decoupling during acquisition since the observed nucleus is 1H. The Ca to N correlation slices along the amide 1H dimension for all the Ala(Me) residues are shown in Fig. 3. All the Ala(Me) a-carbons resonate in a very narrow range of 57.3-58.1 ppm suggesting that the conformations of all the Ala(Me) residues are very similar. This conclusion is in accord with the high helical propensity of Ala(Me) (Basu and Kuki, 1993) and the separation in the chemical shifts of the Ala(Me) f-carbons (Yee et al., 1995; Leibfritz et al., 1992). The 3D 1H-13C-15N correlation experiment also confirmed the non-Ala(Me) a-carbon assignments (see Table 1).

13C Chemical Shift (ppm)

<table>
<thead>
<tr>
<th>Residue</th>
<th>δ for 13Ca</th>
<th>δ for 13C</th>
<th>JCN (Hz)</th>
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</thead>
<tbody>
<tr>
<td>Ac</td>
<td>-</td>
<td>172.55</td>
<td>13.3</td>
</tr>
<tr>
<td>B1</td>
<td>57.46</td>
<td>175.64</td>
<td>14.3</td>
</tr>
<tr>
<td>P2</td>
<td>65.68</td>
<td>175.74</td>
<td>14.3</td>
</tr>
<tr>
<td>B3</td>
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<td>15.4</td>
</tr>
<tr>
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<td>53.87</td>
<td>177.21</td>
<td>15.1</td>
</tr>
<tr>
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<td>57.29</td>
<td>177.86</td>
<td>14.8</td>
</tr>
<tr>
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<td>53.72</td>
<td>178.05</td>
<td>15.7</td>
</tr>
<tr>
<td>Q7</td>
<td>58.04</td>
<td>175.82</td>
<td>14.7</td>
</tr>
<tr>
<td>B8</td>
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<td>15.8</td>
</tr>
<tr>
<td>V9</td>
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<td>175.28</td>
<td>n.d.</td>
</tr>
<tr>
<td>B10</td>
<td>57.65</td>
<td>179.01</td>
<td>15.3</td>
</tr>
<tr>
<td>O11</td>
<td>44.78</td>
<td>173.01</td>
<td>17.0</td>
</tr>
<tr>
<td>L12</td>
<td>53.82</td>
<td>175.90</td>
<td>16.1</td>
</tr>
<tr>
<td>B13</td>
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<td>176.43</td>
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</tr>
<tr>
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<td>64.10</td>
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<td>57.58</td>
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<tr>
<td>Q19</td>
<td>55.56</td>
<td>174.02</td>
<td>18.0</td>
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Table 1. 13C assignments and coupling constants. Carbonyl and Ca 13C chemical shifts and JCN heteronuclear coupling constants of alamethicin dissolved in CD,OH at 300 K. The internal reference was the 13CD,OH solvent multiplet which was set to 49.0 ppm; n.d., not determined.

1JCN and hydrogen bond strength. Fig. 2 shows a 13C-15N correlation spectrum of alamethicin in CD,OH acquired with protons decoupled but 15N coupled. The observed crosspeak splitting is due to couplings of the 13C resonances to 13Ca and 15N resonances which are indicated in Fig. 2 for Gly11 and Gln19, respectively. The long-range JNC are too small to be observed even in the 13C-decoupled spectrum (not shown). The 1JCN are listed in Table 1; the average value is 15.3 ± 1.1 Hz.

Juranić et al. (1995) have proposed that strong hydrogen bonding at the carbonyl elevates 1JCN whereas strong hydrogen bonding at the amide proton decreases 1JCN, both effects being due to changes in the amide bond order. They also suggest that 1JCN values can be used to distinguish intramolecular and
intermolecular hydrogen bonding in proteins because the intramolecular bonds are weaker than those between amides and solvents such as water and methanol (Juranic et al., 1995; Walter and Wright, 1979). For example, in a short helix in human ubiquitin, the N-terminal residues have smaller than average \( J_{CN} \) (14 Hz) and the C-terminal residues have larger than average \( J_{CN} \) (16.5 Hz); the N-terminal residues are hydrogen bonded to solvent at their amide hydrogens and intramolecularly hydrogen bonded at their carbonyl oxygens whereas at the C-terminus the carbonyl oxygens are hydrogen bonded to solvent and the amide hydrogens are hydrogen bonded intramolecularly. A similar pattern is evident in alamethicin (Table 1); the N-acetyl has a smaller than average \( J_{CN} \) (13.3 Hz) which suggests that theHN of Ala(Me)1 is strongly hydrogen bonded to methanol whereas the carbonyl of the N-acetyl is weakly hydrogen bonded to the peptide. Gln19 has a larger than average \( J_{CN} \) (18.0 Hz) which suggests that the C'O of Gln19 is strongly hydrogen bonded to the solvent whereas an intramolecular hydrogen bond to solvent through its NH and C'O or that the intramolecular bonds are weaker than those between amides and proteins because the carbonyl carbon chemical shifts. The addition of Me\( _2 \)SO to alamethicin dissolved in methanol results in a shift to higher field of all the carbonyl resonances except those of Pro2 and Leu12 which move slightly to lower field (see Fig. 4A). The largest shifts observed are for the carbonyls of Gly11 and Gln18, followed by Ala(Me)10, Ala(Me)17, Gln19 and Pro14. These results correlate strongly with the hydrogen bonding states and calculated solvent accessibleibilities of the carbonyls in the crystal structures (see Fig. 4B and Fox and Richards, 1982). For example, in the crystal, the carbonyl oxygens of Gly11, Gln18, and Gln19 are highly accessible to solvent (Fig. 4B) and are not intramolecularly hydrogen bonded. The carbonyl of Ala(Me)10 is intramolecularly hydrogen bonded in only one of the three structures in the crystal and the carbonyl groups of Ala(Me)10 and Gly11 in one molecule are hydrogen bonded to methanol. The carbonyl of Pro14 is intramolecularly hydrogen bonded in two of the three structures and is moderately sensitive to perturbation by Me\( _2 \)SO (Fig. 4A and B). The correlation is poorest for the carbonyl of Ala(Me)17 which is hydrogen bonded to the hydroxyl of phenylalaninol-20 in two molecules and to the amide hydrogen of phenylalaninol-20 in the other (Fox and Richard, 1982). However, no NOEs have been detected supporting such an interaction in solution and the multiplicity of conformations in the crystal may reflect conformational flexibility in solution. The accessibility of the Ala(Me)17 carbonyl to Me\( _2 \)SO supports other evidence that the C-terminal helix is frayed at the end (see below and

Solvent dependence of the carbonyl carbon chemical shifts. The addition of Me\( _2 \)SO to alamethicin dissolved in methanol results in a shift to higher field of all the carbonyl resonances except those of Pro2 and Leu12 which move slightly to lower field (see Fig. 4A). The largest shifts observed are for the carbonyls of Gly11 and Gln18, followed by Ala(Me)10, Ala(Me)17, Gln19 and Pro14. These results correlate strongly with the hydrogen bonding states and calculated solvent accessibleibilities of the carbonyls in the crystal structures (see Fig. 4B and Fox and Richards, 1982). For example, in the crystal, the carbonyl oxygens of Gly11, Gln18, and Gln19 are highly accessible to solvent (Fig. 4B) and are not intramolecularly hydrogen bonded. The carbonyl of Ala(Me)10 is intramolecularly hydrogen bonded in only one of the three structures in the crystal and the carbonyl groups of Ala(Me)10 and Gly11 in one molecule are hydrogen bonded to methanol. The carbonyl of Pro14 is intramolecularly hydrogen bonded in two of the three structures and is moderately sensitive to perturbation by Me\( _2 \)SO (Fig. 4A and B). The correlation is poorest for the carbonyl of Ala(Me)17 which is hydrogen bonded to the hydroxyl of phenylalaninol-20 in two molecules and to the amide hydrogen of phenylalaninol-20 in the other (Fox and Richard, 1982). However, no NOEs have been detected supporting such an interaction in solution and the multiplicity of conformations in the crystal may reflect conformational flexibility in solution. The accessibility of the Ala(Me)17 carbonyl to Me\( _2 \)SO supports other evidence that the C-terminal helix is frayed at the end (see below and

Esposito et al., 1987; Yee and O’Neil, 1992; Franklin et al., 1994; Yee et al., 1995).

The carbonyl chemical shift changes induced by Me\( _2 \)SO could have resulted from a change in through-space shielding and/or a solvent-mediated change in the charge distribution of the carbonyl (Howarth and Lilley, 1978; Urry et al., 1974; Khaled et al., 1979). Alternatively, the change in solvent from a strong proton donor to a strong proton acceptor might have affected the carbonyl chemical shifts by inducing conformational changes in the peptide. To investigate this possibility we monitored the changes in \( J_{CN} \) and \( J_{CN} \) upon addition of up to Me\( _2 \)SO to the peptide dissolved in methanol. Only the Gin7 \( J_{CN} \) changed substantially, dropping from 5.1 Hz to 2.9 Hz, indicating that the N-terminal helix may be stabilized by Me\( _2 \)SO. All other \( J_{CN} \) changed by less than 1 Hz. The pattern of NH chemical shift perturbation by Me\( _2 \)SO (Fig. 4C) is nearly identical to that of the carbonyl carbons. The most strongly perturbed NH resonances are those of residues 12 and 19, followed by residues 11, 18, and 20. These are the NH which are directly hydrogen bonded to the solvent accessible carbonyls in the crystals (Fox and Richards, 1982) and in solution (Fig. 4A and B). The implication is that Me\( _2 \)SO can disrupt intramolecular hydrogen bonds when the adjacent carbonyl carbon is not involved in a stable intramolecular bond. Interestingly, the chemical shift of
the NH of Ala(Me)1 is unperturbed by Me₂SO whereas the ¹J_C-N data and hydrogen exchange data (Dempsey, 1995) indicate a strong interaction with methanol. As expected from the data of Juranic et al. (1995), additions of Me₂SO to methanol solutions of alamethicin reduce the ¹J_C-N at all residues, the greatest reductions being observed at Gly11, Gln18, and the N-acetyl (data not shown). An additional observation is that the NH of Ala(Me)3 appears inaccessible to solvent both by Me₂SO perturbation and ¹J_C-N but is not hydrogen-bonded in the crystal structure as the N-terminus is ³α-helical (Fox and Richards, 1982). One possible explanation is that the N-terminus is a ³α helix in solution, in which case the amide would be hydrogen-bonded.

**Temperature dependence of the carbonyl chemical shifts.**

The detection by NMR of specific peptide—solvent interactions, also observed in the crystal structures, is surprising for such a short peptide and suggests that alamethicin is a significantly more stable molecule than indicated by the NOE-derived structure determinations (Yee et al., 1995; Franklin et al., 1994). To investigate the stability of the peptide we measured the temperature dependence of the carbonyl chemical shift. All the carbonyl resonances exhibit a linear dependence on temperature from -23°C to +57°C. Fig. 5A shows the temperature dependence of the chemical shifts of the carbonyl resonances of the Ala(Me) residues. The temperature coefficients (Δδ/ΔT) of all residues are summarized in Fig. 5B. The carbonyls of the N-terminal acetyl and Ala(Me) residues 1, 3, 5, and 13 move to higher field as the temperature is raised while all the non-Ala(Me) residues and Ala(Me) residues 8, 10, 16, and 17 move downfield. Both positive and negative Δδ/ΔT were also reported in basic pancreatic trypsin inhibitor (Tuchsen and Hansen, 1988).

For the non-Ala(Me) residues postulated to be in a helical conformation at -23°C on the basis of the carbonyl chemical shift (2, 4, 6, 7, 11, 14, 18) the temperature-induced change in the shift is in the direction expected if increasing temperature decreases the time spent in the helical conformation. However, the total change in shift over the entire temperature range is at most 0.6 ppm (Gly11) for any residue. This compares to the difference in carbonyl chemical shift between the average random coil and ³α-helix values which ranges over 1–2.2 ppm in the data compiled by Wishart et al. (1991) and ranges over 2–3 ppm for the residues in short Ala-based peptides studied by Shalongo et al. (1994a,b). The largest temperature coefficients observed are for Gln18 and Gly11, residues which are also the most sensitive to Me₂SO perturbation (Fig. 4A). This suggests that the peptide is flexible in those regions where solvent interacts with the backbone. At +57°C the carbonyl chemical shifts of Gly11 and Gln18 move to less than 0.7 ppm of their random coil values suggesting that the peptide helix is unfolded in the middle and at the C-terminus of the peptide at the elevated temperatures. Flexibility of the peptide backbone has been used to explain interesting observations of paramagnetic enhancements of nuclear relaxation in alamethicin (North et al., 1994). The data presented here identify two sites of flexibility (Gly11 and residues 17–20) which, in combination, might help to explain the relaxation data.

In contrast to the non-Ala(Me) residues which shift upfield, Fig. 5B shows that several Ala(Me) ¹³C resonances and that from the N-acetyl shift downfield as the temperature is increased. Shalongo et al. (1994a) have pointed out that a major component of the observed temperature dependence of peptide ¹³C-carbonyl chemical shifts is the temperature dependence of the lock signal which is about +10 ppb/°C for D₂O (Glase, 1974). For example, in unstructured peptides they observed the temperature dependence of carbonyl resonances to range over about +5–10 ppb/°C (Shalongo et al., 1994a). The range in thermal dependence reflects differences in nuclear shielding among the atoms in the more populated excited rotational-vibrational states when the temperature is raised (Jameson, 1980; Schneider and Freitag, 1976). In their alanine-based peptides at low temperatures, where the helix is fully formed and stable, the thermal dependence of the carbonyl resonances is about +10 ppb/°C (Shalongo et al., 1994a). Conformational changes due to increased temperature were readily apparent as non-linear shifts to higher fields and slopes as high as -40 ppb/°C. The
Ala(Me)-based peptide alamethicin is dramatically different. The most thermal-sensitive residue, Gly11, exhibits a linear shift of only \(-7\) ppm/°C. The small (+1 to +3 ppm/°C), linear thermal dependence of the N-acetyl carbonyl and the carbonyls of Ala(Me) residues 1, 3, 5, and 13 suggest that, over the 80°C temperature range studied, these residues are the most conformationally stable in the peptide. As most of these residues occur in the N-terminus, this interpretation is in agreement with the data which suggests that the N-terminus of alamethicin is more stable than the C-terminus (Esposito et al., 1987; Yee and O’Neil, 1992; Franklin et al., 1994; Yee et al., 1995) and that Ala(Me) residues can give remarkable conformational stability to helical peptides (Augspurger et al., 1995). The presence of an N-acetyl group may add additional stability to the N-terminus of the helix (Chakrabarty et al., 1993; Jung et al., 1983).

Temperature dependence of the molar ellipticity. The temperature dependence of the CD spectrum and the molar ellipticity at 222 nm are shown in Fig. 6. Based on comparison to the ellipticities of alanine-based peptides (Augspurger et al., 1995, and references therein), the data suggest that alamethicin is about 50% helical at -5°C and about 30% helical at +57°C. The low linear thermal dependence of the helix unfolding and the measurable helicity at high temperature are in agreement with the thermal unfolding measured by the \(^{13}\)C chemical shifts.

Folding of Ala(Me) peptides. In linear helical peptides the usual model for conformational change with temperature is the two-state helix/coil transition. This model has been used successfully to explain cooperative transitions observed by CD in model alanine-based peptides (Scholtz et al., 1991). Although the present data suggest that alamethicin can be induced to partially unfold over the 80 K temperature range studied, we fail to see a sharp transition between helix and coil for any of the residues. The all-or-none cooperativity of helix formation can be explained in terms of the relatively high entropic cost of helix initiation compared to that associated with helix propagation (Cantor and Schimmel, 1980). The lack of cooperativity in the folding/unfolding of alamethicin is due, at least in part, to the high helix propensity of the Ala(Me) residues (Basu and Kuki, 1993). Steric hindrance in \(\alpha\)-methylated amino acids such as Ala(Me) offsets the entropic gain of unfolding these peptides (see Augspurger et al., 1995). Thus, the peptide can be viewed as permanently initiated for helix formation at several sites throughout its length [Ala(Me) residues]. The temperature dependence of the carbonyl chemical shifts suggests that the unfolding of the peptide is slightly greater among the non-Ala(Me) residues than the Ala(Me) residues. Interestingly, Ala(Me)10 and Ala(Me)17 appear to have about the same thermal sensitivity as the majority of non-Ala(Me) residues. The sensitivity of their carbonyls to Me,SO suggests that intramolecular hydrogen bonding is comparatively weak in those regions of the peptide.

The results of Augspurger et al. (1995) indicate that an Ala(Me)-rich (77%) nonapeptide is \(3_{10}\) helical and thermally stable up to 150°C in both chloroform and Me,SO. The thermal behaviour of alamethicin (40% Ala(Me)) in methanol is intermediate between the cooperative unfolding of alanine-based peptides in water and the thermal insensitivity of high-Ala(Me) peptides dissolved in non-polar solvents. This suggests that the closed (bent) conformation proposed for alamethicin by North et al. (1994, 1995) may be a rare occurrence rather than the dominant conformation in solution. As organic solvents are known to reduce the cooperativity of peptide and protein folding (Thomas and Dill, 1993), it remains to be determined to what extent the heat stability and lack of cooperative folding in these peptides is due to solvent (Augspurger et al., 1995). An answer to this question will determine the extent to which \(\alpha\)-methylated amino acids can increase the heat stability of designed proteins.

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Supplementary material. The interactions with solvent, heat stability and $^{13}$C-labelling of alamethicin, an ion-channel-forming peptide. Spin-echo difference experiments, Chemical shift index for $^{13}$C carbonyl resonances, Deuterium isotope effect. Fig. S1: a spin-echo difference spectrum. Fig. S2: $^{13}$C carbonyl chemical shift index. Fig. S3: $^{13}$C-$^1$N correlation spectrum. This information is available, on request, from the Editorial Office. Six pages are available.