Solvent effect on the conformation and far UV CD spectra of gramicidin

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Solvent Effects on the Conformation and Far UV CD Spectra of Gramicidin

Abstract: *Solvent effects on the far-uv CD spectra of the polypeptide gramicidin have been studied systematically in a series of alcohols of increasing chain length, ranging from methanol to dodecanol. The effects observed are of two types: primary, involving a change in the equilibrium mixture of conformers present, and secondary, involving a shift in the spectral peak positions as a function of solvent polarizability.*

To quantitate the primary effect, the ratio of the individual conformers present was estimated by deconvolution of the spectra into their component species. For short chain length alcohols, both parallel and antiparallel double helices are found in considerable abundance. As the solvent chain length is increased and its polarity is decreased, the left-handed antiparallel double helical species is favored. For all alcohols with chain lengths of four or more carbon atoms, the ratio of the conformers present remains relatively constant.

To quantitatively examine the secondary effect, the magnitudes of the spectral shifts on the dominant conformer (species 3) have been correlated with the dielectric constants and refractive indices of the solvents, thereby indicating what underlying physical properties are responsible for these shifts.

This work thus demonstrates that for gramicidin, a flexible polypeptide, the solvent effects on the CD spectra can be resolved into two types: changes due to the mixture of conformers present and shifts in the spectral characteristics. Both effects need to be considered when interpreting CD spectra in terms of secondary structure for this and other polypeptides in nonaqueous solutions. © *1997 John Wiley & Sons, Inc. Biopoly* ***42:*** *771–781, 1997*

Keywords: *secondary structure; CD spectroscopy; ion channel; double-helical conformations; solvent polarity; conformational change; polypeptide structure; nonaqueous solvents*

INTRODUCTION

Gramicidin is a polypeptide antibiotic that forms ion channels in phospholipid membranes. It is a flexible molecule, and depending on its environment, can form either helical dimers or double helices. Veatch et al.[1] showed that in a number of organic solvents, gramicidin exists as a mixture of interconvertable conformers. The kinetics of interconversion depend on the solvent type and polypeptide concentration.[2,3] Individual conformers can be isolated chromatographically. Using ir, CD, and

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one-dimensional nmr spectroscopies, Veatch et al.[1] were able to propose that the structures of the four principal conformers were all double helices that differed in the relative directions of their polypeptide chains (parallel or antiparallel), the handedness of the helices, left or right, and the stagger between the chains. CD spectra of the individual isolated species were obtained in isopropanol and dioxane, solvents where the interconversion is slow. Most of the reported CD spectra of gramicidin in other solvents are the net spectra of the mixture of conformers present at equilibrium,[4,5] and although the spectra of the individual conformers do not appear to vary significantly with solvent,[1,6] the net spectra are very different in different solvents.[6,7] Two-dimensional nmr spectroscopic studies[8] of the mixture of conformers present in ethanol have distinguished the individual conformers and confirmed them as being the structures originally proposed by Veatch et al.[1] In addition, species 3 (a left-handed antiparallel double helix) could be isolated, and its high resolution structure has been determined by both nmr and x-ray crystallography.[9–12] Likewise, species 4 (a right-handed parallel double helix) has been isolated and its structure determined by nmr.[13] In this study, we use CD spectroscopy to identify and quantitate the individual species present under a given set of conditions in a series of alcohol solvents and to examine the solvent effects on the spectral characteristics of gramicidin in a systematic manner.

The influence of solvent on a CD spectrum can be generally classified into two types: primary effects and secondary effects. A ''primary effect'' is due to a change in the chromophores induced by the solvent and reflects a change in the structure of the molecule. This type of effect produces significant alterations in both the relative intensities and position of the peaks, because the entire electronic configuration is rearranged, but there is no direct correlation between solvent properties and the spectral characteristics. This type of effect should produce differences in the calculated secondary structures derived from the CD spectra. A ''secondary effect'' is caused by the influence of the electronic charge distribution of the solvent molecules on dissolved chromophores that have fixed configurations or conformations. This solvent–chromophore interaction causes a shift in the transition energy between the ground state and the excited state by altering its electron density distribution, and hence polarizability. The secondary effect produces minor variations in positions of the peak maxima, the magnitudes of the shifts being linearly related to the physical properties of the solvent. The change in an electronic transition can be due to interactions of either permanent or induced dipoles with the ground state or the excited state. We have shown that for another polypeptide (alamethicin) in a series of alcohol solvents, a red shift occurs in both the n-π^* and π-π^* peptide transitions as a function of decreasing dielectric constant and increasing refractive index.[14] Secondary effects should not, in principle, produce a difference in the calculated secondary structure if the conformation is unchanged, but can if the shifts in the peak positions are not taken into account in the analyses.

In this study we have investigated solvent effects on the spectra of gramicidin and have attempted to separate those due to primary and secondary effects. For this study, a series of alcohol solvents that have a range of refractive indices and dielectric constants and are chemically related, i.e., have the same chemically reactive group with different length aliphatic chains, has been utilized.

MATERIALS AND METHODS

Materials and Sample Preparation

Gramicidin D was purchased from ICN Biochemicals (USA). This is a natural mixture of gramicidin A (approx. 80%), gramicidin B (approx. 5%), and gramicidin C (approx. 15%).[15] Methanol and n-hexanol were purchased from Aldrich Chemical Co. Ltd. (UK). Ethanol, n-propanol, isopropanol, n-butanol, isobutanol, n-pentanol, n-octanol, n-decanol, and n-dodecanol were purchased from BDH Merck Ltd. (UK). The n-octanol, n-decanol, and n-dodecanol were commercial grade. All other solvents were spectrophotometric or analytic grade.

Gramicidin was dissolved in the series of alcohol solvents at concentrations of 5 mg/mL. All samples were centrifuged for 4 min at approximately $1300\times\ g$ prior to spectroscopic measurements to remove any insoluble material present.

Spectroscopic Measurements

All CD measurements were made in quartz Suprasil cells (Hellma Kuvetten, Mullheim/Baden, Germany). The path lengths of cells used ranged from 0.05 to 0.001 cm and were chosen to minimize solvent absorption while providing a sufficiently large signal. CD spectra were recorded on an AVIV 62DS spectropolarimeter. The spectropolarimeter was calibrated using benzene vapor for wavelength, and d-10-camphoursulphonic acid for optical rotation at 192.5 and 290 nm. Data in the wavelength range from 185 to 300 nm were collected at 0.2 and 0.05 nm intervals. For every set of conditions, at least three individual samples and five repeated measurements on each sample were obtained and averaged. Averaged base-

lines of the appropriate solvents were subtracted from the averaged sample spectra, and the resultant spectra were smoothed using a Savitsky-Golay filter.[16]

The uv absorption spectra were measured in a 0.05 cm cell over the wavelength range from 190 to 300 nm at 0.5 nm intervals using a Cary 13 (Varian) spectrophotometer. The spectrophotometer was calibrated for wavelength using benzene vapor. All spectroscopic measurements were carried out at a temperature of 25 ± 0.2°C. The positions of the peaks in both the CD and uv absorption spectra were determined using first- or second-derivative methods.

Spectral Analyses

Since the four types of gramicidin conformers present in organic solvents have distinct CD spectra,[1] any spectrum of a mixture of the conformers can be represented as a linear combination of three different CD spectra (conformers 1 and 2 have essentially identical spectra). By a modification to the data base used with the linear least-squares algorithm for the calculation of secondary structure,[17] the observed ellipticity (θ) of any gramicidin spectrum can be analyzed in terms of the individual spectra of the three distinct species, in a similar manner to that used to analyze for helix, sheet, and random components present in protein samples. In this case, the equation is written as follows:

$$\theta_{obs}(\lambda) = f_{1+2}\theta_{1+2}(\lambda) + f_3\theta_3(\lambda) + f_4\theta_4(\lambda)$$

where $\theta_{obs}(\lambda)$ is the observed ellipticity as a function of wavelength, $\theta_{1+2}(\lambda)$, $\theta_3(\lambda)$, and $\theta_4(\lambda)$ are the standard reference ellipticities for species 1 + 2, 3, and 4 at a given wavelength, and f_{1+2}, f_3, and f_4 are the fractions of species 1 + 2, 3, and 4, respectively. The series of simultaneous equations obtained for each wavelength was solved by minimization using a normalized constrained method based on a least-squares fit.[17,18] The CD standard reference data set was constructed using spectra of the individual species, which were separated by thin layer chromatography and measured in isopropanol.[1] A normalized root mean standard deviation (NRMSD) was calculated to indicate the quality of the fit of the calculated structures to the observed spectrum such that:

$$\text{NRMSD} = [(\theta_{obs}(\lambda) - \theta_{cal}(\lambda))^2/\theta_{obs}(\lambda)^2]^{1/2}$$

where $\theta_{obs}(\lambda)$ and $\theta_{cal}(\lambda)$ are the observed and calculated ellipticities, respectively. Higher values of NRMSD correspond to poorer fits, and would suggest that the reference data set is not representative of the conformers present. However, in these studies, NRMSD values of ≤ 0.08 were found for all solvents other than methanol, thus indicating the reference data set used contains the spectra of the types of conformers found in all of the solutions.

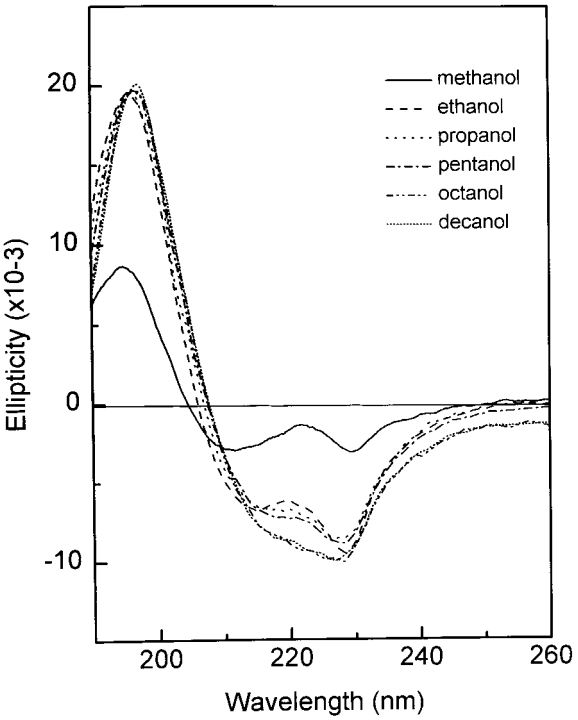

FIGURE 1 The far-uv CD spectra of gramicidin in methanol, ethanol, n-propanol, n-pentanol, n-octanol, and n-decanol.

RESULTS

Far-UV CD Spectra of Gramicidin

The far-uv CD spectra of gramicidin in various alcohol solvents are shown in Figure 1. It is apparent that the waveforms of the CD spectra of gramicidin in methanol and ethanol are significantly different from the waveforms in the other solvents: the two negative peaks are more pronounced and in different ratios in the methanol and ethanol solvents. For the other alcohol solvents, the CD spectra have similar shapes and magnitudes, although the positions of the peak maxima differ slightly. The different behaviors in different alcohols suggests that both primary and secondary solvent effects need to be considered.

In any organic solvent, the net gramicidin spectrum measured is the sum of a mixture of four double-helical species, with possibly a minor amount of monomers (found only in the most polar solvents such as methanol), present in solution weighted by their relative abundance. Species 1 and 2 are left-handed parallel double helices that differ only in the stagger between their two chains, species 3 is a left-handed antiparallel double helix and species 4

Table I The Estimated Contents of the Four Double-Helical Conformers in a Series of Alcohols

Solvent	Species 1 + 2 (%)	Species 3 (%)	Species 4 (%)	NRMSD
Ethanol	41	57	2	0.07
Propanol	29	64	7	0.08
Butanol	23	65	12	0.08
Pentanol	21	69	10	0.04
Hexanol	24	68	8	0.07
Octanol	22	62	16	0.08
Decanol	20	65	15	0.08
Dodecanol	22	68	10	0.08

is a right-handed parallel double helix.[8,19] Veatch and Blout[2,6] showed that the relative populations of the different species present under the same conditions were indistinguishable for gramicidin A and the gramicidin D mixture. The conformers can interconvert with each other on a time scale of seconds to hours depending on the solvent system used.[2,3] Gramicidin has a relatively low dimerization constant in methanol ($2 \times 10^{-3} M$),[1] which means a significant amount of monomers exists in methanol at the concentration used. The gramicidin dimerization constant rapidly increases with alcohol chain length,[1] so for all other solvents used in this study, the amount of monomers present will be insignificant. As a result, the data obtained from methanol is anomalous and will not be examined further in this study, because the reference data do not contain a representative monomer spectrum (as no accurate CD spectrum of a pure monomer exists), so this component can not be analyzed. For all other solvents, essentially only dimeric species should be present at the concentrations used.

The ratios of the four conformers present in each solvent were determined by linear deconvolution of the spectra. The individual spectra of the conformers in isopropanol were used as the reference data set. Veatch and Blout[2,6] showed that although the net spectra in two different solvents, dioxane and isopropanol, were very different, the spectra of the individual conformers in these solvents (and also of the isolated species 3 in ethanol) did not vary significantly with the solvent used. It is noteworthy in this regard that the fits (NRMSDs) obtained are good for all solvents, an indication that the reference data set used is appropriate in each case.

Table I summarizes the estimated proportions of the species present in each solvent. The variations between different preparations of the same sample suggest confidence levels on the order of ±2%. In all solvents, species 3 is the dominant contributor. It was found that species 1 and 2, left-handed parallel double helices, also make a significant contribution to the CD spectra of gramicidin in ethanol. The fraction of species 1 + 2 decreases in alcohols of longer chain length. There is a reasonably constant ratio of the various species present in all the alcohols in the series from butanol to decanol.

To examine the effect of solvent properties on the spectrum of a single component in the mixture, the CD spectra were further processed to eliminate the contributions from species 1 + 2 and 4 to produce Figure 2, the net spectrum for species 3 in each solvent. This was done as follows:

$$\theta_3(\lambda) = \frac{\theta_{\text{obs}}(\lambda) - f_{1+2}\theta_{1+2}(\lambda) + f_4\theta_4(\lambda)}{f_3}$$

From Figure 2, it can be seen that three characteristic peaks are found in all spectra. They are an intense positive peak at around 196 nm and two negative peaks located at about 215 and 228 nm respectively. The two low wavelength peaks are assumed to be the π-π^* perpendicular and π-π^* parallel transitions, respectively, while the peak near 228 nm is recognized as the n-π^* transition.[2] It can be seen that the shapes of the CD curves are generally unaltered, but the positions of the peaks are slightly shifted to longer wavelengths as the carbon chain length of the solvent increases (Table II).

UV Absorption Spectra of Gramicidin

The wavelength shift effect was further investigated using uv absorption spectroscopy. These spectra should primarily reflect the secondary solvent ef-

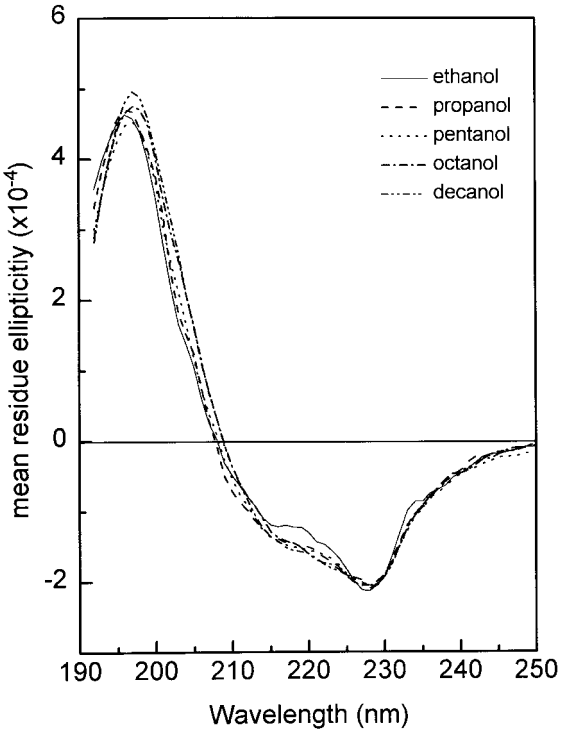

FIGURE 2 The corrected far-uv CD spectra for species 3 of gramicidin in ethanol, n-propanol, n-pentanol, n-octanol, and n-decanol after subtraction of the spectra of species 1 + 2 and 4.

fects since, unlike the CD spectra, they exhibit little dependence on conformation. The uv absorption spectra for gramicidin in the wavelength range between 190 and 300nm were measured independently in two different ways: in a standard uv absorption spectrophotometer and from the dynode voltage records of the CD experiments. Two intense positive bands near 190 and 223 nm and two weak peaks around 280 nm were observed in each case. The positions of the bands were determined from the first and second derivative spectra.

Table II The Wavelengths of the Three Transition Bands of the CD Spectra for Species 3 of Gramicidin

Solvent	$\pi - \pi^*$ ($\perp$) Transition	$\pi - \pi^*$ ($\parallel$) Transition	$n - \pi^*$ Transition
Ethanol	195.87	215.27	227.53
Propanol	196.26	215.34	227.60
Butanol	196.49	215.63	227.88
Pentanol	196.78	216.07	228.11
Hexanol	197.08	216.18	228.20
Octanol	197.17	216.23	228.33
Decanol	197.22	216.32	228.51
Dodecanol	197.34	216.50	228.63

Table III The Wavelength Maxima for Gramicidin Tryptophans from UV Absorption Spectra

Solvent	Wavelength (nm)
Ethanol	221.5, 281.0, 289.1
Propanol	221.8, 281.3, 289.6
Butanol	222.6, 282.0, 290.0
Pentanol	222.6, 282.5, 290.2
Hexanol	222.6, 282.0, 290.8
Octanol	222.7, 282.3, 290.8
Decanol	222.8, 282.6, 291.3
Dodecanol	223.0, 282.8, 291.4

In general, the uv absorption spectra for gramicidin show a red shift with increasing solvent chain length, as was observed for the CD spectra. The peaks near 190 and 223 nm are both shifted to shorter wavelengths with increasingly polar solvents. The intensity is reduced as the polarity of the solvent decreases. The two weak peaks at around 280 nm in the near uv are the characteristic feature of the spectral fine structure of the tryptophan chromophores (Table III). These peaks are assigned to π-π* transitions, and are shifted to slightly longer wavelengths compared with spectra obtained for tryptophans in water.[20] The contributions to the intense peak at around 223 nm may consist of two parts: the major contribution is from the aromatic π-π* transition of the tryptophan side chains; the other contributions are from the π-π* parallel and n-π* transitions of the peptide backbone. The π-π* parallel transition is an exciton splitting band, and the n-π* transition for the amide bond is an electronically forbidden, but magnetically allowed, band. Therefore, it is expected that these latter two contributions will be very weak compared to those of the four tryptophan side chain chromophores. From Beer's law, taking extinction coefficients of 33,884 for tryptophan (220 nm) and 389 for the amide bond (225 nm) in water, respectively,[21] it is found that tryptophans provide 99% of the contribution to this peak. Hence, this peak in the uv absorption spectra can be assigned as mainly due to the tryptophan residues. Such is not the case in the CD spectra, where this transition is a low dichroic one, and the observed 228nm peak is due almost entirely to the peptide transition. All three tryptophan peaks shift to longer wavelengths with increasing solvent chain length (Table III).

The Relationship Between Wavelength Shift and Solvent Properties

In order to determine if there is a general relationship between the solvent properties and the extent of the wavelength shift, the peak positions for the different CD peptide transition bands for the individual species 3 spectra were plotted versus refractive indices, dielectric constants and dipole moments (Figure 3).

It appears that there is a linear correlation between the wavelength shift, and both the refractive indices and dielectric constants in this series of alcoholic solvents. The correlation coefficients for the different plots vary from 0.96 to 0.98. It can be seen that all three characteristic transition bands shift to longer wavelengths with increasing refractive index and decreasing dielectric constant. In contrast to the refractive index and the dielectric constant, no significant relationship (correlation coefficients ranging from 0.38 to 0.50) is found between the wavelength shift and the dipole moments of the alcohol solvents.

Figure 4 depicts the plots of wavelength shift vs refractive index, dielectric constant, and dipole moment for the tryptophan chromophores in the near-uv absorption spectra. It can be seen that the wavelengths of the aromatic transition bands also increase with increasing solvent refractive index and with decreasing dielectric constant (correlation coefficient ≥ 0.95). There is, once again, no apparent relationship between dipole moment and wavelength (correlation coefficient < 0.40). The same phenomenon has been observed in studies of model compounds, N-acetyl ethyl esters of tryptophan,[22] where the wavelength shifts for those tryptophan derivatives were shown to move to longer wavelength with increasing solvent refractive indices.

DISCUSSION

The ratio of gramicidin species present varies with solvent in the short chain alcohols, with species 1 + 2 being relatively more abundant in ethanol, and to some extent in propanol. The amount of these species decreases with longer solvent chain length. However, the ratios of the species present remain essentially constant in alcohols with chain lengths longer than three carbons, the antiparallel species 3 apparently being stabilized under these conditions. One might speculate that this is because species 3, the only species with no net (helix) dipole, is more favored in the less polar solvents. The observed variation in equilibrium mixture composition is a primary effect of the solvent.

The mechanism of the secondary effect—that is, the wavelength shift and hence the change of electron density distribution in different energy states by the interaction between solvent and chromophore molecules—is determined both by the nature of the electronic transition and by the relative contributions of chromophore-solvent interactions to the ground and excited states. Since species 3 of gramicidin is a relatively regular β-helical structure, the secondary solvent effects on all of its amide groups should be more similar to each other than for globular proteins, which contain a mixture of secondary structural types, thus making it a simpler system for studying the secondary solvent effects.

For the far-uv CD spectra of flexible polypeptides, interpretation of the solvent effects is complex, since they arise from both primary and secondary effects. In the case of gramicidin, however, the distinction between primary and secondary effects can be made by deconvoluting the spectra into their component parts. Initially the spectra of the mixtures of conformers appeared to show red shifts for the π-π* transitions of the backbone and the tryptophan side chains, but blue shifts for the n-π* transitions. This was not consistent with our observations on alamethicin and other polypeptides in nonaqueous solvents.[14,23] However, this discrepancy is attributable to the primary effect, i.e., conformation changes, and the secondary solvent effect on the uv CD spectra of gramicidin was apparently masked by the primary effect. Consequently, the spectra of only a single conformation (species 3—the predominant one) were analyzed for solvent effect. When this was done, the tendency of the wavelength shift for the n-π* transition also showed a red shift with increasing refractive index and decreasing dielectric constant, and was consistent with previous observations.

At least the two parameters, refractive index and dielectric constant, are associated with the solvent shift and thus must be considered together. Although several more complicated empirical expressions have been proposed to account for these effects,[24–26] we have used a model based on an empirical quantum mechanism proposed by Bakhshiev[26] to further analyse this system. The relationship is as follows:

$$\frac{\Delta\nu}{\gamma} = C_1 + C_2 \frac{\sigma}{\gamma}$$

where $\Delta\nu$ is the shifted peak maximum relative to a selected standard solvent in frequency units, C_1

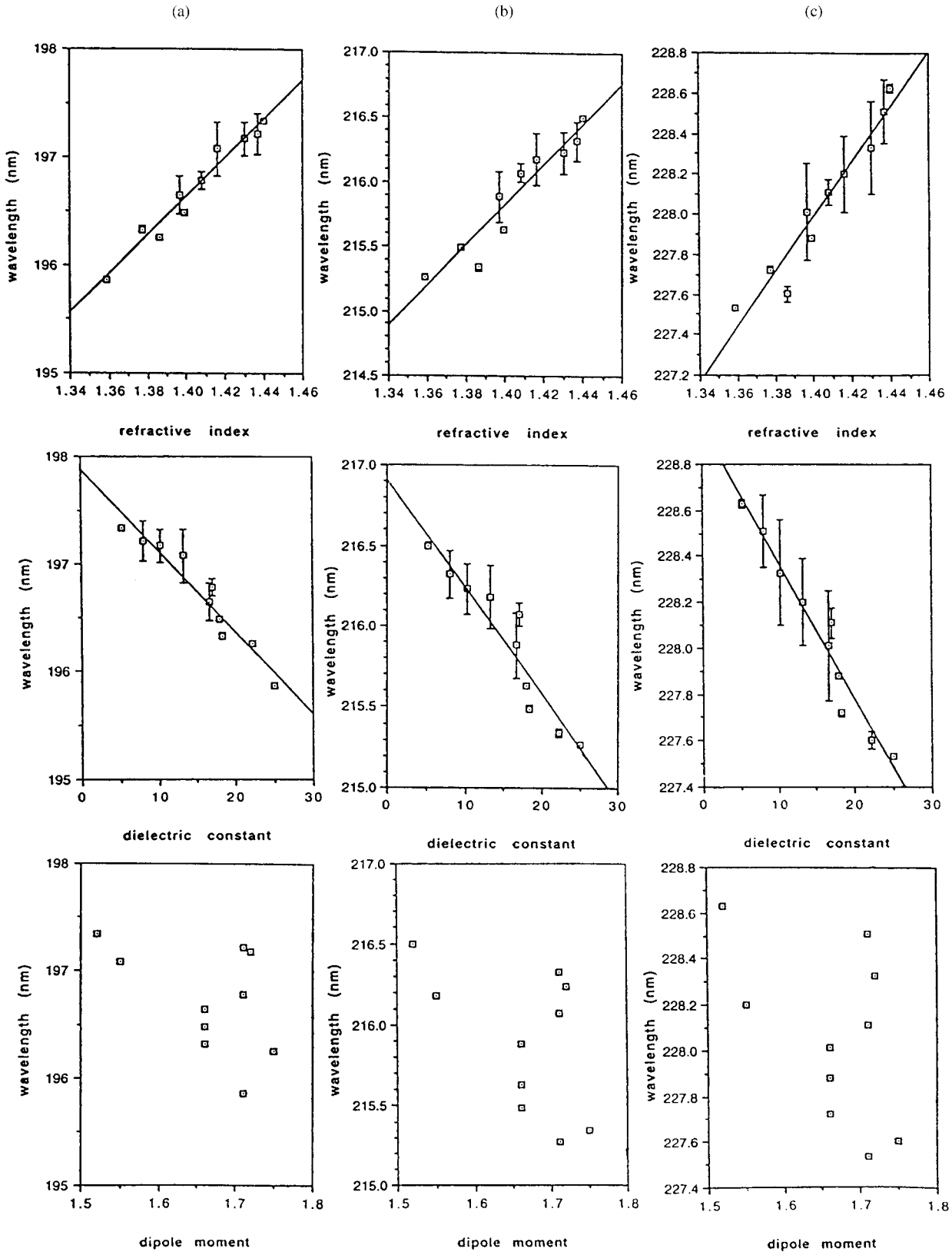

FIGURE 3 Plots of wavelength vs refractive index, dielectric constant, and dipole moment for the peptide transition bands of gramicidin species 3: (a) π-$\pi_{\perp}^{*}$, (b) π-$\pi_{\parallel}^{*}$ and (c) n-π^{*} transitions.

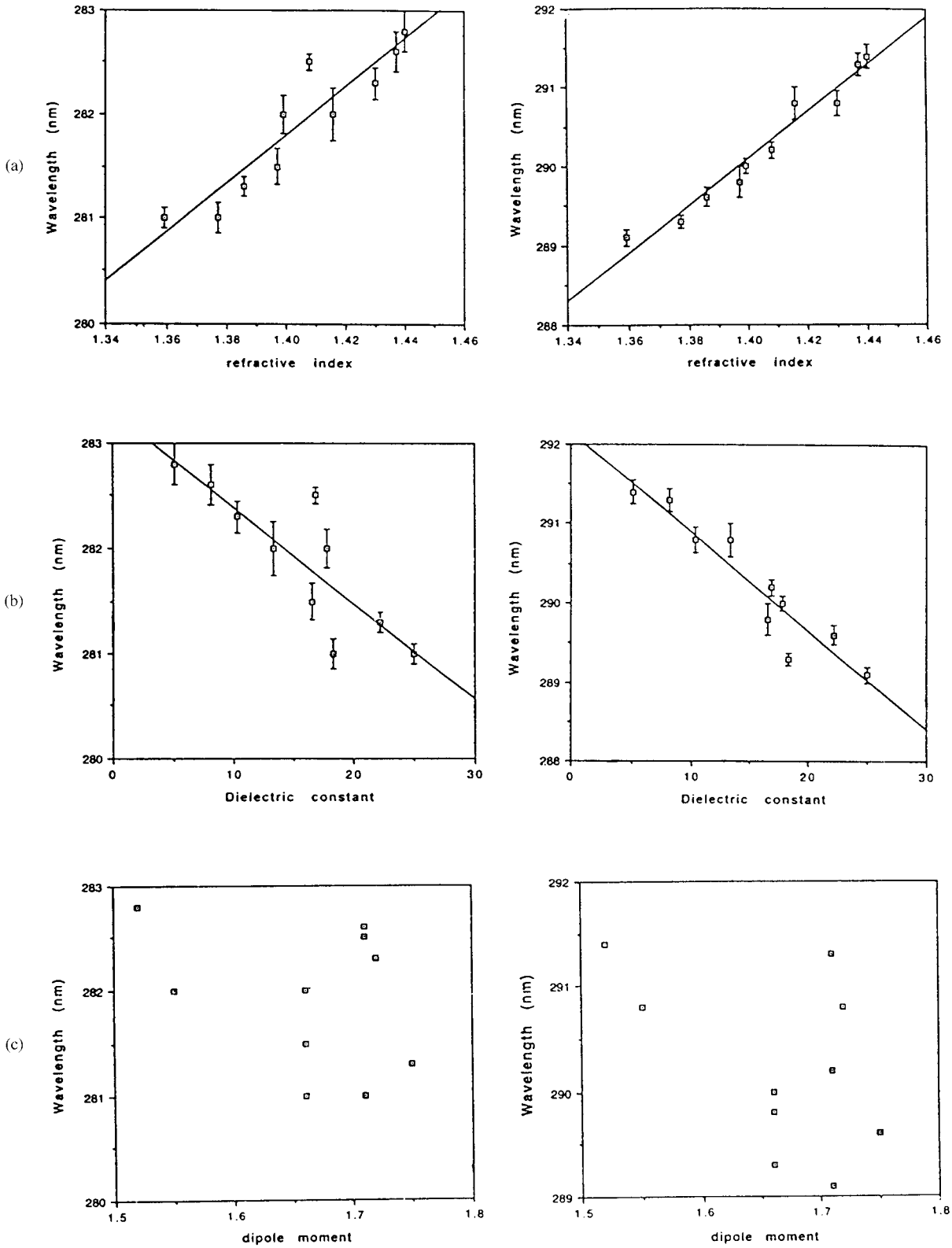

FIGURE 4 Plots of wavelength vs (a) refractive index, (b) dielectric constant, and (c) dipole moment for the near-uv transitions of tryptophan.

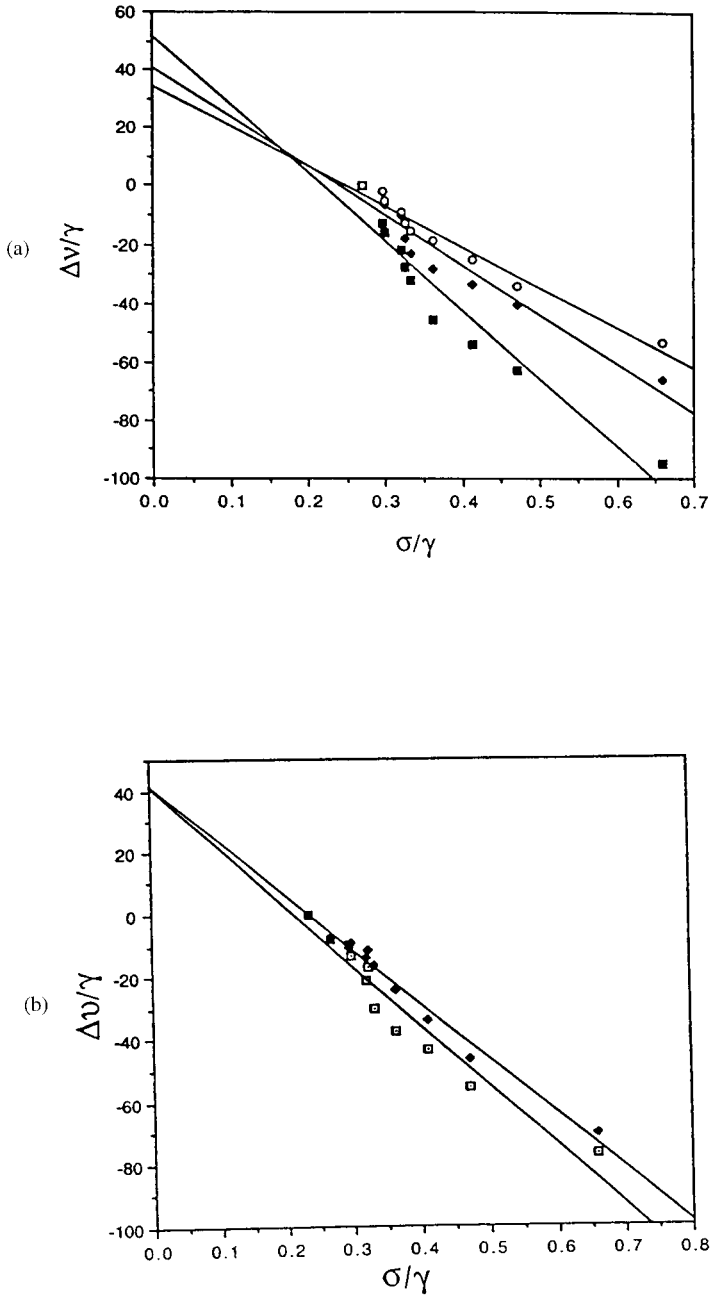

FIGURE 5 Modified Bakhshiev plots obtained from the CD data for (a) backbone transitions (■) π-$\pi^{\perp}$, (◆) π-$\pi^{\parallel}$, and (○) n-π^*; and (b) tryptophan transitions (⊡) (^1L_a) and (◆) (^1L_b).

and C_2 represent the orientation and polarization effects on the spectrum, and γ and σ are defined respectively as

$$\gamma = \left(\frac{2n^2 + 1}{n^2 + 2}\right)\left[\left(\frac{\varepsilon - 1}{\varepsilon + 2}\right) - \left(\frac{n^2 - 1}{n^2 + 1}\right)\right]$$

$$\sigma = \left(\frac{n^2 - 1}{n^2 + 2}\right)$$

and n and ε are the refractive indices and dielectric constants of the solvents, respectively. Figure 5a shows the plot of $\Delta\upsilon/\gamma$ vs σ/γ for the polypeptide

Table IV Parameters of the Bakhshiev Fit for the Gramicidin Backbone and Tryptophan Transitions

	$\pi - \pi^*_\perp$ Transition (^1L_a)[a]	$\pi - \pi^*_\parallel$ Transition (^1L_b)[a]	$n - \pi^*$ Transition
Gramicidin backbone			
C_1	34.45	40.69	51.45
C_2	−138.00	−169.00	−234.77
R	0.99	0.96	0.97
Gramicidin tryptophans			
C_1	40.61	41.45	
C_2	−191.42	−175.47	
R	0.97	0.99	

[a] The corresponding $\pi - \pi^*$ transition bands for tryptophan.

backbone and Figure 5b shows it for the tryptophan side chains. It can be seen that there is a linear relationship. The correlation coefficients (R) are all greater than 0.96. The parameters for the amide backbone π-$\pi^*_\perp$, π-$\pi^*_\parallel$, and n-π^* transitions are summarised in Table IV. The value of C_2 is larger than C_1, thus, the polarization effects appear to be the dominant factor in causing the red shift. This suggests interactions with the permanent dipoles rather than induced dipoles are the principle source of the secondary effects.

CONCLUSIONS

There are two separable solvent effects that can be detected for gramicidin in alcohols of increasing chain length. The primary effect is that the relative proportions of the different species present in the equilibrium mixture change with short chain length alcohols, initially having both the parallel and antiparallel double helices in roughly equal amounts, but shifting in favor of the antiparallel (species 3) with increasing chain length. For solvent chain lengths longer than three carbons, the proportions remain relatively constant, suggesting that the less polar solvents tend to stabilize the antiparallel form. It is interesting to note that species 3 is also the stable form found in crystals made from both methanol and ethanol, although in these solvents the other species are also found in abundance in solution. The results of this study clearly demonstrate the environment-dependent nature of the conformation of this small and relatively flexible polypeptide.

It should be noted, however, that although the ratio of the species present changes in different solvents, even in the most extreme case, where species 3 is approximately eight times as abundant as species 4, and slightly greater than twice as abundant as species 1 + 2, this corresponds to a relative stabilization energy of less than 1 kcal/mol between the most and least stable forms. This is a very small value, indeed, and means that all species are of nearly comparable stability. This result is not terribly surprising, however, if we consider that all the dimeric species have the approximately same number (but different positions) of intermolecular hydrogen bonds contributing to their stability.

In addition to the primary solvent effect, a secondary solvent effect has been observed for gramicidin, which results in a shift in the wavelengths of the uv and CD peak maxima. That shift is correlated with solvent polarity for the longer chain length alcohols. To simplify the interpretation, only the predominant species 3 has been examined (the contributions of the other species being removed computationally). The red shift detected for all the transitions correlates well with the observations we have made for other polypeptides in nonaqueous solvents.[14,23]

In summary, both primary and secondary solvent effects influence the CD spectra of gramicidin in nonaqueous solvents.

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