Resonance Raman Studies of the Purple Membrane†

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ABSTRACT: The individual resonance Raman spectra of the PM568 and M412 forms of light-adapted purple membrane from *Halobacterium halobium* have been measured using the newly developed flow technique. For comparison purposes, the Raman spectra of the model chromophores, *all-trans*- and 13-*cis* retinal *n*-butylamine, both as protonated and unprotonated Schiff bases, have also been obtained. In agreement with previous work, the Raman data indicate that the retinal chromophore is linked to the purple membrane protein via a protonated Schiff base in the case of the PM568 and an unprotonated Schiff base for the M412 form. The basic mechanism for color regulation in both forms appears to be electron delocalization. The spectral features of the two forms are different from each other and different from the model compound spectra.

The purple membrane protein of *Halobacterium halobium* absorbs visible light and uses this energy to actively transport protons across the cell membrane (Oesterhelt and Stoeckenius, 1973). The protein is similar to the visual pigments in that it has the same chromophore, retinal (Oesterhelt and Stoeckenius, 1971), and undergoes similar light-induced spectral changes (Lozier et al., 1975). For this reason, the pigment has often been called bacteriorhodopsin although its physiological role, conversion of light energy to chemical energy, is quite different from that of the visual pigments. In its light-adapted form, the purple membrane protein initially has a broad absorption band centered around 568 nm. Upon absorption of light, this pigment, PM568, is converted to its primary photoproduct which absorbs at longer wavelengths than the pigment and is called the bathoproduct. The bathoproduct is stable at 77 K, but upon warming it goes through a series of thermal (dark) reactions to an intermediate absorbing at 412 nm, M412. This intermediate eventually cycles back to PM568 (Lozier et al., 1975). During this cycle, a proton is actively pumped across the cell membrane by an unknown mechanism (Oesterhelt and Stoeckenius, 1973).

We have studied the vibrational spectrum of the chromophores of the PM568 and M412 forms of light-adapted purple membrane using resonance Raman spectroscopy. The advantage of this technique is that the Raman spectra obtained are characteristic of the chromophore only and not the apoprotein or other colorless purple membrane components. Thus, specific information on the conformation of the in situ chromophore can be obtained (for recent reviews see Callender and Honig, 1977; Warshel, 1977). A major difficulty with this technique, when applied to photosensitive materials like the purple membrane, is that the quantum efficiency of photoconversion of the pigment system by the incident laser light used to stimulate the Raman scattering is many orders of magnitude greater than the quantum efficiency to give inelastically scattered Raman photons. Thus, the measuring light can seriously perturb the composition of the sample. In the experiments performed here, this problem has been overcome using a flow technique that has been recently developed (Callender et al., 1976; Mathies et al., 1976).

Several previous resonance Raman studies of the purple membrane have been performed by Lewis et al. (1974) and Mendelsohn and coworkers (Mendelsohn, 1973, 1976; Mendelsohn et al., 1974). The chief result of these studies has been the demonstration that the PM568 chromophore is linked to the protein by a protonated Schiff base whereas the M412 linkage is unprotonated. The present study augments and expands these studies in that Raman spectra over an expanded spectral range have been obtained, and these spectra are of essentially pure PM568 and M412 forms of the pigment. In addition, Raman data on model chromophores, namely *all-trans*- and 13-*cis*-retinal *n*-butylamine, both as protonated and unprotonated Schiff bases, are presented and compared to the purple membrane results (see also Mathies et al., 1977, for previous Raman measurements of retinal isomers as protonated Schiff bases).

Materials and Methods

Cultures of *H. halobium* were grown and the purple membrane purified according to the procedures of Becher and Cassim (1975). *all-trans*- and 13-*cis*-retinal *n*-butylamine were made by combining the corresponding retinal isomers (Sigma Chemical Co.) with an excess of *n*-butylamine in ethanol. This solution was mixed at 0 °C for 2 h under a stream of nitrogen and freeze-dried. The Schiff bases were protonated by adding to an ethanol solution of retinal *n*-butylamine an aliquot of ethanol saturated with hydrogen chloride. The absorption maximum then shifted to ca. 450 nm, indicating the formation of the protonated Schiff base.

The Raman detection and flow system used for these spectra has been described previously (Callender et al., 1976). The closed flow system described by Callender et al. (1976) for measuring Raman spectra of compounds that isomerize in light was slightly modified for the purple membrane spectral measurements. For measurement of PM568, purple membrane suspended in distilled water was maintained at approximately 5 °C by cooling the sample reservoir with a Lauda Super K-2/R circulator. The pigment in this reservoir was kept in the light-adapted state (PM568) by irradiating with light from a 60-W incandescent bulb. After leaving the reservoir, the sample was in the dark for about 6 s until it reached the exciting laser beam. The 568.2-nm laser line of a coherent Radiation Model 52 krypton ion laser was used at a power level

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of 1.5 mW. The bulk flow velocity was 600 cm/s. Under these conditions less than 5% of the sample was photoconverted during irradiation with the laser (see Callender et al., 1976).

To record the M412 spectrum, NaCl was added to the purple membrane to make a 4.3 M solution and the pH was raised to approximately 10, greatly increasing the lifetime of this intermediate (Becher and Ebrey, 1977). The tubing from the sample reservoir to the Raman measuring capillary tube was shortened, reducing the transfer time to approximately 3 s. The same lamp used for light adapting the purple membrane pigment was used to convert PM568 to M412, but now the sample reservoir was covered with yellow cellophane to remove most of the shorter wavelength light. The 454.5-nm laser line from a Spectra Physics Model 165 argon ion laser was used at a power level of 2.6 mW to measure the Raman spectra of M412. Under these conditions less than 5% of the sample was photoconverted from M412 during irradiation with the laser. After the M412 Raman spectrum was recorded, the membrane suspension was placed in the dark for 30 min and then the Raman spectrum recorded with the 568.2-nm laser line. This spectrum was indistinguishable from the spectrum of the PM568 without NaCl and high pH.

Under the above conditions, the sample is not completely converted to M412. In order to determine the sample composition, the absorption spectra of the high salt, high pH purple membrane suspension were measured under conditions identical with that of the Raman experiments except that a 10-mm flow spectrophotometer cuvette replaced the capillary tube of the Raman system. We estimate that a minimum of 80% of the pigment is in the M412 form. If opal glass is used to minimize the light scattering changes, a clear isosbestic point is obtained indicating that only PM568 andM412 are present. Since the 454.5-nm laser irradiating line is much closer to resonance with the M412 absorption band than the PM568 absorption band, it can be expected that the M412 spectral features will dominate the composite Raman spectra more than its minimum composition of 80% would indicate. Examination of spectral features of the data (see below) shows that the residual PM568 makes almost no contribution to this Raman spectrum.

Although the growth procedures for the bacteria are under conditions which minimize the amount of carotenoids present and nearly all of the carotenoids associated with the purple membrane are removed in the purifying procedures, it cannot be assumed that the sample contains no carotenoid. Because of its visible absorption any carotenoid present would give rise to spurious Raman scattering. The possible presence of carotenoid bands was assayed in the following manner. Raman spectra were taken of the sample washings, and the carotenoid present in these samples was found to be the (2,2/2,2) type as measured by Rimai et al. (1973) which has strong Raman bands at 1154 and 1510 cm^{-1}. These lines were not observed in the PM568 spectrum but were weakly present (less than 5% of the ethylenic intensity) in the M412 spectrum. To examine the carotenoids, stationary double beam measurements (see Oseroff and Callender, 1974) of purple membrane (as used in the PM568 experiment above) were performed with the probe laser beam at 454.5 nm and a pump laser beam at 568.2 nm. With no pump beam present the resulting spectrum was essentially that of PM568, and with the pump beam the spectrum of essentially M412 was obtained. However, the small Raman structure at 1154 and 1510 cm^{-1} was found not to change in these two runs. Thus, we conclude that these two lines found in the M412 spectrum are due to the carotenoid present in the sample; these two lines have been computer subtracted from the M412 spectrum presented below.

The flow Raman spectra of the retinal model compounds (dissolved in ethanol) were taken with lines from the krypton ion laser. The 476.2-nm line at a power level of 2 mW was used for both the 13-*cis*- and *all-trans*-retinal *n*-butylamine and 530.8- and 647.1-nm lines at powers of 3 mW were used for the protonated 13-*cis*- and *all-trans*-retinal *n*-butylamine, respectively.

The flow Raman spectra for 13-*cis*-retinal *n*-butylamine and 13-*cis*-retinal *n*-butylamine HCl were measured with 100 mL of a 2.6×10^{-3} M solution of each of the compounds. The starting solution for each of the all-trans compounds was 100 mL of a 3.5×10^{-3} M solution. High-pressure liquid chromatography assays of the retinals (Chan et al., 1974) showed all samples had an isomeric purity of better than 95%. The ethanol background spectrum has been computer subtracted from the model compound spectra.

Results and Discussion

Figures 1A and 2A show the resonance Raman spectra of the light adapted PM568 and the intermediate M412 chromophores of the purple membrane, respectively. From previous experimental work on model compounds and theoretical calculations (see reviews of Callender and Honig, 1977; Warshel, 1977), general mode assignments can be made. The lines at 1644 cm^{-1} in the PM568 spectrum and at 1623 cm^{-1} in the M412 spectrum correspond to protonated and unprotonated Schiff base (C=N) modes, respectively. The strongest lines in the spectra at 1532 cm^{-1} for PM568 and 1572 cm^{-1} for M412 correspond to ethylenic (C=C) stretching vibrations of the retinal polyene chain. The important information on conformation is contained in the region from 1100 to 1400 cm^{-1}. These vibrations have been primarily assigned to the C-C single bond stretches and the C-C-H bonds, and the bonds near 1440 cm^{-1} to C-H bending within the retinal methyl groups (Rimai et al., 1971; Warshel and Karplus, 1974).

For comparison purposes, flow resonance Raman spectra were taken of the protonated and unprotonated Schiff bases of *all-trans*- and 13-*cis*-retinal. Figures 1B and 1C show these model systems as protonated Schiff bases, and Figures 2B and 2C show them as unprotonated Schiff bases. By comparing the results of the purple membrane Raman spectra to that of these model systems, a great deal can be said concerning the important chromophore properties of this system.

Retinal–Protein Linkage. In agreement with previous resonance Raman experiments with the purple membrane (Lewis et al., 1974; Mendelsohn, 1976), the data in Figures 1A and 2A show that the chromophore of the PM568 form is linked to the protein through a protonated Schiff base while the M412 linkage is unprotonated. The inference is made from the respective positions of the Schiff base mode in the spectra of the two chromophores, namely at 1644 cm^{-1} for the PM568 form and at 1623 cm^{-1} for the M412 form, and by comparison to the line positions of the corresponding spectral region in the protonated (Figure 1) and unprotonated (Figure 2) model compound data. Previous Raman studies by Lewis et al. (1974) have shown the line position of the 1644-cm^{-1} band moves to approximately 1622 cm^{-1} upon deuteration. This change in frequency is expected from a simple reduced mass calculation where hydrogen of the HC=NH stretching mode is replaced by deuteron; Lewis et al. associated the 1644-cm^{-1} line with PM568 and the 1623-cm^{-1} line with M412 by varying the composition of the PM568/M412 in mixtures and by measurements of extinction profiles. In the present study, where

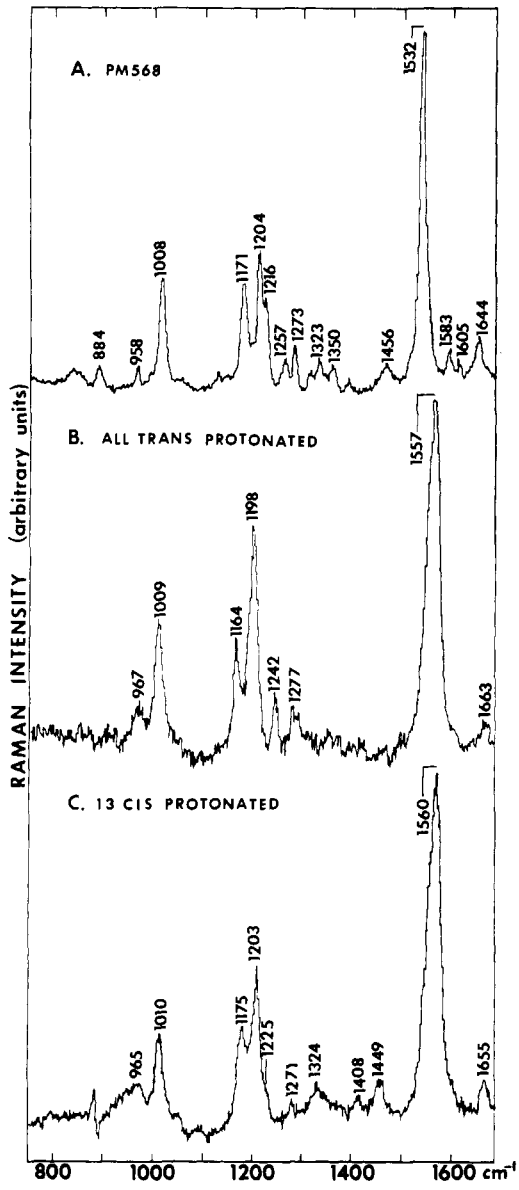

FIGURE 1: Resonance Raman spectra of (A) PM568 light-adapted purple membrane chromophore, (B) *all-trans*-retinal *n*-butylamine HCl, and (C) 13-*cis*-retinal *n*-butylamine HCl. The spectrometer resolution is (A) 7 cm^{-1}, (B) 4 cm^{-1}, and (C) 6 cm^{-1}.

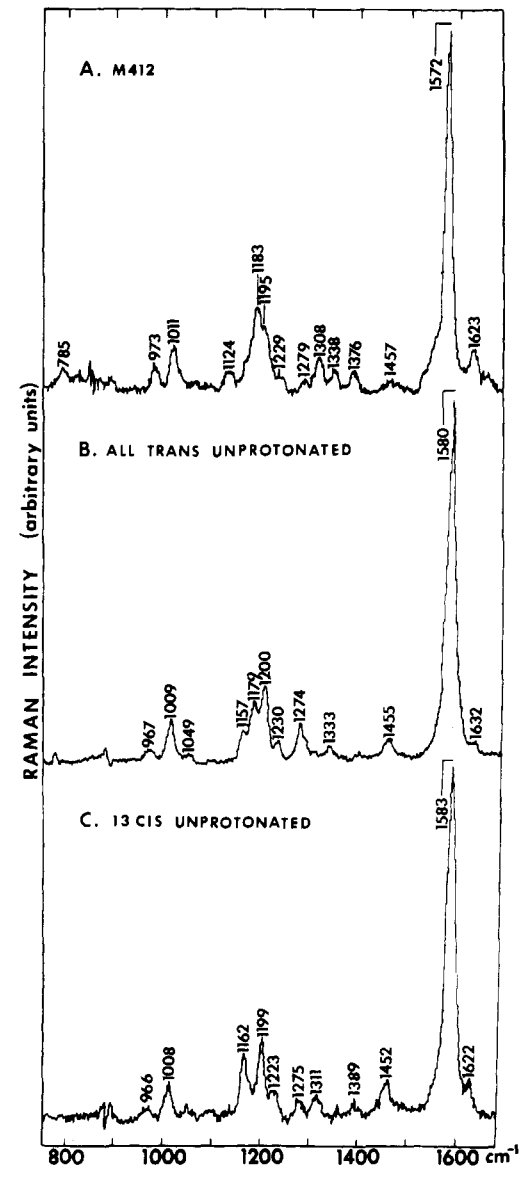

FIGURE 2: Resonance Raman spectra of (A) M412 light-adapted purple membrane chromophore, (B) *all-trans*-retinal *n*-butylamine, and (C) 13-*cis*-retinal *n*-butylamine. The spectrometer resolution is (A) 11 cm^{-1}, (B) 7 cm^{-1}, and (C) 7 cm^{-1}.

each species is separately measured, this assignment is confirmed.

Color of the Purple Membrane Protein. The mechanism for wavelength regulation of the absorption maxima in the visual pigments appears to be through control of the extent of electron delocalization in the protonated Schiff base chromophore (see discussion in Honig et al., 1976). As a consequence of this delocalization, the first excited state is lowered in energy more than the ground state resulting in a bathochromic shift in λ_{max}. Increasing electron delocalization results in a decrease of electron density in essential double bonds and an increase in essential single bonds. Thus, as first pointed out by Rimai and coworkers (1971), the line position of the ethylenic (C=C) stretching mode is a sensitive indication of electron delocalization since with increased delocalization the bond order is decreased resulting in a shift to lower frequency of the C=C band. Figure 3 shows that there is a good correlation between the λ_{max} values of both protonated and unprotonated Schiff bases, as well as rhodopsin and isorhodopsin, and their C=C stretching frequency (see also Rimai et al., 1971). An alternative mode of wavelength regulation in visual pigments, twisting about double bonds (Kakitani and Kakitani, 1975), also would cause red shifts by essentially raising the energy of the ground state, but appears to be excluded (see also Honig et al., 1976). In this case the double bond twist would cause a significant decrease in bond order and thus a very large lowering of the C=C frequency. Thus, in contradiction to Figure 3, none of the pigment C=C data would correlate with the model compound data, where double bond twisting is extremely unlikely.

The mechanism regulating the color of the protonated Schiff base chromophore in the purple membrane also appears to involve electron delocalization. The ethylenic frequencies in both the PM568 and the M412 forms are shifted to lower frequency with the red shift in their absorption maxima compared to their respective model chromophores of Figures 1 and 2. This is a very good correlation between the C=C vibration predicted from the delocalization model for a chromophore

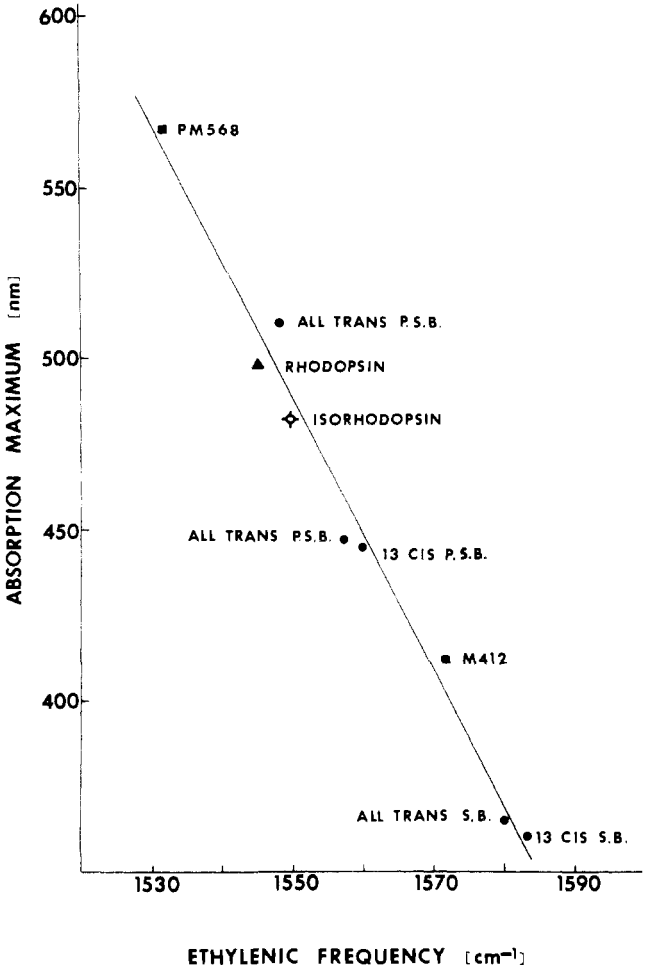

FIGURE 3: Correlation of ethylenic (C=C) stretching frequency of retinal-based structures with their absorption maxima. All data are from Figures 1 and 2 except the all-trans-protonated Schiff base absorbing at 512 nm (Heyde et al.,1971) and rhodopsin and isorhodopsin (Oseroff and Callender, 1974; Callender et al., 1976): p.s.b. = protonated Schiff base; s.b. = Schiff base.

absorbing at 568 nm (see Figure 3) and that actually observed. This strongly favors electron delocalization as the mechanism through which the protein controls the absorption maximum of the protonated Schiff base chromophore in the purple membrane. Other mechanisms, such as twisting about double bonds of the chromophore, can only play a secondary role in determining ethylenic band position.

The major source of the wavelength shift of the pigment from 568 to 412 nm is the deprotonation of the Schiff base which causes an electron localization in the essential C=C bands. (We (Becher and Ebrey, 1977) have not found a band at 520–550 nm as reported by others (Lozier et al., 1975) as being part of the 412-nm spectrum.) As can be seen in Figure 3, there is a good correlation between the λ_{max} of M412 and its ethylenic frequency as predicted on the assumption it is due just to electron delocalization. This does not imply that M412 is formed just by deprotonation of PM568; in fact other factors controlling electron delocalization, such as the positions of the amino acids around the chromophore, probably also have changed.

The Conformation of the Protein-Bound Chromophore. As mentioned above, the fingerprint region, 1100–1400 cm^{-1}, is sensitive to the conformation of retinal. In work on the visual pigments, it has been shown that the resonance Raman spectra of rhodopsin and isorhodopsin correspond closely to the Raman spectra of their respective model chromophores (Mathies et al., 1977).

The apparent conformational properties determined by Raman spectra of the chromophore in the purple membrane do not seem to be as clear. In chemical extraction experiments, PM568 has been found to have an all-trans conformation (Oesterhelt et al., 1973; Pettei et al., 1977). Comparing the PM568 Raman spectrum with the protonated retinal data of Figure 1, the PM568 data are not in exact correspondence with either the all-trans- or 13-cis-protonated retinal Schiff base spectra. The set of three lines in PM568 at 1171, 1204, and 1216 cm^{-1} corresponds rather closely to a similar set found in the 13-cis- but not in the all-trans-protonated Schiff base spectrum; in addition, the PM568 spectrum generally has more structure in the 1250–1400-cm^{-1} region than the protonated *all-trans*-retinal spectrum. On the other hand, many of these lines have no correspondence in the 13-cis spectrum either. It should also be pointed out that the PM568 spectrum is quite different from previous measurements of 11-cis- and 9-cis-protonated Schiff bases of retinal (Mathies et al., 1977). Thus, the conformational properties of the chromophore of PM568 do not bear an exact resemblance to those of the protonated 13-*cis*- or the *all-trans*-retinal. From these results, it appears that the conformation of the protein-bound chromophore of PM568 does not have the conformation of any of the isomers of retinal-protonated Schiff bases found in solution but rather has a conformation that relaxes to all-trans when the chromophore is extracted.

Similar observations apply to the M412 chromophore. The M412 spectrum does not correspond exactly to those of either the all-trans- or the 13-cis-unprotonated Schiff bases (Figure 2). Overall there seems to be a closer correspondence of the 13-cis-unprotonated Schiff base to M412. However, the line at 1183 cm^{-1} in the M412 spectrum is either not present or is shifted to 1162 cm^{-1} in the 13-cis model compound. Under some conditions, chemical extraction of the retinal of M412 yields approximately a 1:1 ratio of *all-trans*- and 13-*cis*-retinal; modification of the conditions for forming the unprotonated intermediate can give almost entirely the 13-cis isomer (Pettei et al., 1977). These observations together with the present Raman spectral results suggest that the conformation of M412 is probably 13-cis but, like that of the PM568 form, the structure in M412 is not identical with the structure found in solution.[1]

Acknowledgments

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[1] The M412 form might exist in two conformations, which lead to either *all-trans*- or 13-*cis*-retinal when the chromophore is extracted. We feel this is unlikely because of the sharpness of the ethylenic line of the M412 spectrum; however, this possibility cannot be ruled out.

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Conformational Prediction for Snake Venom Toxins and Laser Raman Scattering of a Cardiotoxin from Taiwan Cobra (*Naja naja atra*) Venom†

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ABSTRACT: Secondary structure regions in snake venom toxins were predicted using the prediction method of Chou and Fasman (Chou, P. Y., and Fasman, G. D. (1974), *Biochemistry 13,* 222) and an averaging scheme assuming structural homology in each type of toxins. The results indicate that, in general, snake toxins contain only some β-sheet regions and β bends. The content of secondary structures thus predicted does vary to some extent. The predicted results correlate well with conclusions from physicochemical studies. Interestingly, β-bend regions predicted for the two types of neurotoxins, short-neurotoxin-type and long-neurotoxin-type, are primarily located in the middle of disulfide loops in spite of large differences in primary sequences. Comparisons between predicted results and the crystal structure of erabutoxin b determined at 2.75 Å resolution suggest that the two types of neurotoxin are both sequencely and conformationally related while cardiotoxins could have an entirely different molecular topology. The Raman spectrum of a Taiwan cobra cardiotoxin indicates that the content of β-pleated-sheet structure could be greater than that in neurotoxins.

Toxins from venom of snake of *Proteroglyphae* suborder are small basic polypeptides devoid of enzymatic activity. One group of these toxins which block the nicotinic acetylcholine receptor of the muscle motor end plate is known as curarimimetic neurotoxins (Lee, 1972; Yang, 1974; Condrea, 1974). Another group, cardiotoxins or cytotoxins, has a relatively low toxicity to animials. This group does induce a variety of effects which are exerted primarily on cellular membrane leading to a disturbance of its organization and function (Condrea, 1974).

Much work has been done over the past decade toward clarification of the structure-functional relationship of these toxins. This work includes sequence determination, chemical modification studies, x-ray structure analysis, and other physicochemical investigations. To date there have been over 62 amino acid sequences determined (Figure 1). These toxins are among the smallest proteins known (60–74 amino acid residues). They are cross-linked by four or five disulfide bonds. Although they are homologous to one another in sequence, the pharmacological and serological properties differ greatly from neurotoxins to cardiotoxins. There are only 11 invariant amino acid residues found from sequence alignment including 8 half-cystines in invariant disulfide bridges. Therefore, these snake venom toxins provide particularly favorable conditions for comparative structural studies, e.g., through conformational prediction, laser Raman scattering, and CD and ORD[1] spectra measurements.

The main purpose of the present work was to predict secondary structure regions in snake venom toxin proteins and to

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[1] Abbreviations used: CD, circular dichroism; ORD, optical rotatory dispersion.