Conformational Studies of Peptide Heart Stimulant Anthopleurin A

LASER RAMAN, CIRCULAR DICHROISM, FLUORESCENCE SPECTRAL STUDIES, AND CHOU-FASMAN CALCULATIONS*

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Sea anemone contain a number of closely related peptide heart stimulants. In the present investigation, the conformation of anthopleurin A from Anthopleura xanthogrammica was investigated by laser Raman, circular dichroism, and fluorescence spectral methods and by the Chou-Fasman method using sequence data. The recent $^{13}$C NMR data of the peptide (Norton, R. S., and Norton, T. R. (1979) J. Biol. Chem., in press) provided useful information for the interpretation of the above-mentioned spectral data. The results from these spectral methods suggested that anthopleurin A and the related sea anemone peptides are roughly spherical in shape due to the presence of some $\beta$-bends, possibly due to a $\beta$-pleated sheet region and due to the 3 cystine residues in the peptide which exist in the gauche-gauche-gauche configuration. The sole tyrosine residue is exposed to the solvent, a finding which has now been confirmed by $^{13}$C NMR. The laser Raman and fluorescence spectral procedures showed that one or more of the tryptophan residues are buried. Interestingly, the reduction of the native protein with dithioerythritol did not change the spherical shape even in the presence of 5 M guanidine HCl and the carboxymethylcysteine derivative of the peptide was folded even in the presence of the denaturing agent, guanidine HCl.

A number of closely related peptides have been isolated from different species of sea anemone (1-3) which act as neuromuscular blocking agents in invertebrates (4), and cause a positive inotropic effect on the heart of vertebrates (5). At about $10^{-8}$ to $10^{-7}$ M concentrations, they are heart toxins and cause heart seizure while at lower concentrations at lo- to lo- M, they are heart stimulants with positive inotropic action but exhibit no chronotropic nor blood pressure effects (3, 5). At lo- M concentration, it has been reported that toxin II, at lo- M, they are heart stimulants with positive inotropic action but exhibit no chronotropic nor blood pressure effects (3, 5). In addition, toxin II has recently been shown to enhance activation of the action potential sodium ionophore of electrically excitable neuroblastoma cells by veratridine and batrachotoxin (7). Thus far, toxins I, II, and III (8) have been isolated from Anthopleura sulcata, Anthopleurin A and B from Anthopleura xanthogrammica, and Anthopleurin C from Anthopleura elegansima (3). The amino acid sequences of some of these peptides have been shown to differ only slightly. Each of the peptides contains 3 cystine residues and 3 tryptophan residues in homologous positions in the peptides (8, 9). The pairing of the cystine residues in inox II have been recently reported (8) and unpublished results from our laboratory indicate analogous pairing of the 6 cysteine residues in AP-A.

For structure-function investigations, it is useful to know the conformations of the peptide heart stimulants. The only conformational information available is a report that toxin II is a random coil peptide which is held together by disulfide bonds (10). Thus, the conformation of AP-A was calculated by the Chou-Fasman procedure from sequence data (11) and was also investigated by the following spectral methods, i.e., laser Raman, circular dichroism, and fluorescence methods, and the results are interpreted in this report.

**EXPERIMENTAL PROCEDURES**

**Materials**—AP-A was isolated and purified as described previously from the sea anemone, A. xanthogrammica (3). The samples were at least 97% pure as judged from amino acid analyses and various chromatographic tests. Sources of the chemicals were: guanidine HCl, ultrapure, Schwarz/Mann; 2-mercaptoethanol, Sigma Chemical Co.; dithioerythritol, Schwarz/Mann; iodoacetic acid, J. T. Baker, which was recrystallized from CHCl$_3$ and D$_2$O, Sigma Chemical Co. All of the common reagents used were of reagent grade quality.

**Methods**—The procedure of Chou-Fasman (11) was used to calculate the conformation of AP-A and toxin II. The carboxymethylcysteine (Cys(Cm)) derivative was prepared by the procedure of Crestfield and Moore (12) as described previously (9). Amino acid analyses were performed on the peptide after 24 h hydrolysis of the peptide in 5.7 N HCl and subsequent analysis in the Beckman model 121 MB automatic amino acid analyzer as described basically by Spackman et al. (13). Spectra were recorded in the Cary model 14 automatic recording spectrophotometer and all pH measurements were made in the Corning model 122 Research pH meter.

**Laser Raman Spectroscopy**—Samples (powder) were excited by the 514.4 nm line of an argon ion laser, Spectrophysics model SP-164, with a green interference filter. The resulting spectra were recorded using a Raman spectrometer (Ramanog 5) manufactured by Spex Industries, Inc.

For deuteration, the sample was dissolved in D$_2$O and was allowed to deuterate at 0°C over 6-8 h.

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2. The abbreviations used are: AP-A, anthopleurin A; Cys(Cm), S-$\beta$-carboxymethylcysteiny1; Gdn-HCl, guanidine hydrochloride.
to stand for an hour or more. The samples were then lyophilized. This procedure repeated twice for completion of deuterium exchange. The lyophilized sample was finally placed in a specially designed, sealed sample chamber which was saturated with D$_2$O. In order to demonstrate reproducibility the spectra were taken many times.

**CD Spectra—**CD spectra were taken in the Cary model 61 spectropolarimeter using 0.1-cm-path length cells for the spectral interval from 190 to 250 nm and 0.5-cm cells from 250 to 310 nm. The slit widths of the instrument were programmed so that a constant 20 Å bandwidth was maintained. The instrument was calibrated with d$_{10}$-camphorsulfonic acid at 290 nm. The data were expressed in terms of mean residue ellipticity (θ), where θ = (θ°C) (Ml cl, where θ°C is the observed ellipticity in degrees at wavelength λ, M is the mean residue weight (104.7 g mol$^{-1}$); l is the path length of the cell in centimeters; and c is the protein concentration in grams per cm$^3$.

**Fluorescence Studies—**All studies were done in 0.1 M sodium phosphate buffer, pH 7.5 and 23°C. The sample was observed at right angles to the exciting beam in a custom-made spectrofluorometer. Polarization spectra were obtained with natural exciting light. Data collection and output was via a PDP-8S computer interfaced to the instrument. Two readings were taken at each wavelength and averaged for the emission spectra. Three readings per wavelength were averaged for each polarized intensity.

**RESULTS AND DISCUSSION**

**Prediction of Conformation of AP-A by Chou-Fasman Calculations—**The amino acid sequences of AP-A and toxin II are shown in Fig. 1. Calculations suggested the presence of β-reverse turns at: residues 7 to 10 (Asp-Ser-Asp-Gly), $\beta_{1-3} = 1 \times 10^{-4}$; residues 20 to 23 (Gly-Thr-Leu-Trp), $\beta_{5-7} = 6.7 \times 10^{-4}$; residues 25 to 28 (Tyr-Pro-Ser-Gly), $\beta_{3-5} = 4 \times 10^{-4}$; residues 29 to 32 (Cys-Pro-Ser-Gly), $\beta_{6-7} = 2.6 \times 10^{-4}$; residues 34 to 37 (His-Asn-Cys-Lys), $\beta_{6-7} = 5.8 \times 10^{-4}$; and residues 40 to 43 (Gly-Pro-Thr-Ile), $\beta_{1-5} = 8.1 \times 10^{-5}$ for AP-A. β-Pleated sheet regions were predicted for residues 2 to 6 (Val-Ser-Cys-Leu-Cys) and residues 45 to 49 (Trp-Cys-Lys-Gln) where the calculated P$_\perp$ values (11) were 1.24 and 1.31, respectively, for AP-A. No α-helical regions were predicted from the calculations. Since Toxin II has a very similar sequence to AP-A, it was of interest to calculate its conformation. β-reverse turns were predicted at residues 7 to 10 (Asp-Ser-Asp-Gly), residues 14 to 17 (Arg-Gly-Asn-Thr), residues 27 to 30 (Cys-Pro-Ser-Gly), residues 32 to 35 (His-Asn-Cys-Lys), and residues 38 to 41 (Gly-Pro-Thr-Ile). β-Pleated sheet regions were predicted in regions occupied by residues 21 to 25 and 42 to 46. No α-helical regions were predicted.

Despite the acknowledged approximation of the calculations due to inherent limitations of the method (11) as well as the steric effects imposed by the presence of three disulfide bonds in a relatively small polypeptide, it is very likely that AP-A consists of β-reverse turns and β-pleated sheet structures although the exact locations and the quantitative aspects of the predicted secondary structure may be in error.

**Laser Raman Spectroscopy—**In studies of peptide backbone conformation, the frequencies of amide I and amide II bands are mainly used. The amide I band arises from the coupled C=O stretching vibrations of peptide linkages. Normally an helix shows the amide I frequencies of 1650 to 1657 cm$^{-1}$, β-sheet structure at 1668 to 1680 and random coil at 1664 to 1668 cm$^{-1}$ (14). Similar assignments were also made by Spiero and Gabor (15). The β-reverse turn structure shown by oxytocin and glycyloxytocin gives the amide I band in the range of 1663 to 1670 (16, 17). In the present study of anthopleurin A a very high amide I band of 1677 cm$^{-1}$ was observed (Fig. 2); this definitely excludes the possibility of α helical and probably also the random coil structures.

The amide III band arises from in-plane vibration of the peptide bond, the main contribution being from the in-plane bending vibration of an amide-NH. Usually β-sheet structures are detected spectrally by a low amide III band at 1235 to 1240 cm$^{-1}$; random coil conformations by the amide III band at 1241 to 1251 cm$^{-1}$ and the α helix by the band at 1261 to 1296 cm$^{-1}$ (14, 15). The β-reverse turn structures show amide III band at 1260 to 1272 cm$^{-1}$ (16, 17). In the case of AP-A, the major band was observed at 1254 cm$^{-1}$ (Fig. 2). When the AP-A was deuterated, the original major peak at 1254 cm$^{-1}$ disappeared (Figs. 3 and 4) suggesting that the 1254 cm$^{-1}$ band is indeed the amide III band. The frequency of 1254 cm$^{-1}$ cannot be explained in terms of pure β-sheet, pure random coil, or pure α helix structures. Since the frequency of

FIG. 1. The amino acid sequences of AP-A (8), toxin I, and toxin II (9). In the figure, ATX-I and ATX-II stand for toxins I and II. Identical sequences are blocked off.
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The gauche-gauche conformation gives $\nu_{s-s}$ (stretching vibration) at 510 cm$^{-1}$, trans-gauche-gauche at 525 cm$^{-1}$, and trans-gauche-trans at 540 cm$^{-1}$. Usually proteins give $\nu_{s-s}$ at 510 cm$^{-1}$. Synthetic peptides such as oxytocin antagonists penicillamine-oxytocin, penicillamine, and leucyl-oxytocin show trans-gauche-gauche and trans-gauche-trans conformations (16, 17).

There is 1 mol of tyrosine in AP-A. From the study of model compounds such as glycyltyrosine, lysozyme, and sea snake neurotoxins (23, 24), it is known that the relative intensities of the Raman lines at 850 and 830 cm$^{-1}$ are related to the environment of the tyrosine side chain. The origin of the doublet is attributed to Fermi resonance between the ring-breathing and out-of-plane vibrations of the $p$-substituted benzene (25). Judging from the intensity ratio of tyrosine bands at 853 and 834 cm$^{-1}$ by the method of Yu and Jo (23), the single tyrosine residue is exposed. There are 3 mol tryptophan/mol of anthopleurin A. The 1361 cm$^{-1}$ band of tryptophan in Raman spectra is a very sensitive indicator of whether the tryptophan is buried or exposed (24). When the indole ring is buried and involved in certain interactions within a protein molecule, the 1361 cm$^{-1}$ band shows a sharp peak. As the indole ring becomes accessible to water molecules, the 1361 cm$^{-1}$ line diminishes (27). Random coiled glucagon does not show a sharp line at 1361 cm$^{-1}$ (28). Since AP-A contains 3 mol of tryptophan/mol of peptide, the interpretation of the Raman spectra becomes more difficult and gives only "averaged information." There is a distinct line at 1360 cm$^{-1}$ (Fig. 2), indicating that at least some of the tryptophan side chains are buried. The tryptophan residues are located at positions 23, 33, and 45 in the sequence of anthopleurin A (9). Since the tryptophan at position 23 is very close to the tyrosine at position 25, which was shown to be exposed, it is logical to conclude that Trp-23 is also exposed but the tryptophan side chains at 33 and 45 are buried or $\sigma$-bonded to the other amino acid residues.

Currently two methods are available in Raman spectroscopy to measure protein secondary structure quantitatively. One method is proposed by Pezolet et al. (29). This method gives only the content of $\beta$-sheet structure in the molecule. Also, analysis by this method requires a distinct amide III band at 1240 f 3 cm$^{-1}$. Since AP-A does not have an amide III band in this region, the method is not applicable.

One advantage of Raman spectroscopy over other techniques such as CD is that the former can analyze the conformation of a disulfide bond and some side chains. The disulfide bond stretching vibration of 514 cm$^{-1}$ indicates that the three disulfide bonds present in anthopleurin A have gauche-gauche-gauche conformation for each of the C$\equiv$C=S=S=C=C networks. It was shown previously by Sugeta et al. (21, 22) using model compounds, that gauche-gauche-gauche conformation gives $\nu_{s-s}$ (stretching vibration) at 510 cm$^{-1}$, trans-gauche-gauche at 525 cm$^{-1}$, and trans-gauche-trans at 540 cm$^{-1}$. Usually proteins give $\nu_{s-s}$ at 510 cm$^{-1}$. Synthetic peptides such as oxytocin antagonists penicillamine-oxytocin, penicillamine, and leucyl-oxytocin show trans-gauche-gauche and trans-gauche-trans conformations (16, 17).

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The amide I band of AP-A excludes the possibility of $\alpha$ helix and probably 100% random coil structures, the amide III band at 1254 cm$^{-1}$ can best be interpreted in terms of a mixture of $\beta$-sheet and $\beta$-reverse turn structure. This explanation is reasonable in light of spectral and structural studies of their proteins. For instance, snake neurotoxins known to have both $\beta$-sheet and $\beta$-reverse turn structure also give high amide III frequencies (18, 19). The conformation analysis by the Chou-Fasman method of (11, 20) shown in this paper also indicated the presence of both $\beta$-sheet and $\beta$-reverse turn structure.

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The second method was developed by Lippert et al. (30); the method yields the proportions of α helix, β-sheet, and random coil in the protein concentration. The equation employed does not include a term for the contribution of regions of β reverse turn structure. Therefore, there is serious question whether it is meaningful to use this equation for a protein-like anthopleurin A, which is predicted to have a high β reverse turn content. Nevertheless, it is of interest to test the validity of this method. The method is to solve the following four simultaneous equations:

\[ C_{\text{protein}}^{\text{protein}} = \alpha I_{1450} + \beta I_{1660} + \gamma I_{1650} \]
\[ C_{\text{protein}}^{\text{protein}} = \alpha I_{1632} + \beta I_{1660} + \gamma I_{1650} \]

In these equations $C_{\text{protein}}^{\text{protein}}$ is a scaling constant. $I_{1450}$ is the intensity of a Raman spectrum of the protonated form of a protein. $I_{1632}$ and $I_{1660}$ are the intensities of a Raman spectrum for the deuterated form of a protein. The $L^*$ and $I^*$ are Raman intensities previously determined for poly(l-lysine) in its various secondary structural forms.

By solving these equations we obtained the results of 21% α helix, 22% β-sheet, and 57% random coil. These results do not agree with the analysis obtained by the Chou-Fasman procedure. The main principle involved in the method of Lippert et al. (30) is that the α helix shows a prominent peak at 1632 cm⁻¹, the β sheet conformation at 1660 cm⁻¹, and the random coil at 1240 cm⁻¹. As can be seen from Fig. 3, AP-A shows a peak at 1254 cm⁻¹ in the difference spectrum. Even at 1240 cm⁻¹, there is substantial Raman intensity which is mainly contributed from the β reverse turn and β sheet structures. This is the probable reason the method yields such a high content of random coil structure (57%).

This result suggests that there is a need to revise the equations of Lippert et al. (30) to include a term for β reverse turns. The revised method would include five simultaneous equations. Thus the data for AP-A is only qualitative.

**CD Spectra of AP-A and Cys(Cm)-AP-A—Conformational data of AP-A can be obtained from circular dichroism measurements. Native AP-A spectra were recorded at pH 2.0 (partially unfolded); pH 7.0 (native); pH 9.5 (possibly partially unfolded, tyrosine phenolate ionization occurring); and in 90% ethanol (testing of the effect of the dielectric constant). The spectrum of the Cys(Cm)-AP-A was taken only in 0.1 M potassium phosphate buffer, pH 7.0, to check the effect of the cleavage of the disulfide bonds. The spectral data were separated into the far ultraviolet wavelength region (Fig. 5) and the near-ultraviolet (Fig. 6) wavelength regions. In the far ultraviolet wavelength region, an extremum was observed at 203 nm which arises from π-π* peptide bond transitions (31). The wavelength of this extremum did not change much with pH or when dissolved in 90% ethanol. However, at pH 2.0, the ellipticity became more negative. The CD spectrum of Cys(Cm)-AP-A in this wavelength region, at pH 7.0, showed a negative extremum at about 194 nm which is indicative of a random coil conformation. The occurrence of the 203 nm extremum can be attributed to the presence of β reverse turns in AP-A. Oxytocin which contains only one β reverse turn shows a similar extremum at 208 nm (17) and Bush et al. (32) have observed that certain peptides with a type II β reverse turn show extrema at 203. In the n-π* peptide bond transition region (31), there was an extremum at about 233 to 234 nm which occurs for peptide with a type II β reverse turn (32). The far ultraviolet CD spectral data qualitatively suggested the possible presence of type II β reverse turns and it is possible that some β pleated sheet structure is present, especially from the extremum observed at 194 nm (33). Also, the n-σ* transitions of the cystine residues appeared to occur in the 230 to 250 nm region.

The near-ultraviolet wavelength CD spectra of AP-A at various pH values and in 90% ethanol showed considerable fine structure. The extrema arise from the presence of 3 cystine residues, 3 tryptophan residues, 1 tyrosine residue. Included are the n-π* transitions of the cystine residues (34), the π-π* transitions of tyrosine residue and the 1$I_1$ and 1$I_2$
vibronic transitions of the tryptophan residues (34). Slight changes in the positions of the extrema and the actual values of the ellipticity changed as the pH was varied. Extrema were observed at 275, 284, 287, 290, and 298 nm under all conditions used in the present investigation. Extrema at about 277.5 and 280 nm were observed at pH values of 2.0 and 7.0. Other extrema were also observed and especially significant was the shift of the CD observed from about 250 to 263 nm at pH 9.5. AP-A loses all activity when incubated at pH 9.5 for 4 days (5) and both the spectral shift and loss in activity may arise from disulfide exchange reactions which are known to occur at alkaline pH values. Although the tyrosine residue can start to ionize at pH 9.5, no clear-cut spectral evidence could be obtained. The CD spectrum of the Cys(Cm) derivative when compared with the native AP-A at pH 7.0 showed large differences. In the region from 240 to 260 nm, the ellipticity values became increasingly negative and the fine structure observed from 263 to 320 nm were largely abolished. This indicated that the aromatic residues were exposed since the conformation of the Cys(Cm) derivative was in random chain conformation. Because of the presence of a multiple number of cysteine and tryptophan residues, it is difficult at the present time to assign the extrema to particular amino acid residues.

Fluorescence Studies—As shown in Fig. 7, the tryptophan fluorescence of AP-A was severely quenched, being only 6% of that of free tryptophan. The emission maximum is shifted approximately 7 nm to the blue (to 358 nm) from that for free tryptophan, indicating some degree of hydrophobic environment. Carboxymethylation shifted the emission maximum to 362 nm, close to that of free tryptophan. Carboxymethylation also liberated considerable fluorescence of Cys(Cm)-AP-A which was about 6.8 times more fluorescent than AP-A. If the Cys(Cm)-AP-A was placed in 5 mM Gdn-HCl, it is important to remember that there are 3 cystine residues.

The secondary structure is still well maintained under these conditions.

The polarization spectrum of L-tryptophan as shown in Fig. 8 was essentially flat and slightly negative, instead of zero, as it should be. This is an indication of a slight negative grating polarization. The data were not corrected for this.

The polarization spectrum of AP-A is essentially the same, whether it is in phosphate buffer, 5 mM Gdn-HCl, or 5 mM Gdn-HCl plus 10^-4 M dithioerythritol, indicating very little, if any, change of conformation under these conditions. The shape of the spectrum is similar to that of tryptophan in a rigid medium, although the absolute values are lower, as would be expected for a molecule of this size at room temperature.

Cys(Cm)-AP-A is seen to have a much lower and flatter polarization spectrum than AP-A (Fig. 9). The Cys(Cm)-AP-A spectrum is unaffected by 5 mM Gdn-HCl. This result indicated that carboxymethylation has a very profound effect on the secondary and tertiary structure of AP-A, whereas 5 mM Gdn-HCl has no effect. The Cys(Cm)-AP-A polarization spectrum indicated a peptide with considerable flexibility, but one which was not yet truly a random coil.

Very surprising was the effect of 10^-4 M dithioerythritol on the polarization spectrum of Cys(Cm)-AP-A in 5 mM Gdn-HCl. From Fig. 9, it can be seen that 10^-4 M dithioerythritol essentially restored the Cys(Cm)-AP-A back to its native conformation, even in the presence of 5 mM Gdn-HCl. Concomitant with this effect was a drastic quenching of the tryptophan fluorescence back to that of native AP-A in 5 mM Gdn-HCl. The presence of dithioerythritol apparently resulted in folding of the derivative back into a conformation similar to the one present in native AP-A. When considering the conformation of AP-A, it is important to remember that there are 3 cysteine residues.
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Fig. 9. Polarization spectra of AP-A and Cys(Cm)-AP-A. Symbols used are: O—O, AP-A; ×—×, Cys(Cm)AP-A; Δ—Δ, Cys(Cm)AP-A in 5 M Gdn. HCl; ○—○, Cys(Cm)AP-A in 5 mM Gdn. HCl + 10⁻⁴ M dithioerythritol. The emission wavelength used was 360 nm.

residues in a small peptide consisting of only 49 amino acids and the close sequence homology of toxin II and AP-A.

Concluding Remarks—An earlier report suggested that toxin II, a sea anemone peptide heart stimulant very similar to AP-A, exists in a random chain conformation (10). The recent ¹³C NMR spectral study of Norton and Norton as well as the results presented in this report lead to the general conclusion that AP-A does not exist in solution as a random coil. The NMR data, i.e. the rotational correlation time, suggested that AP-A has a roughly spherical shape. The mere presence of three disulfide bonds in native AP-A are sufficient to cause the molecule to be spherical. The conformational data presented here suggest that AP-A qualitatively folds up to cause the molecule to be spherical. The conformational presence of three disulfide bonds in native AP-A are sufficient to cause the molecule to be spherical. The conformational presence of three disulfide bonds in native AP-A are sufficient to cause the molecule to be spherical.

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Conformational studies of peptide heart stimulant anthopleurin A. Laser Raman, circular dichroism, fluorescence spectral studies, and Chou-Fasman calculations.

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