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FOR THE RECORD

Multiple conformations of the sea anemone polypeptide anthopleurin-A in solution

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Abstract: Anthopleurin-A (AP-A) is a member of a family of sea anemone-derived polypeptides that interact with sodium channels in a voltage-dependent manner, producing a positive inotropic effect on the mammalian heart. There has been considerable interest in this molecule as a lead compound for the development of novel therapeutic agents. Earlier attempts to define the 3-dimensional structure of AP-A were complicated by the fact that it was found to exist in 2 conformations in solution. Using ^{1}H- and ^{13}C-NMR spectroscopy, we have now shown that this conformational heterogeneity arises from *cis–trans* isomerization about the Gly 40–Pro 41 peptide bond and that in the major form of the protein this peptide bond adopts a *cis* conformation. Furthermore, the increased sensitivity afforded by higher-field NMR has allowed identification of additional minor conformations of AP-A, the origin of which is presently unknown. We believe there will be many more examples of the detection by high-field NMR of previously unobserved minor conformations of proteins in solution.

Keywords: cardiac stimulant; *cis* peptide bond; multiple conformations; NMR; toxin

Anthopleurin-A (AP-A) is a member of a family of sea anemone-derived polypeptides that interact with the voltage-gated sodium channel (Tanaka et al., 1977; Norton, 1991). In the mammalian heart this results in delayed inactivation of the channel, producing a positive inotropic effect without affecting blood pressure or heart rate (Blair et al., 1978; Scriabine et al., 1979). Although these polypeptides have limited potential as therapeutic agents due to their immunogenicity and lack of oral activity, a knowledge of their 3-dimensional structure may provide a lead in the development of new agents for the treatment of cardiac conditions. Initial NMR studies of the conformation of AP-A were complicated by the fact that several protons were found to resonate at 2 different chemical shifts (Gooley et al., 1984; Gooley & Norton, 1985; Mabbutt & Norton, 1990). Subsequently it was shown that this splitting was due to conformational rather than chemical heterogeneity in the sample (Gooley et al., 1988). Comparison of the NMR spectra of AP-A and the related polypeptide toxins ATX I and ATX II led to the suggestion that this heterogeneity arose from *cis–trans* isomerization of the Gly 40–Pro 41 peptide bond (Gooley et al., 1984, 1988). Because the resonances of Pro 41 in AP-A were only partially assigned in earlier studies, however, no direct evidence for the conformation of this peptide bond could be obtained. In this report we describe ^{1}H-NMR studies of AP-A at 600 MHz that have allowed completion of the assignments of all resonances in the major conformation of AP-A and the majority of resonances in the minor conformation. Sequential connectivities observed in NOESY spectra of AP-A confirm that the heterogeneity does indeed arise as a result of *cis–trans* isomerization of the Gly 40–Pro 41 peptide bond and that in the major conformer this bond is *cis*. In addition, further splitting of resonances has been identified in the spectra of AP-A at 600 MHz, with as many as 4 distinct resonance positions being observed for several protons. Because this splitting is present in both native and synthetic AP-A (Pennington et al., 1994), it appears to represent yet another form of conformational heterogeneity in the molecule.

Sequence-specific resonance assignments for AP-A were made using standard methods (Wüthrich, 1986). Two distinct resonance positions were observed for most protons. For 13 of the 44 amide protons the chemical shifts in the 2 conformers differed by >0.1 ppm (Fig. 1), suggesting that there may be differences in their structures. Figure 2 shows the region of a double quantum spectrum (Wagner & Zuiderweg, 1983) of AP-A containing the remote NH to $C^{\alpha}H_1 + C^{\alpha}H_2$ cross peaks of the Gly residues. The presence of more than 1 resonance for each of the residues is clearly evident. Additional unassigned peaks, presumably arising from further conformations (see below) of 1 or more of the Gly residues, are also present.

The conformation of an X–Pro peptide bond can be determined on the basis of sequential NOEs to the Pro $C^{\alpha}H$ and $C^{\delta}H$ resonances (Wüthrich, 1986). Figure 3A shows part of the aliphatic region of a NOESY spectrum (Anil-Kumar et al., 1980; Macura et al., 1981) of AP-A. In the major conformer, cross peaks are observed between the $C^{\alpha}H$ resonances of Gly 40 and Pro 41, indicating that the peptide bond is *cis*, whereas in the minor conformer, cross peaks are observed between the reso-

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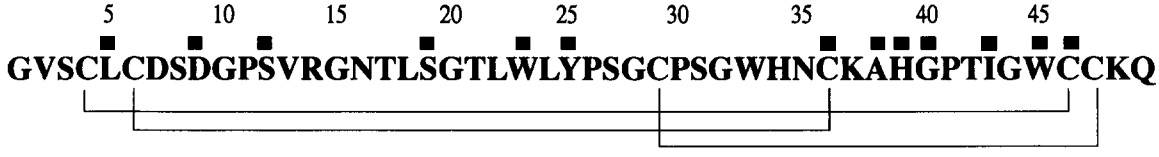

Fig. 1. Amino acid sequence of AP-A. Lines indicate the position of the 3 disulfide bonds. Those residues for which the amide proton chemical shift differs by more than 0.1 ppm between the major and minor conformations are indicated (■).

nances of Gly 40 $C^{\alpha}H$ and Pro 41 $C^{\delta}H$, consistent with a *trans* conformation. Studies of small peptides have established the ^{13}C chemical shift ranges for proline carbons in *cis* and *trans* environments (Dorman & Bovey, 1973; Wüthrich, 1976). The most sensitive indicator of the X-Pro peptide bond conformation is the Pro C^{β} resonance, with chemical shifts of 33.1 and 30.6 ppm in the *cis* and *trans* forms, respectively. Figure 3B shows part of a natural abundance heteronuclear multiple quantum coherence (HMQC) spectrum (Bax et al., 1983) of AP-A. The ^{13}C chemical shifts of the Pro 41 C^{β} resonances in the major and minor conformations are 33.5 and 30.2 ppm, respectively, confirming that the Gly 40–Pro 41 peptide bond is *cis* in the major form of AP-A and *trans* in the minor.

In a NOESY spectrum of AP-A acquired at 315 K, a chemical exchange cross peak is observed between the major and minor resonances of the well-resolved Cys 46 NH proton. This cross peak is absent from spectra acquired at 300 K. An estimate of the barrier to interconversion between the 2 forms can be obtained from the equation (Gunther, 1980)

$$\Delta G\ddagger = 19.14T_c[10.32 - \log(k/T_c)],$$

where T_c is the coalescence temperature and k is the rate of interconversion at this temperature. Because this equation assumes exchange between 2 equally populated states, a correction has to be applied to reflect the relative populations of the interconverting species (Martin et al., 1980). In AP-A, the ratio of major to minor conformations is approximately 2:1, in which case the rate of interconversion, k, can be obtained from the expression

$$k = \delta\nu/0.672,$$

where $\delta\nu$ is the chemical-shift difference in Hertz between the 2 states. In AP-A, multiple peaks are still observed for a number of proton resonances even at temperatures as high as 358 K (Gooley et al., 1988). On the basis of this observation, the $\Delta G\ddagger$ value is >78 kJ mol^{-1}. Values of $\Delta G\ddagger$ for *cis–trans* isomerization of X-Pro peptide bonds in short peptides have been shown to range from 68 to 83 kJ mol^{-1}, with the dipeptide Gly-Pro having a value of 77 kJ mol^{-1} (Grathwohl & Wüthrich, 1981).

These observations clarify the origin of conformational heterogeneity observed previously in spectra of AP-A. ^{1}H-NMR spectra recorded at 600 MHz, however, contain a number of low-intensity peaks that could not be clearly identified as spin systems from native AP-A in spectra obtained at lower frequencies. Because these peaks are present in spectra of both AP-A purified from *Anthopleura xanthogrammica* and synthetic AP-A (Pennington et al., 1994), it is unlikely that they are chemical contaminants or denatured protein. Rather, they appear to reflect additional conformational heterogeneity in the native molecule. As shown in Figure 4, certain well-resolved resonances

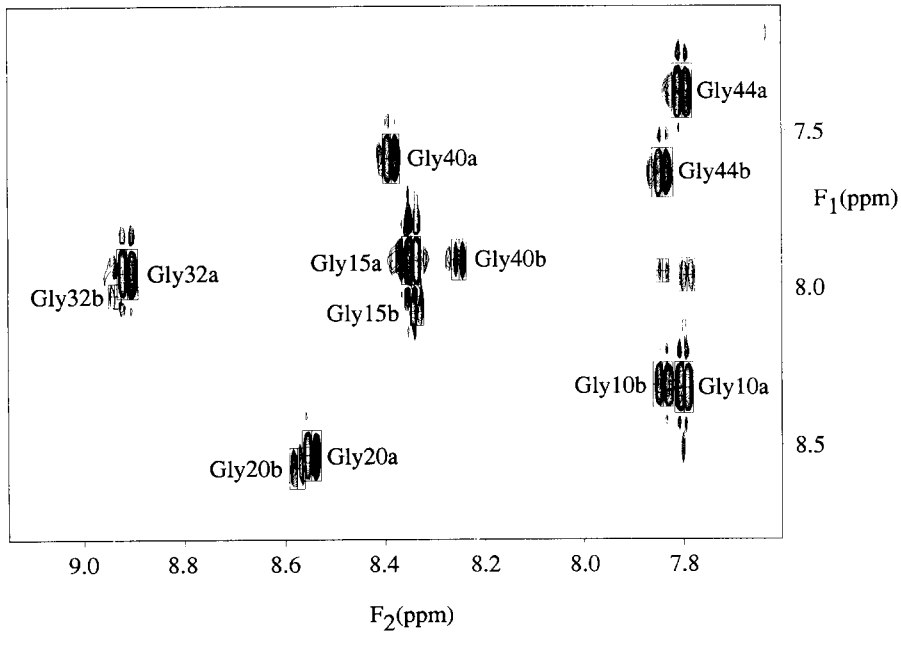

Fig. 2. Region of the double-quantum coherence spectrum of AP-A in 90% H_2O/10% D_2O at pH 4.8 and 300 K, acquired with a total multiple-quantum preparation time of 40 ms, showing the remote NH to $C^{\alpha}H_1$ + $C^{\alpha}H_2$ cross peaks from resonances of the major (a) and minor (b) conformations of the Gly residues. For all NMR experiments the protein concentration was ca. 3.5 mM. Remote peaks arising from Gly 28, which has an NH resonance position of 6.94 ppm, are not shown.

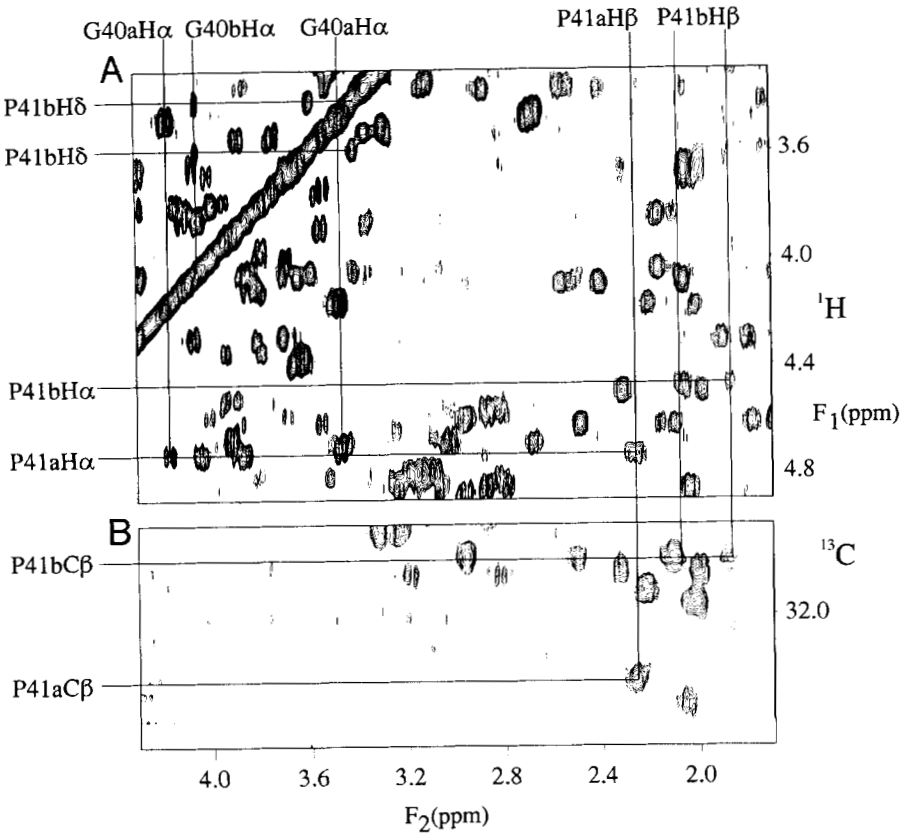

Fig. 3. A: Part of the aliphatic region of the 200-ms mixing time NOESY spectrum of AP-A in D_2O recorded at 600 MHz, pH 4.8, and 300 K. Sequential connectivities between proton resonances of Gly 40 and Pro 41 in both major (a) and minor (b) conformations are highlighted. In the minor conformer (b), cross peaks are observed to only 1 of the Gly 40 $C^{\alpha}H$ protons. The intraresidue $C^{\alpha}H_1$ to $C^{\alpha}H_2$ cross peak for the minor conformation of Gly 40 at 4.08/3.81 ppm is obscured by overlap with other peaks. **B:** Natural abundance ^1H-^{13}C HMQC spectrum of AP-A in 90% H_2O/10% D_2O recorded at 500 MHz, pH 4.8, and 298 K, showing the C^{β}-H^{β} cross peaks of Pro 41 in the *cis* (a) and *trans* (b) forms.

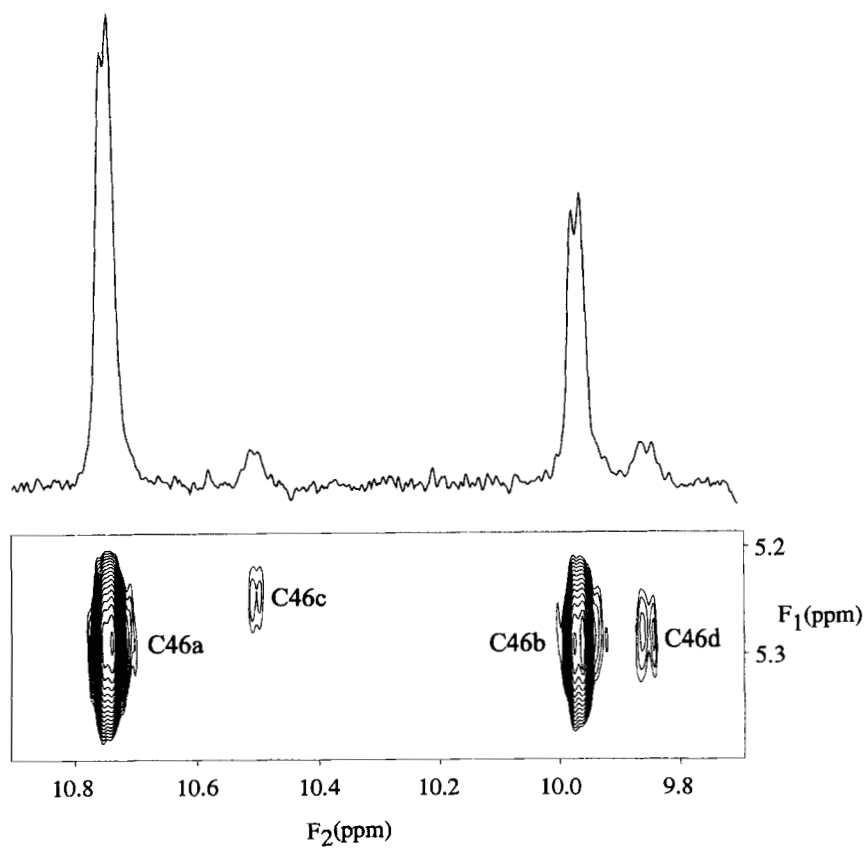

Fig. 4. Part of the amide to aliphatic region of the total coherence spectrum of AP-A in 90% H_2O/10% D_2O at pH 4.8 and 300 K, recorded with a spin-lock period of 70 ms. Cross peaks arising from the 4 distinct conformations of Cys 46 NH are labeled. The projection was calculated by summing all rows of the displayed region.

have as many as 4 distinct resonance positions for a single proton, with relative intensities of approximately 18:9:1:1. At present the cause of this further heterogeneity is unknown, but, because AP-A contains a total of 4 Pro residues as well as 3 disulfide bridges (Fig. 1), it could arise from *cis–trans* isomerization of one of the remaining X-Pro peptide bonds or as a result of isomerism of one of the disulfides. Recently, the existence of a minor conformation caused by disulfide isomerization has been identified in bovine pancreatic trypsin inhibitor (Otting et al., 1993). It is likely that the presence of such minor conformers of proteins in solution is more common than previously recognized and that high-field NMR may identify many other examples.

A high-resolution structure of AP-A is essential as a basis for the rational design of mimics of the native protein, which may have potential as novel therapeutic agents. However, structure determination is complicated by the existence of multiple conformations in solution. Although conformational heterogeneity caused by *cis–trans* isomerization of prolyl peptide bonds is not uncommon, it is somewhat unusual for the *cis* conformer to dominate in a native globular protein in solution (Evans et al., 1987; Stanczyk et al., 1989; Santoro et al., 1993). Clearly it will be necessary to determine the structure and activity of the different conformers as part of the process of designing synthetic analogues. Work is currently underway in this laboratory to calculate structures of AP-A based on NMR data and to determine the structural implications of the observed conformational heterogeneity. In addition, AP-A is being expressed in bacteria with a view to undertaking site-directed mutagenesis studies. Substitution of Pro 41 would be expected to remove the *cis–trans* isomerization observed in native AP-A, thereby addressing the question of how this conformational difference affects cardiac stimulatory activity. It may also provide insight into the cause of the additional heterogeneity observed in the conformation of this protein.

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