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Secondary solvent effects on the circular dichroism spectra of polypeptides in non-aqueous environments: influence of polarisation effects on the far ultraviolet spectra of alamethicin

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Abstract

Secondary solvent effects on the far ultraviolet circular dichroism spectra of the polypeptide alamethicin have been studied systematically in a series of alcohols. The magnitudes of the shifts have been correlated with the physical properties of the solvents in an attempt to discover the underlying physical principles responsible for these shifts. The solvent effect in non-aqueous solvents generally produces spectral transitions with peaks found at longer wavelengths than those in aqueous solution, and is correlated with increasing refractive indices and with decreasing dielectric constants of the solvents. It appears that polarisation effects are the major contributors to the interactions between the chromophore and solvent molecules, and hence give rise to the red shift. It is clear that this secondary solvent effect is an important factor which should be considered in the examination and estimation of polypeptide secondary structures in non-aqueous solvents and membranes.

Keywords: Secondary structures; Membrane proteins; Non-aqueous solvents; Dielectric constant; Solvent polarity; Spectroscopy

1. Introduction

Circular dichroism (CD) spectroscopy directly measures the differential absorption coefficients between left- and right-handed circularly polarised radiation. For biopolymers, the CD spectrum is a function of the asymmetry of the chromophores which have intrinsic optical activity. The CD waveform of a protein in the far ultraviolet region arises primarily from the amide backbone absorption, whilst the aromatic chromophores mainly produce the spectrum in the near ultraviolet range. As periodic structures, i.e. α-helix, β-sheet and β-turn, have distinct spatial arrangements of the ϕ and ψ angles between adjacent peptide bonds, these give rise to different characteristic CD spectra. Hence, CD spectroscopy has been used extensively in protein chemistry for the measurement of secondary structure and the detection of conformational changes. Since it is a technique which is usually applied to solutions, the solvent effects can play an important role in these applications.

The effects of solvent on the CD spectrum can be generally classified into two types, primary and secondary effects. A "primary effect" is due to the

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conformational change in the chromophores induced by the solvent. In this case, the spectrum shows significant alterations in waveform, that is, in the shape and relative intensities and positions 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 a rather fixed configuration or conformation. This solvent–chromophore interaction causes a shift in the transition energy between the ground state and the excited state by altering its electronic density distribution, and hence dipole moment and polarisability. The secondary effect produces a minor variation in wavelength maxima, and possibly absorbance intensity, compared to the primary effect, its magnitude being linearly related to the physical properties of the solvent. The change in an electronic transition could be due to interactions of either permanent or induced dipoles with either the ground state or the excited state [1–3]. It should not, in principle, produce a difference in the calculated secondary structure, but will if the changes in the peak positions are not taken into account in the analysis.

The secondary solvent effect has been broadly investigated for a number of simple chemical compounds and aromatic amino acid residues [4,5]. The wavelength shift can be large in small chemical compounds but is relatively minor for amino acid residues. In proteins, however, the interpretation is complicated by the diversity of environments of the intrinsic chromophores. For smaller peptides, the primary and secondary effects are usually observed together due to the flexible nature of peptides, which can readily undergo conformational changes in different environments.

In general, the solvent effects on the CD spectra of proteins have been observed to produce red shifts in apolar solvents and blue shifts in polar solvents relative to their positions in aqueous solutions [6–10]. It was also recognised by Chen et al. [11] that the different environments of exposed and buried amino acid residues could shift the positions of the transition bands, and this potential factor might affect the estimation of protein secondary structure. Some studies have attempted to correct for the solvent influences by either shifting the whole reference data set in positive or negative wavelengths [12], or by deconvoluting the experimental spectra into individual Gaussian peaks [7,9,10]. Particularly for membrane proteins and peptides which are functionally stable in more hydrophobic environments, the direct application of CD reference spectra based on water-soluble proteins whose secondary structures are known from X-ray diffraction studies has been found to be inadequate [9,10,12,13].

In this study, the aim has been to focus on the potential secondary solvent effects on CD spectra when non-aqueous solvents are used, and in particular by systematic variation, to determine the underlying physical properties responsible for the observed changes. In order to eliminate the primary solvent effects resulting in conformation changes, some experimental criteria are hence required: (1) the polypeptide under study should be able to dissolve in a wide range of solvents, (2) the conformation of this peptide should be rather fixed in the various solvent systems, especially in a series of chemically-related solvents, (3) the polypeptide should be small enough so that essentially all of its peptide bonds are exposed to solvent, (4) the conformation of the polypeptide under investigation should be primarily of one secondary structural type to simplify interpretation, and (5) the peptide should contain no aromatic side chains in order to eliminate contributions from these chromophores to the far ultraviolet CD spectrum. Alamethicin, which is a polypeptide antibiotic that forms voltage-dependent channels in membranes [14–16] fully fits these criteria, since it contains no aromatic amino acids and has a simple but fixed (helical) conformation in a variety of alcohol solutions [17].

The criteria for the choice of solvents are that: (1) the series of solvents should have a range of refractive indices and dielectric constants and (2) the solvents should be chemically related, i.e. have the same chemically reactive group with different length aliphatic chains. For this study, we have chosen a series of aliphatic alcohols, with increasing chain length as our solvent system in which alamethicin is highly soluble. The interactions between chromophores and solvent molecules are mainly donor–

acceptor interactions (i.e. electron or hydrogen donor–acceptors). These forces are additive. The effects are usually described by means of average physical characteristics of the environment such as refractive index or dielectric constant. In the case of alamethicin, since much of the secondary structure of the peptide is helical in nature, the characteristic bands seen in the spectra are the three π–$\pi^*_\perp$, π–$\pi^*_\parallel$ and n–π^* transitions, located at approximately 190–195, 207–215 and 222–230 nm, respectively. In this study, the influences of the different solvent molecules on these transition bands are discussed.

2. Materials and methods

2.1. Materials

Alamethicin was purchased from Sigma Chemical Co. (St. Louis, MO). This alamethicin is the R_f50 version with Gln at position 18. The sequence is heterogeneous with respect to position 6. Ala and Aib (α-aminoisobutyric acid) occur in approximately equal proportions as confirmed using HPLC and fast atom bombardment mass spectrometry. Methanol, *n*-hexanol and trifluoroethanol were purchased from Aldrich Chemical Co. (UK). All other solvents were purchased from BDH Merck (UK). *n*-Octanol, *n*-decanol and *n*-dodecanol were commercial grade. All others were spectrophotometric or analytic grade.

2.2. Sample preparation

Alamethicin was dissolved in a series of alcohol solvents at concentrations ranging from 0.5 to 5 mg/ml due to the different solubilities of the peptide in the various solvents. All samples were centrifuged for 4 min at approximately $1300 \times g$ prior to spectroscopic measurements to remove any insoluble materials.

2.3. Spectroscopic measurements

All CD measurements were made in quartz Suprasil cells (Hellma Kuvetten, Mullheim/Baden, Germany). The pathlengths of cells used, ranging from 0.05 to 0.001 cm, were chosen to minimise solvent absorption while providing a sufficiently large signal. CD spectra were recorded on an AVIV 62DS spectropolarimeter with a large angle detector geometry. The spectropolarimeter was calibrated using benzene vapour for wavelength, and (+)-d-10-camphorsulphonic 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 taken and averaged. Average baselines of the appropriate solvents were subtracted from the average sample spectra, and the resultant spectra were smoothed using a Savitsky–Golay filter.

The UV absorption spectra were measured in a 0.05 cm cell over the wavelength range from 190 to 300 nm at 1 nm intervals using a Cary 13 (Varian) spectrophotometer. The spectrophotometer was calibrated for wavelength using benzene vapour. All spectroscopic measurements were carried out at a temperature of 25 ± 0.2°C. The positions of peaks in both the CD and UV absorption spectra were determined using first or second derivative methods.

3. Results

3.1. Far ultraviolet (UV) CD spectroscopy

The X-ray structure of alamethicin shows it to be a mixed conformation of α- and 3_{10}-helices in crystals prepared from an acetonitrile/methanol mixture [16]. NMR studies in methanol [18–21] and in dimethyl sulphoxide [22], and Raman [23] and Fourier transform-IR [24] spectroscopic studies in dimethyl sulphoxide have shown a similar proportion of helical structure. In a wide range of organic solvents, the structure of alamethicin is seen to be relatively constant and dominated by helix, but is calculated to be only 20–40% helical from CD spectroscopic studies [17,25,26].

Fig. 1 shows the far UV CD spectra of alamethicin in ethanol, *n*-butanol, *n*-octanol and *n*-decanol in the wavelength range from 185 to 250 nm. In all these CD spectra, there is a positive peak near 194 nm and two negative peaks located at about 209 and 223 nm. These peaks are characteristic of helical secondary structures. They are assigned as the π–π^* perpendicular, π–π^* parallel and n–π^* transitions,

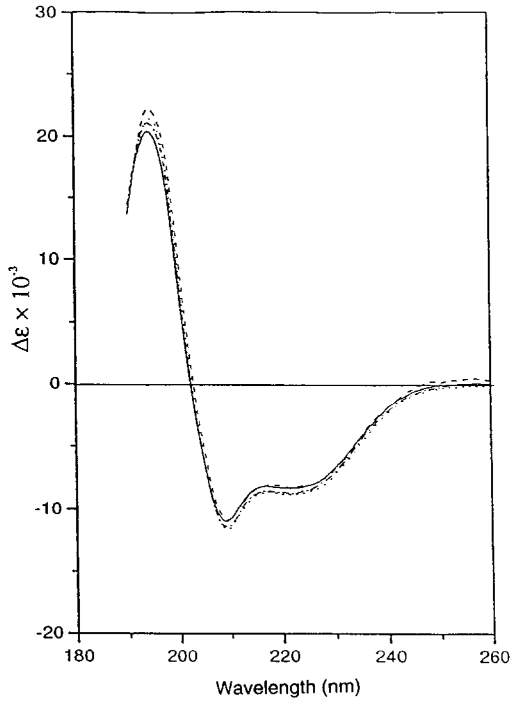

Fig. 1. Far-ultraviolet CD spectra of alamethicin in ethanol (————), butanol (— · —), octanol (· · · ·), and decanol (— — —).

respectively [27]. Since alamethicin contains no aromatic groups other than the C-terminal phenylalaninol, which has a very low ellipticity, no meaningful signals in the near UV CD spectra can be observed.

It can be clearly seen that the spectra are very consistent in terms of the magnitude of the peaks and their spectral waveforms in all the solvents, so the secondary structure of alamethicin must be very similar in these alcohols. In Fig. 1, it can be seen that all the transition bands, π–π^* perpendicular, π–π^* parallel and n–π^* bands, are shifted to longer wavelengths with the decreasing polarity of solvents from methanol to the longer chain alcohols. The shift of the π–π^* perpendicular transition bands at 194 nm is less obvious than the π–π^* parallel transition peak at 209 nm. The location of the peak maxima and minima are summarised in Table 1.

3.2. Far UV absorption spectroscopy

Fig. 2(A) and Fig. 2(B) show the absorption and dynode voltage spectra (the latter are similar to UV absorption records and are obtained from the spectropolarimeter during the CD measurements), for alamethicin in the series of alcohol solvents. UV

Table 1
Peak maximum (nm) in the CD spectra of alamethicin in a series of alcoholic solvents

Solvent	π–π^*(⊥) transition	π–π^*(‖) transition	n–π^* transition
methanol	194.09	209.00	223.36
ethanol	194.13	209.03	223.43
propanol	194.21	209.03	223.55
butanol	194.24	209.06	223.86
pentanol	194.37	209.13	223.90
hexanol	194.43	209.15	223.92
octanol	194.61	209.24	223.95
decanol	194.58	209.34	224.03

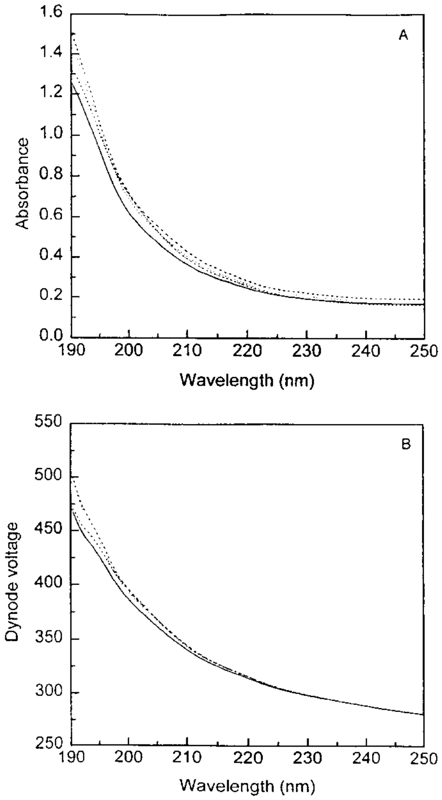

Fig. 2. (A) UV absorption spectra of alamethicin in ethanol (————), butanol (— · —), octanol (· · · ·), and decanol (— — —). (B) Dynode voltage records of alamethicin in ethanol (————), butanol (— · —), octanol (· · · ·), and decanol (— — —).

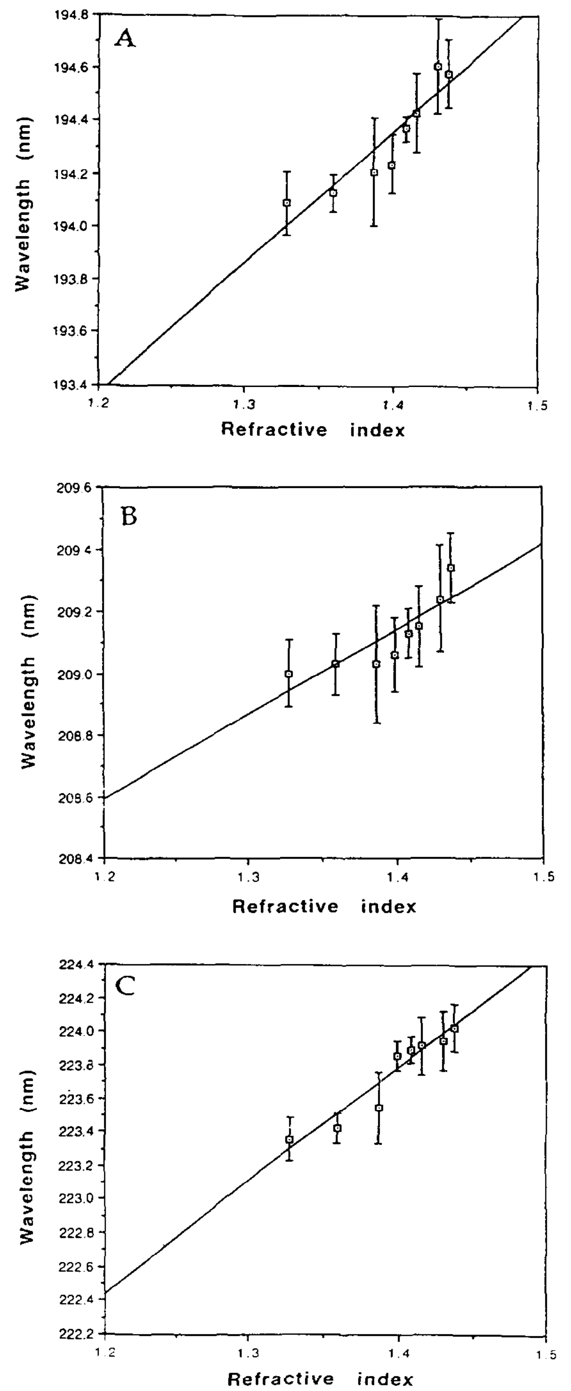

Fig. 3. Plots of wavelength versus refractive index for the three transition bands of alamethicin, (A) $\pi-\pi^*$ ($\perp$), (B) $\pi-\pi^*$ ($\|$) and (C) $n-\pi^*$ transitions.

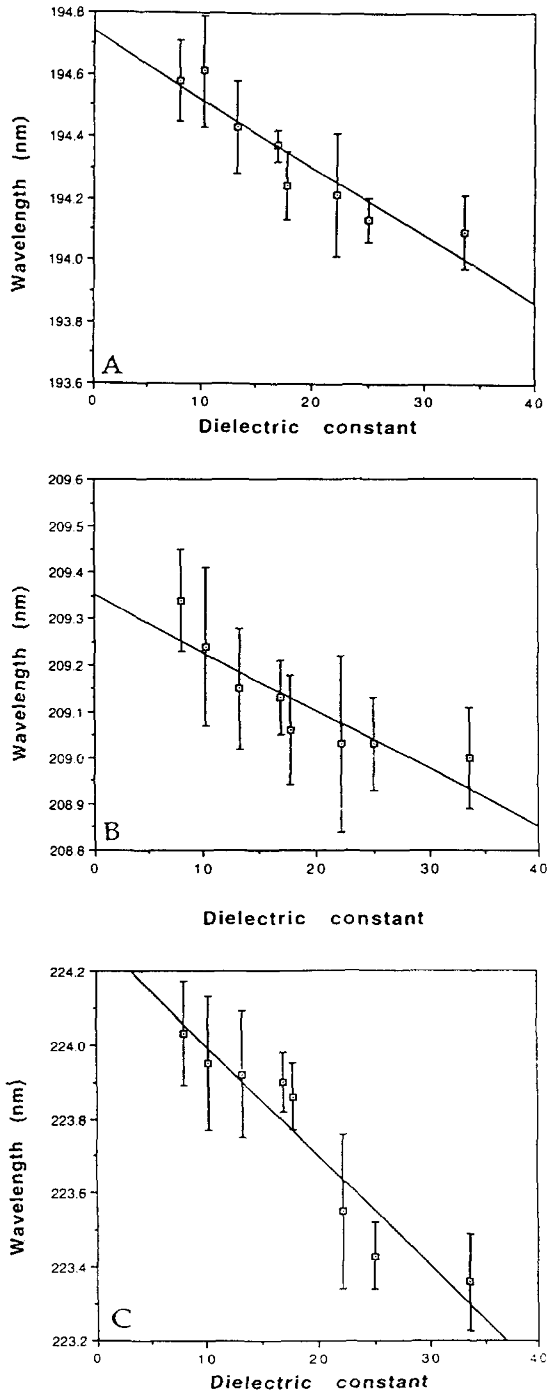

Fig. 4. Plots of wavelength versus dielectric constant for the three transition bands of alamethicin, (A) $\pi-\pi^*$ ($\perp$), (B) $\pi-\pi^*$ ($\|$) and (C) $n-\pi^*$ transitions.

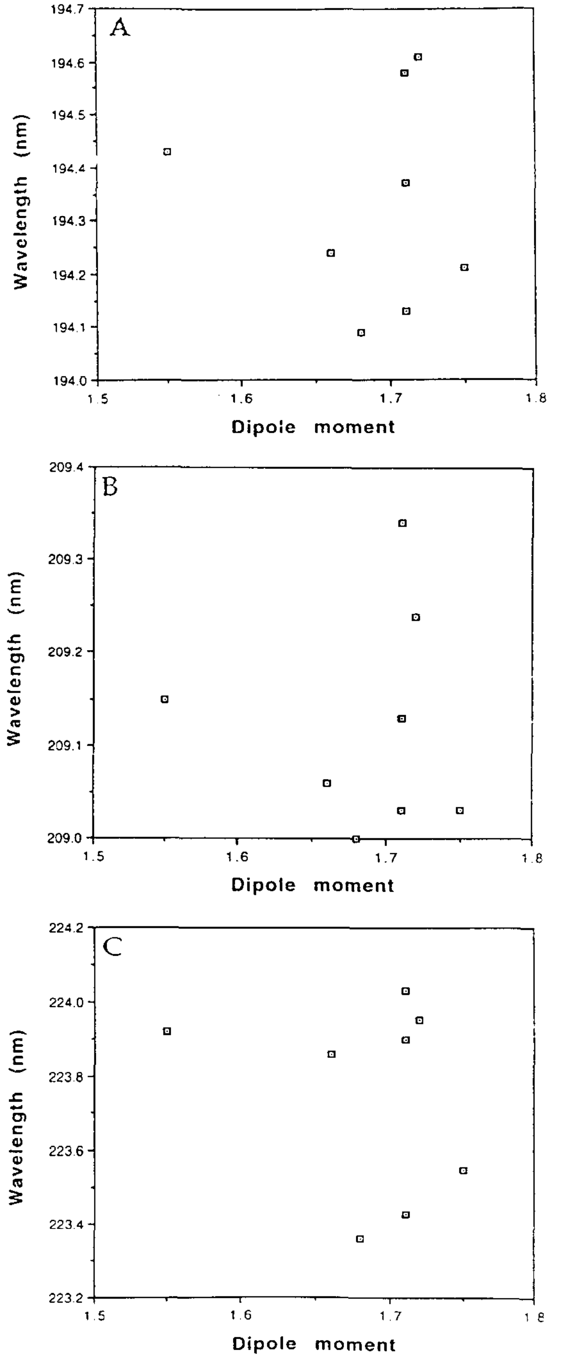

Fig. 5. Plots of wavelength versus dipole moment for the three transition bands of alamethicin, (A) $\pi-\pi^*$ ($\perp$), (B) $\pi-\pi^*$ ($\|$) and (C) $n-\pi^*$ transitions.

spectra are in general less sensitive to conformational changes than CD spectra, but they still exhibit the solvent effects. Unlike the CD spectra, these spectra show no peaks but a maximum absorption at about 190 nm. The weak shoulder at around 220 nm which often appears in long-chain helical biopolymers such as poly-L-alanine is not obvious in this case. This may be due to the fact that alamethicin is a simple peptide with only 19 amino acids, and its conformation has a limited amount of regular helical structure, on average. As observed in the CD spectra, the $\pi-\pi^*$ transitions in the UV spectra shift to longer wavelengths with decreasing polarity of solvents.

3.3. 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 transition bands were plotted versus refractive indices, dielectric constants and dipole moments for the series of chemically-similar solvent species. Figs. 3, 4 Figs. 5 show the plots of wavelength versus refractive indices, dielectric constants and dipole moments, respectively.

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 are all very high and vary from 0.9 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 refractive index and dielectric constant, no relationship has been found between the wavelength shift and the dipole moments of the alcoholic solvents.

4. Discussion

For alamethicin, the $\pi-\pi^*$ and $n-\pi^*$ transition bands all show a red shift with increasing refractive index and with decreasing dielectric constant. No significant alteration in the intensity of the CD data has been observed. Since alamethicin contains no

Trp, Phe, Tyr amino acids, any possible effect from aromatic side chains can therefore be excluded, thus making this a simple peptide system. As the UV spectra show the same trends as the CD spectra, the differences in the CD spectra are unlikely to be due to differences in conformation. All evidence suggests that the conformation is constant in this series of alcoholic solvents, so the spectral changes should reflect only the secondary effects (i.e. those due to solvent properties). The energy change between ground and excited states, ΔE, produces the change in frequency of the peak maxima. Hence, the secondary solvent effect should be the dominant factor in the changes in the CD spectra of alamethicin.

From studies of model organic compounds and their derivatives [3–5,28], it has been suggested that $n-\pi^*$ transitions are red-shifted as the refractive index of the solvent increases. In the $n-\pi^*$ transitions, the non-bonding orbitals obviously have more electron density than the anti-π configurations. When the chromophores are transferred into a more polar solvent, the solvent molecules interact more strongly with the polar ground state, and therefore, stabilise the non-bonding electronic distribution more than the π^* bonding orbital. So, the energy difference is decreased when the solvent is less polar, and the spectral shift is to longer wavelengths as the refractive index of the solvent system increases.

In contrast to the $n-\pi^*$ transition, the excited state of the $\pi-\pi^*$ transitions are more polar than the ground state. Studies of a series of simple amide chromophores have suggested that there is a large blue shift for primary amides, a smaller blue shift for secondary amides and a slight red shift for tertiary amides when they are transferred from water into cyclohexane due to the latter's lack of ability to form hydrogen bonds between chromophores and solvent molecules [29]. The red shift of the $\pi-\pi^*$ transition observed for the alamethicin peptide backbone with the decreasing polarity of the alcohol solvents is consistent with expectations based on the small molecule compounds, since the amide NH's are hydrogen-bonded to the amide C=O groups in helical structures but not the polar (aqueous) solvent. If there is no major conformation change involved, the amide N–H groups will be mostly insulated from direct contact with polar solvent molecules. So, the $\pi-\pi^*$ transitions for the peptide amide backbone produce a red shift similar to that of tertiary amides [29].

The mechanism of 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. The interpretation of secondary solvent effects is made difficult because they are usually small and not easy to measure precisely, and also because they are often the result of several individual effects which sometimes reinforce one another and sometimes cancel out. For proteins and peptides, the situation is more complicated than simple model chromophores, since the interactions between solvent molecules and each chromophore are varied, and the internal environments of individual chromophores are inhomogeneous.

The equilibrium configuration of the solvent molecules surrounding the chromophore groups differs in the ground and excited states. According to the Franck–Condon principle for intermolecular interactions, the nuclear motions are very slow compared with the changes in the electronic distribution in a molecule. So, molecules have no time to change orientation and to take the equilibrium configuration which corresponds to the energy minimum during the period of light absorption. This makes the interactions different in the ground and excited states. Therefore, the absorption spectrum shift is proportional to a change in the difference of stabilisation energies of the ground and excited states. In principle, four factors contribute to this: orientation effect, dispersion interaction, and polarisation effect by transition dipole moment or by permanent dipole moment.

The orientation effect is due to the solvent molecules adjacent to the chromophores absorbing radiation. They may be excited by some interactions amongst themselves, or interactions between them and chromophore molecules such as hydrogen bonding. This displacement is caused by the interactions between oriented chromophore and solvent molecules. An orientation effect may be expected when solute and solvent molecules are both polar, and when solute dipole moment changes during the transition. The induced shift of the spectrum by this effect can be either red or blue, depending on whether there is an increase or decrease in the chromophore's dipole moment.

The dispersion effect is caused by the polarisation of the electronic distribution of solvent molecules by alterations in the electron distribution within the chromophores, and vice versa. The polarisation effect is induced by dipole moment. When it is induced in the chromophore by exciting radiation, i.e. transition dipole moment, it is called polarisation by transition dipole moment. On the other hand, if it is induced by the permanent dipole moment of the chromophores, both in the ground and excited state, the effect is called polarisation by permanent dipole moment. The former polarisation effect is smaller than the latter but is the dominant factor when both solute and solvent are non-polar. This polarisation effect always leads to a red shift of the spectrum with increasing refractive index, and is generally called the polarisation red shift. The effect of permanent dipole moment on the shift of spectrum can be either blue or red, depending on whether the dipole moment of the solute is decreased or increased, respectively.

Calculations of the effects of interactions between solvent molecules and chromophores must recognise there is no single parameter-dependent relationship which can describe the extent of the different effects on the absorption spectra of chromophores. At least the two parameters, refractive index and dielectric constant, must be considered together. Although several more complicated empirical expressions have been proposed to account for these effects [2,30,31], the model based on the empirical quantum mechanism proposed by Bakhshiev [31] has been used to further analyse this system, since we have been able to show the correlation with refractive index and dielectric constant. The most appropriate equation is as follows:

$$\Delta\nu = C_1\left(\frac{2n^2+1}{n^2+2}\right)\left[\left(\frac{\varepsilon-1}{\varepsilon+2}\right)-\left(\frac{n^2-1}{n^2+1}\right)\right] + C_2\left(\frac{n^2-1}{n^2+2}\right)$$

where $\Delta\nu$ is the shifted value relative to a selected standard solvent in frequency units, and n and ε are the refractive indices and dielectric constants of the solvents, respectively. The first term represents the contribution from orientation interactions, and the second term includes all other polarisation and dispersion factors.

A modified equation following the above function can allow us to extrapolate the parameters, C_1 and C_2, for the orientation and polarisation effects on the spectrum. The equation is described as:

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

where

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

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

Fig. 6 shows the plots of $\Delta\nu/\gamma$ vs. σ/γ for the transition bands of the alamethicin polypeptide backbone, where the spectrum in methanol is taken as the standard. It can been seen that there is generally a linear relationship between the frequencies and solvent properties. The correlation coefficients are all greater than 0.92. For these transition bands, the C_2 parameters are negative and significantly larger than those of C_1. The values for the amide backbone $\pi-\pi^*_\perp$, $\pi-\pi^*_\parallel$ and $n-\pi^*$ transitions are summarised in Table 2.

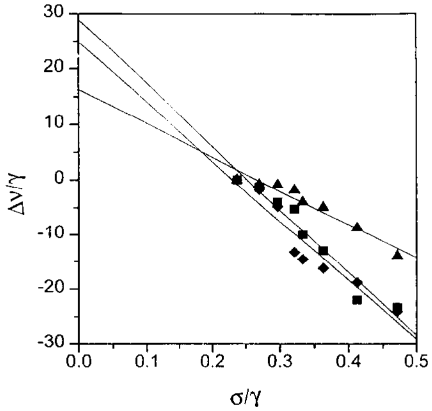

Fig. 6. Modified Bakhshiev plots for alamethicin, (■) $\pi-\pi^*$ ($\perp$), (▲) $\pi-\pi^*$ ($\parallel$) and (◆) $n-\pi^*$ transitions.

Table 2
Parameters estimated from Bakhshiev plots for the three transition bands

	$\pi-\pi^*(\perp)$ transition	$\pi-\pi^*(\parallel)$ transition	$n-\pi^*$ transition
C_1	28.83	16.24	27.76
C_2	−114.51	−61.08	−107.81
R	0.97	0.97	0.96

According to the Bakhshiev function, the large C_2 value demonstrates that the polarisation effects are the dominant factors in causing the red shift in wavelength of the far UV CD spectra of alamethicin. This red shift is most likely to be a general red shift induced by the transient dipole polarisation effect.

In this case, the influences from the orientation effect and hydrogen bonding are less important in the more hydrophobic solvents. Although the transition dipole-induced polarisation effect dominates the solvent effect in the far UV CD spectra of alamethicin, the parameter C_1 shows that there also exists a minor orientation effect. This may arise from two possible sources: (1) Since alamethicin is mainly helical, the dipole moment and orientation of the helical structure may contribute to the orientation effect, and (2) the carboxyl groups may still dynamically form hydrogen bonds with apolar solvent molecules such as methanol, hence the linear relationship with dielectric constant.

5. Conclusions

We have previously noted [9,10,12] the influence of solvent environment on the CD spectra of polypeptides in non-aqueous environments. The present study has examined the solvent effects on the three peptide backbone transitions and correlated the physical properties of solvents with the nature of the solvent–chromophore interactions. From our studies, it is clear that non-aqueous solvents shift spectral peak positions. These shifts can be either red-shifts or blue-shifts, depending on the nature of the chromophores and the interactions between the solvent molecules and chromophores. It is not necessary that the magnitude and direction of spectral shifts be homogeneous, and the treatments of the transition shifts could be different depending on their electronic configurations. Some studies have also suggested that at least the $n-\pi^*$ and $\pi-\pi^*$ perpendicular and parallel transitions need to be treated differently [1,9,10].

Since alamethicin is primarily a helical structure, the secondary solvent effects on all its amino acids have similar patterns. Such effects may also be expected for β-sheet structures because of their intramolecular hydrogen bonding, which may prevent the interaction of NH groups with polar solvents. For more open structures such as β-turns and random structures, different behaviours may be expected. It is worth considering these other types of conformations individually in the future.

Finally, our studies have focused on a small peptide with a highly helical conformation. The alteration in its electronic transitions is rather smaller than in the simple molecule cases studied previously [5,29]. This is due to the more complex microenvironments and interactions with each chromophore in the peptide. Hence, we can expect that distinguishing the secondary effects will be more difficult for the large proteins due to their different environments. The solvent molecules may interact differently with different chromophores, and different directions of solvent effects may even cancel each other out.

CD studies on membrane peptides and proteins are becoming increasingly important. However, there are a number of phenomena that arise due to the particulate nature and non-aqueous environment present in phospholipid membranes, which must be considered when secondary structural analysis methods, originally developed for aqueous-soluble proteins, are employed on membrane proteins. These potential artifacts include differential scattering, due to the large size of the membrane particles relative to the wavelength of light used [32], and absorption flattening, due to the high concentration of proteins sequestered into the membrane particles [33], as well as the solvent shift phenomenon examined in this work. It is clear that in the case of alamethicin the non-aqueous solvent effects on the far UV CD must be taken into account when considering its spectral properties [9,10,12]. This is likely to be true in the analysis of secondary structure for many membrane polypeptides by CD, since this class of protein usually exists in a hydrophobic non-aqueous environment. Thus, this study represents one aspect of a continuing effort to aid in the elucidation of protein and peptide structures in membrane and non-aqueous environments by CD spectroscopy.

Acknowledgements

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