DETERMINATION OF β-TURN CONFORMATION BY LASER RAMAN SPECTROSCOPY

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ABSTRACT To correlate the Raman frequencies of the amide I and III bands to β-turn structure, three peptides shown to contain β-turn structure by x-ray diffraction and NMR were examined. The compounds examined were tertiary

butyloxycarbonyl—Cys—Pro—Val—Cys—$CONHCH_3$,
(S—S bridge between the two Cys residues)

benzyloxycarbonyl–Aib–Pro–Aib–Ala–$COOCH_3$,

and benzyloxycarbonyl–Aib–Pro–$CONHCH_3$.

The amide I band of these compounds is seen at 1,668, 1,665, and 1,677 cm^{-1}, and the amide III band appears at 1,267, 1,265, and 1,286 cm^{-1}, respectively. Thus, it is concluded that the amide I band for type III β-turn structure appears in the range between 1,665 and 1,677 cm^{-1} and the amide III band between 1,265 and 1,286 cm^{-1}.

INTRODUCTION

Laser Raman spectroscopy has been used successfully to study the conformation of polypeptides and proteins, especially for the elucidation of α-helix, β-sheet, and random coil (1–4). This is based on the frequencies of the amide I and III bands that arise from in-plane vibrations of the peptide bond. The amide I band is due to the coupled C═O stretching vibration, whereas the amide III is mainly due to the N—H in-plane bending vibrational mode. Two methods were proposed to quantify protein conformation. The method of Pezolet et al. (5) is used only for the determination of β-sheet content, whereas the equation developed by Lippert et al. (6) is used to calculate the content of α-helix, β-sheet, and random coil.

As Lord (7) pointed out, conformation of the protein backbone is related to the torsional angles ϕ and ψ about the C^α—N bond and the C′—C^α bond (Ramachandran angles), thus making it possible to establish experimental or theoretical means for correlation.

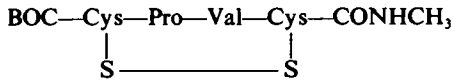
It has been recognized that β-turns, which permit polypeptide chain reversals, constitute important conformational components of protein structure (8). Consecutive type III β-turn structures lead to the formation of 3_{10} helical segments (9). Woody (10) carried out theoretical calculations on a wide variety of β-turn types and proposed the theoretical β-turn circular dichroism (CD) curve. This illustrates an effort to find a method to determine the β-turn structure from a CD study. Recently there has been a great effort to pursue this problem by the Raman spectroscopic method (11). The β-reverse turn structure found in

cyclic model compounds such as oxytocin and gly-oxytocin gives an amide I band in the range between 1,663 and 1,670 cm^{-1} and an amide III band in the range between 1,260 and 1,272 cm^{-1} (12, 13). The β-turn structure of oxytocin was established by NMR (14).

To identify and characterize further the β-turn conformation by the Raman spectroscopic method, three peptides shown to contain type III β-turn structure by NMR and x-ray diffraction studies were examined, and the results of the investigation are presented in this paper.

METHODS

Specific procedures for the synthesis of Z—Aib—Pro—Aib—Ala—$COOCH_3$ and Z—Aib—Pro—$CONHCH_3$ have been published (15, 16). Z refers to the benzyloxycarbonyl group, while Aib refers to α-aminoisobutyric acid.



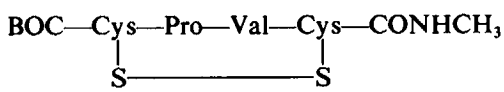
was synthesized by solution phase methods with dicyclohexyl-carbodiimide mediated couplings. BOC represents the tertiary butyloxycarbonyl group. A 2 + 2 coupling procedure was adopted. The benzyl group was used for thiol protection. Its removal was effected with Na/liquid ammonia. Oxidation to the disulfide was carried out in dilute aqueous $K_3Fe(CN)_6$. The disulfide was purified by silica-gel chromatography and characterized by its mass spectrum and by 270 MHz ^1H and 67.89 MHz ^{13}C NMR spectroscopy. Detailed synthetic procedures will be reported elsewhere (Venkatachalapathi and Balaram, manuscript in preparation). Deuterium oxide was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J. All compounds were chromatographically homogeneous on silica gel. For Raman spectroscopic analysis, powder samples were used.

Raman spectra of the peptides were obtained using a Spex Industries, Inc. (Metuchen, N.J.) Ramalog 5 spectrometer equipped with a computer (Spex SCAMP), and excitation at the 514.5-nm line of an argon ion laser (Spectrophysics, model SP-164) with a green filter. Capillary cells of 1.0 mm Diam were used to fill the samples. To perform deuterium exchange experiments, the peptides in D_2O were kept at room temperature for 24 h and then lyophilized. This process was repeated two to three times. The spectra of deuterated samples were obtained in sealed capillary cells saturated with D_2O vapor.

RESULTS

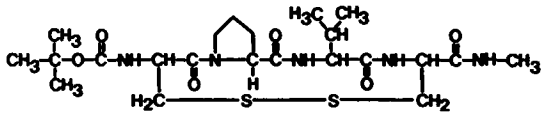
The chemical structures of three compounds investigated are shown in Fig. 1.

The amide I band of

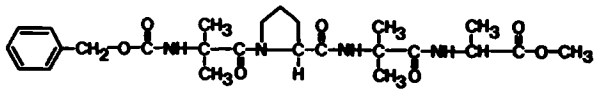


is clearly shown at 1,668 cm^{-1}, which shifts only slightly to 1,663 cm^{-1}, after deuteration (Fig. 2). The degree of deuteration was estimated quantitatively by measuring the intensity ratio of the 1,267-cm^{-1} and normalizing it against the 1,451-cm^{-1} line of the C—H bending vibration which is insensitve to structural change. By comparing the $I_{1,267}/I_{1,451}$ ratio before and after the deuteration, it is concluded that the 1,267-cm^{-1} band is indeed the amide III band while the 1,249-cm^{-1} band is not. Deuteration is an essential procedure to ascertain which is the amide III band, since this region contains highly mixed vibrational modes. The disulfide bond stretching vibration is clearly seen at 522 cm^{-1} (Fig. 2), which indicates that the conformation

1 BOC-CYS-PRO-VAL-CYS-CONHCH$_3$



2 Z-AIB-PRO-AIB-ALA-COOCH$_3$



3 Z-AIB-PRO-CONHCH$_3$

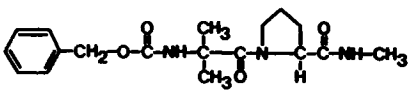
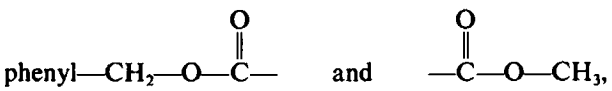


FIGURE 1 The chemical structure of the peptides containing β-turn structure.

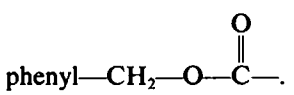
of C—C—S—S—C—C is of the *trans-gauche-gauche* type. The S—S stretching band is not entirely symmetrical and has a distinct shoulder in the 505–510-cm^{-1} region. This suggests that the compound may have another conformation of the *gauche-gauche-gauche* type.

As can be seen from Fig. 3, there are high frequency bands occurring at 1,752 and 1,706 cm^{-1} for Z—Aib—Pro—Aib—Ala—$COOCH_3$. These can be assigned to the ester C=O vibration of



since the ester C=O vibration is known to appear at a higher frequency than the peptide C=O vibration. The amide I band occurred at 1,665 cm^{-1} and remained at the same frequency after deuteration. Quantitative measurement of intensity ratios, $I_{1,265}/I_{1,455}$ and $I_{1,254}/I_{1,455}$, indicates a decrease in the intensity of these bands of 35.8 and 11.3%, respectively. This confirms that the 1,265-cm^{-1} band is the amide III band.

The amide I band of Z—Aib—Pro—$CONHCH_3$ is clearly shown at 1,677 cm^{-1} (Fig. 4). The small band at 1,693 cm^{-1} is probably due to the ester C=O vibration of



There are three bands in the region of 1,240–1,300 cm^{-1}. To ascertain which of these three bands is a real amide III band, the deuterium isotopic exchange in D_2O was performed. The intensity ratios of $I_{1,245}/I_{1,450}$, $I_{1,264}/I_{1,450}$, and $I_{1,286}/I_{1,450}$ were measured before and after the D_2O treatment. The 1,286-cm^{-1} band was reduced 29% in intensity by adding deuterium

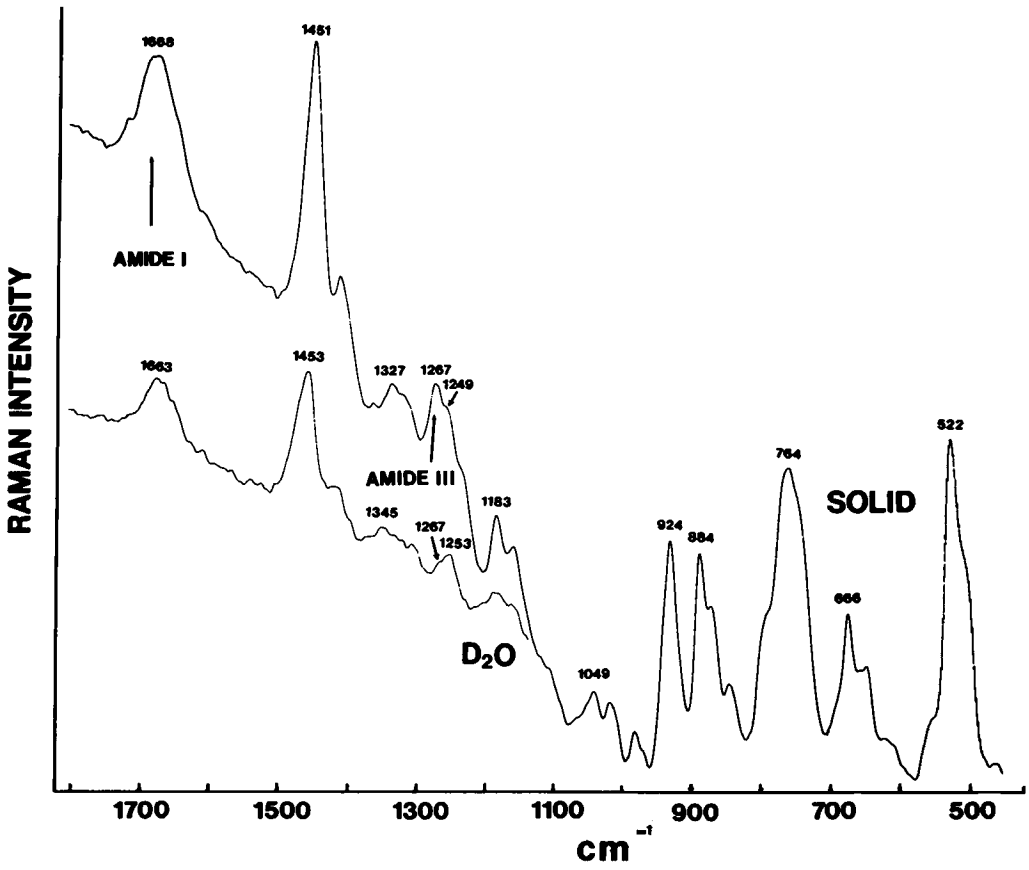
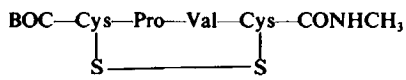


FIGURE 2 Raman spectra of



in solid (top) and D_2O (bottom). Experimental conditions: radiant power, 350 mW (solid), 400 mW (D_2O); integration time, 0.5 s; scale, 5,000 pulses/s (solid), 1,000 pulses/s (D_2O); slit width, 400 μm; scan increment, 0.5 cm^{-1}; number of scans, 5 (solid), 10 (D_2O).

oxide, whereas those of 1,245- and 1,264-cm^{-1} bands remained unchanged with respect to 1,449 cm^{-1} C—H bending vibrations, which indicates that the 1,286-cm^{-1} band is the amide III band. The same technique was used by Han et al. (17) for [Leu^5]-enkephalin. They found that, whereas the 1,268-cm^{-1} band normalized against the 1,445-cm^{-1} band did not decrease its intensity, the normalized 1,344-cm^{-1} band was reduced 25%. By this technique they concluded that the 1,344-cm^{-1} band was the amide III band.

DISCUSSION

Amide I and Amide III Raman bands are particularly useful in assigning peptide backbone conformations in proteins. The amide I band for the α-helix, β-sheet, and random coil conformations occurs in the regions between 1,646 and 1,658 cm^{-1}, 1,665 and 1,680 cm^{-1}, and 1,660 and 1,666 cm^{-1}, respectively (18). The amide I band for proteins known to have

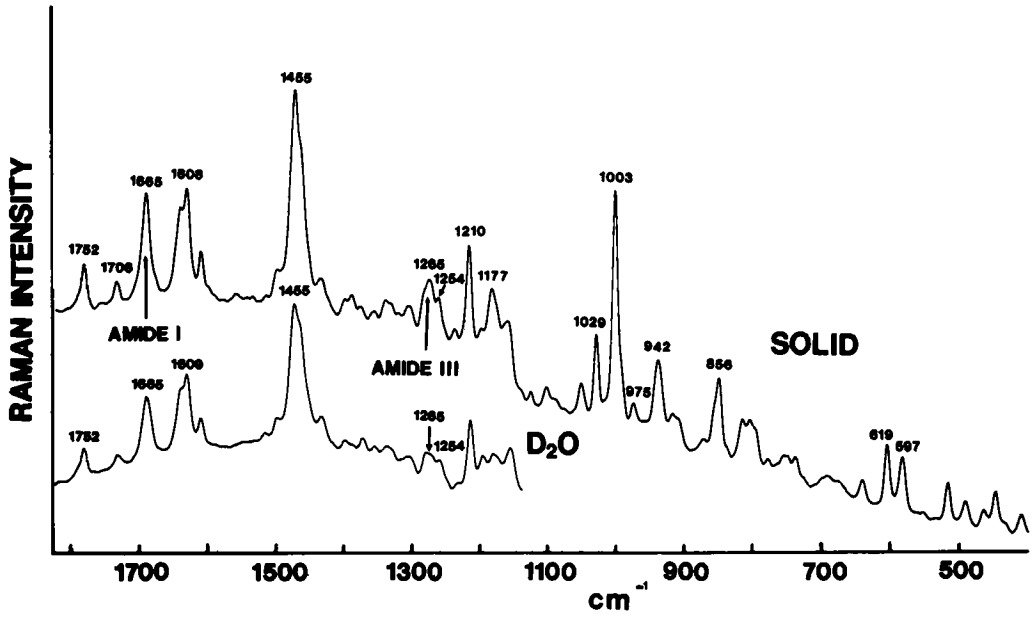


FIGURE 3 Raman spectra of Z—Aib—Pro—Aib—Ala—$COOCH_3$ in solid (top) and D_2O (bottom). Experimental conditions: radiant power, 100 mW (solid), 200 mW (D_2O); integration time, 0.5 s; scale, 2,000 pulses/s; slit width, 300 μm (solid), 350 μm (D_2O); scan increment, 0.5 cm^{-1}; number of scans, 5 (solid), 10 (D_2O).

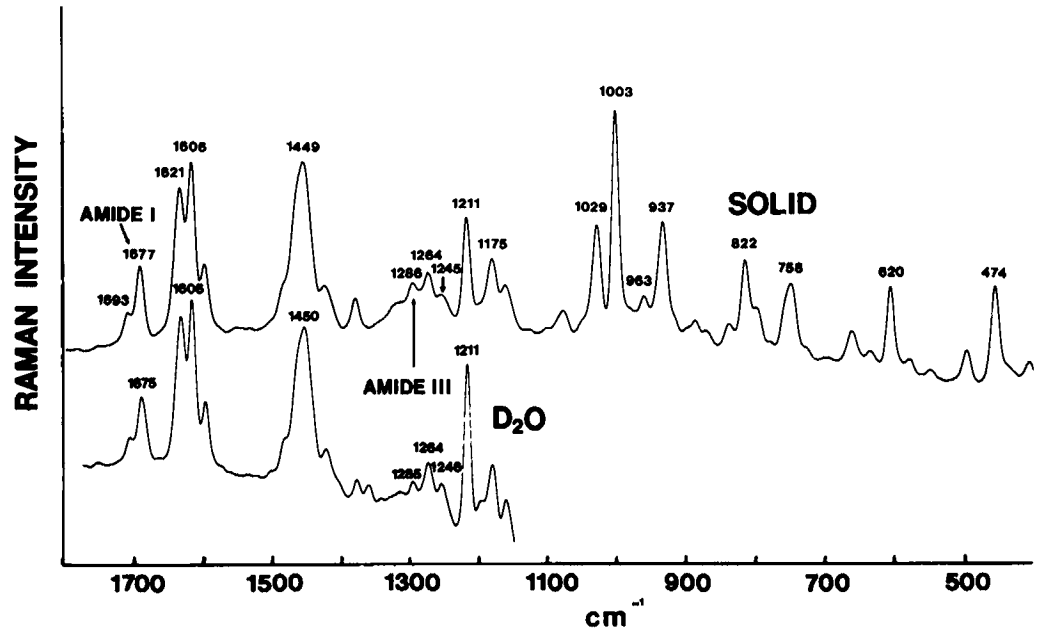
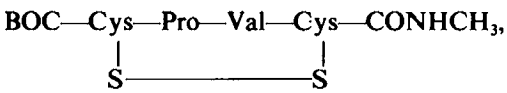


FIGURE 4 Raman spectra of Z—Aib—Pro—$CONHCH_3$ in solid (top) and D_2O (bottom). Experimental conditions: radiant power, 100 mW (solid), 200 mW (D_2O); integration time, 0.5 s; scale, 2,000 pulses/s (solid), 1,000 pulses/s (D_2O); slit width, 300 μm (solid), 400 μm (D_2O); scan increment, 0.5 cm^{-1}; number of scans, 5 (solid), 10 (D_2O).

β-turn structure was observed at 1,662–1,672 cm^{-1} (12, 13, 19). The frequencies of the amide III vibration of the α-helix, β-sheet, and random coil conformations are characterized by the Raman bands in the regions between 1,260 and 1,310 cm^{-1}, 1,235 and 1,242 cm^{-1}, and 1,240 and 1,250 cm^{-1} (18). To establish Raman characteristics of β-turn, three model peptides have been examined in this study. The choice of peptides was dictated by results of earlier studies, which strongly favor β-turn conformations in these compounds.

Stereochemical constraints can be introduced in acyclic peptide sequences of the presence of α-aminoisobutyric acid (Aib), resulting in a restriction of conformational freedom. Excellent correlations have therefore been obtained between NMR and IR studies in solution and x-ray diffraction studies in the solid state for Aib containing peptides (15, 20, 21). Aib peptides are, therefore, useful models in the development of spectroscopic methods of peptide conformational analysis. The major impetus for their study has been derived from the extensive occurrence of Aib residues in the membrane active polypeptide, alamethicin (22), and related microbial products (23). Z—Aib—Pro—$CONHCH_3$ and Z—Aib—Pro—Aib—Ala—$COOCH_3$, chosen as model compounds for establishing the Raman characteristics of β-turns, have been shown to possess Type III β-turn conformations in the solid state by x-ray diffraction (16, 24). These folded structures are maintained in solution and evidence for the presence of 4 → 1 intramolecular hydrogen bonds has been obtained from NMR (15) and infra-red studies (20, 21). In Z—Aib—Pro—$CONHCH_3$ a single Aib—Pro β-turn is present, whereas in Z—Aib—Pro—Aib—Ala—$COOCH_3$ a consecutive β-turn conformation is adopted with Aib(1)—Pro(2) and Pro(2)—Aib(3) as the corner residues. The Aib(3) and Ala(4) NH groups participate in intramolecular hydrogen bonding. An interesting feature of both peptides is the observation of the X_2—Pro_3 β-turn, a feature seldom observed in small peptides.

In the cyclic disulfide



the presence of the Pro—X sequence and the constraints of forming the 14-member disulfide ring favor the Pro(2)—Val(3) β-turn. From temperature dependence studies of NH chemical shifts in benzene and dimethylsulfoxide, the involvement of the Cys(4) NH in an intramolecular hydrogen bond has been established, providing support for the β-turn structure in apolar solvents. Detailed ^1H NMR studies also suggest involvement of Val(3) NH in a hydrogen bond. A conformation compatible with NMR and CD data would involve a consecutive β-turn conformation, with Cys (1)—Pro(2) and Pro(2)—Val(3) as the corner residues (Venkatachalapathi and Balaram, manuscript in preparation).

The Raman frequencies of the amide I and III bands observed in these peptides are shown in Table I. The amide I and III bands are found between 1,665 and 1,677 cm^{-1} and 1,265 and 1,286 cm^{-1}, respectively. It has been shown that Z—Aib—Pro—$CONHCH_3$ contains one intramolecular hydrogen bond (16), whereas Z—Aib—Pro—Aib—Ala—$COOCH_3$ (15) and

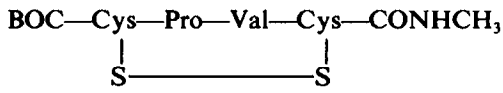


TABLE I
CHARACTERISTIC RAMAN LINES OF PEPTIDES KNOWN TO CONTAIN β-TURN STRUCTURE

Compound	Type of β-turn	Amide I	Amide III	Reference
		(cm^{-1})		
BOC—Cys—Pro—Val—Cys—CONHCH$_3$ (S—S)	II	1,668	1,267	This paper
Z—Aib—Pro—Aib—Ala—COOCH$_3$	III	1,665	1,265	This paper
Z—Aib—Pro—CONHCH$_3$	III	1,677	1,286	This paper
Oxytocin	*	1,666	1,260	12
Gly-oxytocin	*	1,670	1,270	13
Pro-Leu-GlyNH$_2$	II	1,650	1,234	25
		1,687	1,268	
[Leu^5]-enkephalin	I′	1,674	1,255	17
			1,271	
			1,282	
			1,325	

*Exact type of β-turn of these compounds is unknown, but the related compound [Pro^3, Gly^4] oxytocin was established by NMR to be type II β-turn structure (29).

(Venkatachalapathi and Balaram, manuscript in preparation) have two hydrogen bonds. It is known that a decrease in the amide I frequency occurs when a peptide C=O is involved in hydrogen bonding. The amide I band for the compound containing one hydrogen bond appears at 1,677 cm^{-1}, whereas the other two compounds having two hydrogen bonds show lower frequencies of 1,665 and 1,668 cm^{-1}, respectively.

The amide I and III frequency band positions of oxytocin, gly-oxytocin, and Pro—Leu—GlyNH$_2$ are also listed for comparison. Oxytocin and gly-oxytocin, which are known to possess β-turn structure (14), give an amide I band in the range between 1,663 and 1,670 cm^{-1} and an amide III band in the range between 1,260 and 1,272 cm^{-1} (12, 13). The Raman spectra of vasopressin (lysyl and arginyl), mesotocin, and vasotocin (19) neurohypophyseal hormones having structural features similar to oxytocin, showed that the amide I and III bands occurred in the ranges observed for oxytocin and gly-oxytocin. This suggests that these cyclic peptides also possess β-turn structure. Recently Hseu and Chang (25) studied the conformation of Pro—Leu—GlyNH$_2$ by Raman spectroscopy, which is known to contain a type II β-turn structure. The amide I bands were observed at 1,650 and 1,687 cm^{-1}, and the amide III band occurred at 1,234 and 1,268 cm^{-1}. The amide I band for [Leu^5]-enkephalin which is known to have Type I′ β-bend is shown at 1,674 cm^{-1} and the amide III bands at 1,255, 1,271, 1,282, and 1,325 cm^{-1} (17).

The range of amide I and III bands for the β-turn structure of three peptides reported in this paper is compared with the results of normal vibrational calculations (26, 27, 28). The theoretical calculation yields the amide I frequency ranges of 1,640–1,645 and 1,685–1,690 cm^{-1} for Type I β-turn, 1,660–1,665 and 1,685–1,690 cm^{-1} for Type II β-turn, and 1,643–1,649 and 1,686 cm^{-1} for Type III β-turn. The amide III frequency range of 1,290–1,330 cm^{-1} is calculated for Types I, II, and III β-turns. It can be seen that the amide

III band of the peptides presently investigated is comparable to, but not in the ranges predicted nor reaching a value as high as, 1,330 cm^{-1}. It is not unusual for observed spectroscopic data of biological compounds to deviate from the theoretical predictions from model compounds.

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REFERENCES

1. Fawcett, V., and D. A. Long. 1976. Biological applications of Raman spectroscopy. *In* Molecular Spectroscopy. R. F. Barrow, D. A. Long, and P. H. Millen, editors. Chemical Society, London. 4:125–195.
2. Spiro, T. G., and B. P. Gaber. 1977. Laser scattering as a probe of protein structure. *Annu. Rev. Biochem.* 46:553–572.
3. Yu, N.-T. 1977. Raman spectroscopy: a conformational probe in biochemistry. *Crit. Rev. Biochem.* 4:229–280.
4. Carey, P. R., and V. R. Salares. 1980. Raman and resonance Raman studies of biological systems. *In* Advances in Infrared and Raman Spectroscopy. R. J. H. Clark and R. E. Hester, editors. Heyden & Son Ltd., London. 7:1–58.
5. Pezolet, M., M. Pigeon-Gosselin, and L. Coulombe. 1976. Laser Raman investigation of the conformation of human immunoglobulin G. *Biochim. Biophys. Acta.* 453:502–512.
6. Lippert, J. L., D. Tyminski, and P. J. Desmeules. 1976. Determination of the secondary structure of proteins by laser Raman spectroscopy. *J. Am. Chem. Soc.* 98:7075–7080.
7. Lord, R. C. 1977. Strategy and tactics in the Raman spectroscopy of biomolecules. *Appl. Spectrosc.* 31:187–194.
8. Chou, P. Y., and G. D. Fasman. 1977. β-Turns in proteins. *J. Mol. Biol.* 115:135–175.
9. Venkatachalapathi, Y. V., and P. Balaram. 1979. An incipient 3_{10} helix in Piv—Pro—Pro—Ala—NHMe as a model for peptide folding. *Nature (Lond.).* 281:83–84.
10. Woody, R. W. 1974. Studies of theoretical circular dichroism of polypeptides: contribution of β-turns. *In* Peptides, Polypeptides, and Proteins. E. R. Blout, F. A. Bovey, M. Goodman, and N. Lotan, editors. John Wiley & Sons, New York. 338–350.
11. Williams, R. W., A. K. Dunker, and W. L. Peticolas. 1980. A new method for determining protein secondary structure by laser Raman spectroscopy applied to fd phage. *Biophys. J.* 32:232–234.
12. Tu, A. T., J. B. Bjarnason, and V. J. Hruby. 1978. Conformation of oxytocin studied by laser Raman spectroscopy. *Biochim. Biophys. Acta.* 533:530–533.
13. Hruby, V. J., K. K. Deb, J. W. Fox, J. B. Bjarnason, and A. T. Tu. 1978. Conformational studies of peptide hormone using Raman and circular dichroism spectroscopy. *J. Biol. Chem.* 253:6060–6067.
14. Nikiforovich, G. V., V. I. Leonova, S. G. Galaktionov, and G. I. Chipens. 1979. Theoretical conformational analysis of oxytocin molecule. *Int. J. Pept. Protein Res.* 13:363–373.
15. Nagaraj, R., N. Shamala, and P. Balaram. 1979. Stereochemically constrained linear peptides. Conformations of peptides containing α-aminoisobutyric acid. *J. Am. Chem. Soc.* 101:16–20.
16. Prasad, B. V. V., N. Shamala, R. Nagaraj, R. Chandrasekaran, and P. Balaram. 1979. Crystal and molecular structure of benzyloxycarbonyl—α—aminoisobutyl—L—prolylmethylamide: the observation of an X_2—Pro_3 type III β-turn. *Biopolymers.* 18:1635–1646.
17. Han, S.-L., E. R. Stimson, F. R. Maxfield, and H. A. Scheraga. 1980. Conformational study of [Leu^5]-enkephalin by laser Raman spectroscopy. *Int. J. Pept. Protein Res.* 16:173–182.
18. Frushour, B. G., and J. L. Koenig. 1975. Raman spectroscopy of proteins. *In* Advances in Infrared and Raman Spectroscopy. R. J. H. Clark and R. E. Hester, editors. W. and J. Mackay, Lordwood, England. 1:35–97.
19. Tu, A. T., J. Lee, K. K. Deb, and V. J. Hruby. 1979. Laser Raman spectroscopy and circular dichroism studies of the peptide hormones mesotocin, vasotocin, lysine vasopressin, and arginine vasopressin. *J. Biol. Chem.* 254:3272–3278.
20. Rao, Ch. P., R. Nagaraj, C. N. R. Rao, and P. Balaram. 1979. Infrared spectroscopy as a probe for the development of secondary structure in the amino-terminal segment of alamethicin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 100:244–248.

21. Rao, Ch. P., R. Nagaraj, C. N. R. Rao, and P. Balaram. 1980. Infrared studies on the conformation of synthetic alamethicin fragments and model peptides containing α-aminoisobutyric acid. *Biochemistry.* 19:425–431.
22. Mueller, P., and D. O. Rudin. 1968. Action potentials induced in biomolecular lipid membranes. *Nature (Lond.).* 217:713–719.
23. Pandey, R. C., C. J. Carter, Jr., and K. L. Rinehart, Jr. 1977. High resolution and field desorption mass spectrometry studies and revised structure of alamethicin I and II. *J. Am. Chem. Soc.* 99:8469–8483.
24. Shamala, N., R. Nagaraj, and P. Balaram. 1977. The crystal and molecular structure of the amino terminal tetrapeptide of alamethicin. A novel 3_{10} helical conformation. *Biochem. Biophys. Res. Commun.* 79:292–298.
25. Hseu, S., and H. Chang. 1980. Laser Raman studies on the conformation of Pro—Leu—Gly—NH_2. *Biochim. Biophys. Acta.* 624:340–345.
26. Bandekar, J., and S. Krimm. 1979. Vibrational analysis of peptides, polypeptides, and proteins: characteristic amide bands of β-turns. *Proc. Natl. Acad. Sci. U.S.A.* 76:774–777.
27. Bandekar, J., and S. Krimm. 1980. Vibrational analysis of peptides, polypeptides, and proteins. VI. Assignment of β-turn modes in insulin and other proteins. *Biopolymers.* 19:31–36.
28. Krimm, S., and J. Bandekar. 1980. Vibrational analysis of peptides, polypeptides, and proteins. V. Normal vibrations of β-turns. *Biopolymers.* 19:1–29.
29. Ballardin, A., A. J. Fischman, W. A. Gibbons, J. Roy, I. L. Schwartz, C. W. Smith, R. Walter, and H. R. Wyssbrod. 1978. Conformational studies on [Pro^3, Gly^4]-oxytocin in dimethyl sulfoxide by ^1H nuclear magnetic resonance spectroscopy: evidence for a type II β-turn in cyclic moiety. *Biochemistry.* 17:4443–4454.