Conformational heterogeneity in polypeptide cardiac stimulants from sea anemones

Paul R. Gooley, John W. Blunt[+] and Raymond S. Norton*

School of Biochemistry, University of New South Wales, PO Box 1, Kensington, NSW 2033, Australia

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High-resolution ^{1}H NMR spectra at 300 MHz of the polypeptide cardiac stimulants anthopleurin-A and *Anemonia sulcata* toxin II reveal conformational heterogeneity in both molecules. The two conformations, manifest in a number of split ^{1}H resonances, are in slow exchange over a wide range of pH and temperature. Heterogeneity affects a region of these molecules containing the structurally and functionally important Asp residues. By comparison with a homologous polypeptide *Anemonia sulcata* toxin I, which does not show this type of heterogeneity, it is suggested that the heterogeneity may originate in *cis-trans* isomerism of the Gly-40 to Pro-41 peptide bond.

Cardiotonic agent *Neurotoxin* *Polypeptide conformation* *^{1}H-NMR* *Proline* *pH titration*

1. INTRODUCTION

Sea anemones contain a number of polypeptides of M_r ~5000, which exhibit strong cardiac stimulatory activity. The best characterised of these are AP-A[2] from *Anthopleura xanthogrammica* [1] and ATX II from *Anemonia sulcata* [2,3], which share extensive sequence homology (fig.1). These polypeptides appear to act by delaying inactivation of the cardiac Na^+ channel [4], thus prolonging the fast inward Na^+ current and increasing the force of cardiac contraction. Because of their potency in this respect their pharmacological and electrophysiological properties are being extensively studied. However, little is known about their structure in solution. AP-A and ATX II have been investigated by a range of spectroscopic techniques [5,6], and their overall structures and ionisation behaviour have been compared using ^{13}C NMR spectroscopy [7–9]. However, more detailed structural information is required as a basis for understanding their mode of action. We are carrying out high-resolution ^{1}H NMR studies of the structures of AP-A, ATX II, and the closely related polypeptide ATX I [10], the sequences of which are shown in fig.1. We have observed major conformational heterogeneity in AP-A and ATX II, but not ATX I. We describe these findings and present a possible explanation in terms of *cis-trans*

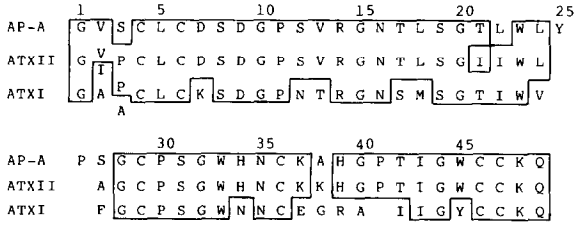

Fig.1. Amino acid sequences of AP-A, ATX II and ATX I [1,3].

[+] Permanent address: Department of Chemistry, University of Canterbury, Christchurch, New Zealand

* To whom correspondence should be addressed

Abbreviations: AP-A, anthopleurin-A; ATX I, *Anemonia sulcata* toxin I; ATX II, *Anemonia sulcata* toxin II; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; COSY, 2-dimensional homonuclear correlated spectroscopy

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isomerism of a Pro peptide bond in the native proteins. This conformational heterogeneity may affect the cardiac stimulatory activities of these molecules.

2. MATERIALS AND METHODS

AP-A was isolated from *A. xanthogrammica* by the methods of [1] or [11], and ATX I and II from *An. sulcata* as in [2,3]. Sample purity was checked by polyacrylamide gel electrophoresis (PAGE) both at pH 4.6 and in the presence of SDS [12]. Densitometer scans of the gels indicated that AP-A contained less than 5% impurity, while ATX II contained up to 18%. AP-A was also pure according to amino acid and N-terminal sequence analyses. ^2H_2O (>99.75% ^2H) was obtained from the Australian Atomic Energy Commission, Lucas Heights, NSW, and ^2HCl and NaO^2H from Merck, Sharp and Dohme, Montreal.

Prior to NMR experiments samples were incubated in ^2H_2O at room temperature and lyophilised, this procedure being carried out at least twice. pH was measured at 22°C using an Activon model 101 pH meter fitted with an Ingold microelectrode, model 6030-02. Titration curves were analysed by a non-linear, least-squares fit, assuming rapid exchange between the acidic and basic extremes of each ionisation.

300 MHz ^1H NMR spectra were recorded in the pulsed Fourier transform mode with quadrature detection on a Bruker CXP-300 spectrometer incorporating an Aspect 2000 computer. Spinning sample tubes (5 mm o.d.) were used. Probe temperature was 27°C. Typical acquisition parameters were: sweep width 3816 or 4000 Hz, 90° RF pulse (5.4–7.3 μs), 2.0 s recycle time, 8192 time-domain addresses. Prior to Fourier transformation, spectra were multiplied by a Lorentz-Gauss window function, and in most cases zero-filled to 32768 addresses. Chemical shifts are expressed relative to DSS, but were measured using a trace of internal dioxane at 3.751 ppm downfield from DSS. A 2-dimensional COSY spectrum [13] was recorded using the pulse sequence t_0-90°-t_1-90°-t_2, with appropriate phase cycling to select N-type peaks. t_1 was incremented 255 times from 0.01–67 ms, t_2 was 0.27 s, sweep width 3800 Hz, and recycle time 1.5 s. Low-power irradiation of the H^2HO resonance was applied continuously during the period t_0. Prior to Fourier transformation the 256 × 2048 data matrix was multiplied by a sine-bell function in t_1 and a sine-squared bell function in t_2, and zero-filled to yield a 1024 × 4096 matrix. The frequency domain spectrum is shown in the absolute value mode, without symmetrisation.

3. RESULTS

^1H NMR spectra of AP-A and ATX II contain many resonances which are split into two components. In the aromatic regions of their spectra, shown in fig.2, this is most clearly seen for the well-resolved $H^{\epsilon 1}$ resonances of the two His residues, near 8.5 ppm. In each spectrum the downfield $H^{\epsilon 1}$ resonance is split into two peaks, 1a and b, with intensities of ~2/3 and 1/3 of a single proton, respectively, in AP-A, and half a proton each in ATX II. The corresponding $H^{\delta 2}$ resonances are also split, as shown by the COSY spectrum of AP-A in fig.3. In this spectrum the existence of spin–spin coupling between two protons is manifested in a pair of off-diagonal cross peaks which form two corners of a square. The other two

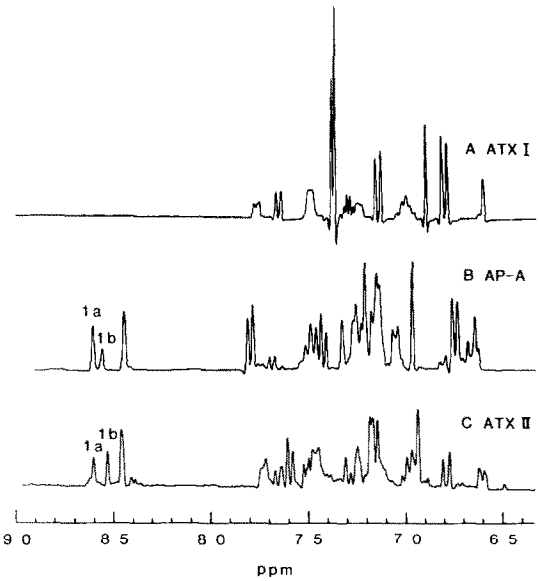

Fig.2. Aromatic regions of 300 MHz ^1H NMR spectra of (A) 2 mM ATX I in ^2H_2O, pH 6.8 (2240 scans); (B) 7 mM AP-A in ^2H_2O, pH 4.0 (3264 scans); (C) 2 mM ATX II in ^2H_2O, pH 4.7 (2000 scans). Other spectral conditions are described in section 2.

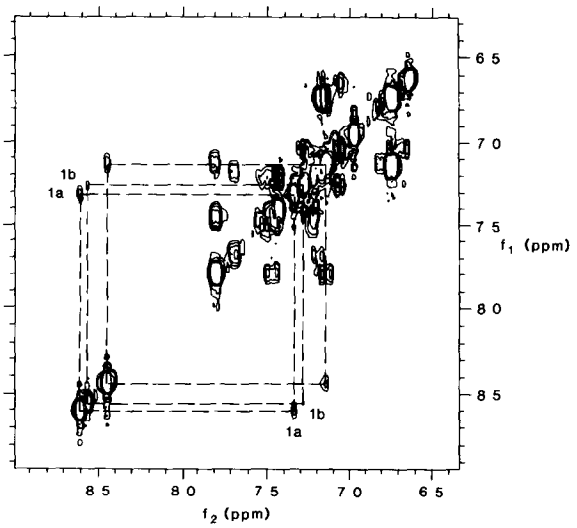

Fig.3. Contour plot of the 6.35–9.0 ppm region of a 300 MHz ^{1}H COSY spectrum of 10 mM AP-A in ^2H_2O, pH 4.7, 27°C. Digital resolution in f_1 is 3.71 Hz/pt and in f_2 1.85 Hz/pt. Spectrum was recorded in ~18 h. Proton-proton J connectivities between His$^{\epsilon 1}$ and His$^{\delta 2}$ for both His are shown.

corners, located on the diagonal, correspond to the chemical shifts of the two coupled protons. In fig.3 the chemical shifts of peaks 1a and b are 8.61 and 8.57 ppm, respectively, and those of their corresponding H$^{\delta 2}$ resonances 7.33 and 7.27 ppm. At the pH values of figs 2 and 3, the H$^{\epsilon 1}$ and H$^{\delta 2}$ resonances of the second His residue show little or no splitting, but clear splitting occurs at other pH values.

Several other resonances in the aromatic and methyl regions of the spectra of these two polypeptides show similar splittings. Low-field ^{13}C NMR spectra of ATX II also showed splitting on one of the Trp C$^{\gamma}$ resonances [9]. The extent of these effects, together with their observation in two polypeptides from different species, suggests that they are not due to contaminants. AP-A is pure by the criteria of amino acid composition, N-terminal sequence analysis and polyacrylamide gel electrophoresis, while ATX II contains a small amount of impurity. In neither case can the splittings be accounted for by impurities.

It appears, therefore, that AP-A and ATX II exist in two conformations in solution. In each molecule these two conformations are in slow chemical exchange over the pH range 1.4–12.8 at 27°C, as indicated by the lack of exchange broadening for pairs of resonances only a few hertz apart (e.g., in ATX II at pH 4 sharp peaks 3 Hz apart are observed for the two components of one His H$^{\epsilon 1}$ resonance, indicating that the lifetime of each conformation is $\gg$50 ms). At low pH there is a decrease in the fraction of one conformation in ATX II (e.g., at pH 1.4–1.7 the ratio of minor to major form is approx. 1:2.6, compared with 1:1.2 at pH 4–5), but not in AP-A. At 67°C (pH 4.0) for AP-A and 75°C (pH 5.0) for ATX II the two conformations are still in slow exchange. Clearly, there is a high activation energy barrier to interconversion.

His-34 and -39 of APA- and ATX II are both affected by the heterogeneity. In addition, both residues sense the ionisation of a carboxylic group at low pH, and the pK_a for the latter differs in the two conformations, as indicated in table 1. Taking a weighted average over the data for H$^{\epsilon 1}$ of His-34 and H$^{\delta 2}$ and H$^{\epsilon 1}$ of His-39 in AP-A, the pK_a of this carboxylic group is 1.7 in conformation *a* and 2.5 in *b*. Corresponding values for His-39 in ATX II are 1.9 and 2.3. Previous ^{13}C NMR studies of AP-A [8] and ATX II [9] showed that one of the Asp residues (at position 7 or 9 in the sequence) has p$K_a \lesssim 2$, and that protonation of this carboxylate causes extensive conformational changes in AP-A and ATX II. The fact that this structurally important Asp residue exhibits different pK_a values in the two forms indicates that its local environment is also affected by the heterogeneity.

4. DISCUSSION

The ^{1}H NMR data presented here indicate that AP-A and ATX II exhibit conformational heterogeneity in solution. This type of conformational heterogeneity is not observed in ATX I (In ATX I methyl resonances of Met-18 and one Thr residue show splitting into two peaks of equal intensity [10]. These splittings collapse at low pH or intermediate temperature, and have a different origin from the major effects observed for AP-A and ATX II.) (see fig. 2) Although there are many amino acid substitutions in ATX I, compared with AP-A and ATX II, we believe that the significant difference is the absence of Pro-41 in ATX I. Thus, it is possible that the peptide bond preceding

Table 1

Titration data for His residues of AP-A (1–2 mM, ^2H_2O, 27°C) and ATX II (0.7–1.5 mM, ^2H_2O, 27°C)

Resonance			pK_{a1}	δ_{A1}	$\delta_{A1}-\delta^B{}_1$	pK_{a2}	δ_{A2}	$\delta_{A2}-\delta_{B2}$
AP-A								
His-34[A]	$H^{\epsilon 1}$	a	1.5 (0.3)	8.524	0.070	6.90 (0.09)[C]	8.456	0.941
		b	2.6 (0.3)	8.498	0.045			
	$H^{\delta 2}$	a/b	2.3 (0.3)[C]	7.091	−0.050	6.82 (0.13)[C]	7.141	0.356
His-39	$H^{\epsilon 1}$	a	1.8 (0.3)	8.603	−0.036	6.65 (0.10)	8.638	0.988
		b	2.27 (0.16)	8.483	−0.099	6.71 (0.08)	8.587	0.938
	$H^{\delta 2}$	a	1.8 (0.3)	7.267	−0.075	6.65 (0.11)	7.339	0.400
		b	2.7 (0.2)	7.218	−0.065	6.77 (0.08)	7.284	0.345
ATX II								
His-34[A]	$H^{\epsilon 1}$	a/b	–[D]			6.91 (0.12)[C]	8.475	0.971
	$H^{\delta 2}$	a	–[E]			6.81 (0.11)	7.150	0.413[F]
		b				6.76 (0.12)	7.173	0.412[F]
His-39	$H^{\epsilon 1}$	a	1.95 (0.17)	8.511	−0.123	6.46 (0.09)	8.637	0.988
		b	2.36 (0.19)	8.406	−0.150	6.50 (0.06)	8.556	0.907
	$H^{\delta 2}$	a	1.9 (0.2)	7.146	−0.176	–[E]		
		b	2.21 (0.15)	7.015	−0.240	–[E]		

[A] Residue numbering system for AP-A is used (fig.1)
[B] Values in parentheses are standard deviations
[C] Average value for forms a and b
[D] Peak moves downfield slightly at pH < 4 and splits into two components
[E] Peak overlap and uncertainties about crossover prevent accurate pK_a determinations
[F] Analysed assuming no crossover of peaks during titration. Peak crossover would change values fo 0.389 and 0.436, respectively, with no effect on pK_a or δ_A

Pro-41 in AP-A and ATX II can exist in either the *cis* or *trans* configuration in the native proteins. Many biologically active peptides contain equilibrium mixtures of *cis* and *trans* peptide bonds preceding Pro residues, and some native proteins, e.g., ribonuclease A and subtilisin, contain *cis* Pro residues [14]. However, we know of only one example of conformational heterogeneity in a native, globular protein ascribed to *cis-trans* isomerism of the peptide bond preceding a Pro residue, viz., the Leu-20 to Pro-21 bond in dihydrofolate reductase [15].

Some support for the proposition that conformational heterogeneity in AP-A and ATX II originates in the Gly-40 to Pro-41 peptide bond comes from the effects on the His $H^{\delta 2}$ and $H^{\epsilon 1}$ resonances. In each polypeptide, the downfield $H^{\delta 2}$ and $H^{\epsilon 1}$ resonances show greater splitting than their upfield counterparts. From analysis of nuclear Overhauser enhancement studies and ionisation behaviour, the downfield $H^{\delta 2}$ and $H^{\epsilon 1}$ resonances are assigned to His-39 and the upfield resonances to His-34 (unpublished). The greater splitting of both the $H^{\delta 2}$ and $H^{\epsilon 1}$ resonances of His-39 compared with His-34 is consistent with heterogeneity in the Gly-40 to Pro-41 peptide bond conformation being the cause of the splittings.

Conformational heterogeneity in AP-A and ATX II, whatever its origin, is of interest not just from the standpoint of protein structure, but because this heterogeneity affects residues thought to be essential for cardiac stimulatory activity. His-39 has been proposed to be part of the 'active site' of AP-A [1]. In addition, arguments based on chemical modification studies and ^{13}C NMR data [7–9] indicate that one or both Asp residues are essential for activity. As the local environments of these functionally important residues are affected by heterogeneity, it is likely that cardiac stimulatory activity is also affected.

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