Analysis of Circular Dichroism Spectra of Oriented Protein−Lipid Complexes: Toward a General Application†

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ABSTRACT: A new application of circular dichroism on oriented films of protein−lipid complexes is presented in this work, which provides quantitative information on the orientation of α-helices with respect to the incident light beam. We used literature reference circular dichroism spectra for the various secondary structures to develop a new set of spectra, where the different directions of absorption within the molecular axis frame for each secondary structure type are taken into account. Using this new set of spectra, we could determine the orientation of the helical part of melittin in oriented films composed of various phospholipids. The orientation of the helix axis is found to be perpendicular to the membrane normal of films of dioleoylphosphatidylcholine, dioleoylphosphatidylglycerol, and dioleoylphosphatidylserine, whereas the helix was aligned preferentially parallel to the membrane normal for membranes composed of dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol at a hydration of approximately 2-4 water molecules per lipid. The orientations found by circular dichroism at this low hydration for the various systems agreed very well with those obtained by Fourier transform infrared measurements on these samples. Upon increasing the hydration of the film (to approximately 20 water molecules per lipid), it is shown by circular dichroism that the orientation of the helix of melittin changes most in films of dioleoylphosphatidylserine, where it adopted, under these conditions, a preferred parallel orientation with respect to the membrane normal. Complications in the analysis of circular dichroism spectra of oriented samples are discussed and illustrated using patches of native purple membrane containing bacteriorhodopsin and films of alamethicin in dioleoylphosphatidylserine membranes.

Compared to soluble proteins, not much is known about the structure of membrane-associated proteins. The commonly applied techniques all have their limitations in studying these complexes. For example, nuclear magnetic resonance (NMR)[1] can give detailed information on an atomic level; however, the particle size is limiting in the analysis of high-resolution NMR data, restricting the approach to small polypeptides in a membrane-mimicking environment, like micelles. Solid state NMR offers the advantage that immobile systems can be studied, but generally requires specific labeling of the protein. For X-ray studies, one needs crystals of the membrane-reconstituted proteins, which are often difficult to obtain.

Two other techniques that do not give atomic resolution, but have been shown to be very useful in studying the secondary structure of proteins in lipid complexes, are infrared (IR) spectroscopy and circular dichroism (CD). Using IR to study films of protein−lipid complexes, one has the additional advantage that one is able to extract information on the orientation of the secondary structure elements with respect to the membrane (Goormaghtigh & Ruysschaert, 1990). However, in order to separate the helical from the random-coiled contributions in the spectra, deuteration of the amides usually is performed. This results in a loss of information for all regions that are loosely folded, irrespective of the secondary structure. Also, with CD information has been obtained on the orientation of secondary structure elements in lipid−protein films (Vogel, 1987; Wu et al., 1990). In these studies, assumptions concerning the secondary structure of the peptides in the film (melittin and alamethicin, respectively) were required to extract CD spectra belonging to the different directions of absorption within the α-helix, as predicted by the Moffit theory (Moffit et al., 1957).

We chose the strategy of deconvoluting reference CD spectra, described in the literature for all four secondary structure elements, in terms of Gaussian absorption bands. Because these absorptions have a defined direction in the molecular axis frame, information on the orientation of secondary structure becomes accessible. From the absorption bands obtained, new reference spectra are composed, cor-

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[1] Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; DOPS, dioleoylphosphatidylserine; DOPG, dioleoylphosphatidylglycerol; DOPC, dioleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DTPC, ditetradecylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; (ATR-FT)IR, attenuated total reflection fourier transform infrared; LUVET, large unilamellar vesicle by extrusion technique.

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responding to the different orientations of the secondary structure element with respect to the light beam. This new set of reference spectra is used to fit experimental CD spectra of various protein−lipid complexes.

In this new method using a CD technique performed on a commercially available spectropolarimeter, we first eliminate possible spectral artifacts, such as the CD contribution of the lipids, linear dichroism due to inhomogeneities in the materials used, or differential light scattering by particles, which is large relative to the wavelength of the incident light. Next, we calculate the individual contributions of the secondary structure elements in oriented systems without the requirement of knowledge of the spectrum of the nonoriented sample. Finally, we calculate the orientation of the α-helical component without making assumptions concerning the secondary structure of the polypeptide. We will illustrate this method using melittin in the presence of various saturated and unsaturated phospholipids. The results will be compared to IR measurements on the same systems. We will also discuss the CD measurements of several other protein−lipid complexes in order to demonstrate the general applicability of this approach [see the accompanying paper: de Jongh et al. (1994)] and to illustrate and discuss complications such as absorption flattening (this paper).

MATERIALS AND METHODS

Materials. Dioleoylphosphatidylcholine (DOPC) was synthesized and purified according to established methods (van Deenen & de Haas, 1964). DOPS, DOPG, DMPC, and DMPG were obtained from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. All anionic lipids were sodium salts. Melittin (Sigma, St. Louis, MO) was purified according to Batenburg et al. (1987).

Sample Preparation. Stock solutions of DOPC, DOPS, DOPG, DMPC, and DMPG were prepared by dissolving appropriate amounts of lipid in chloroform. After evaporation of the solvent under reduced pressure, the lipid film was suspended in H_2O. By applying the extrusion technique at room temperature using polycarbonate filters of 400 nm, as described by Hope et al. (1985), large unilamellar vesicles (LUVETs) were obtained, and after the lipid concentration was determined according to Rouser et al. (1970), the volume was adjusted, resulting in stock solutions of 24 mM.

To 10 μL of a melittin stock solution in water (1 mg/mL) was added 8.8 μL of a lipid stock solution to obtain final lipid to protein molar ratios of 60. After incubation for 10 min at room temperature, one-half of the sample was used to record a CD spectrum (the so-called solution spectrum), after its volume was adjusted to 40 μL using demineralized water, in a 0.02 cm cuvette on a Jasco-J600 spectropolarimeter at room temperature. In the 190−260 nm region, 64 scans were accumulated with a scan speed of 50 nm/min and a time constant of 0.125 s and were subsequently averaged. The other half was placed on a circular quartz plate (thickness of 1 mm and a diameter of 18 mm) and dried under a continuous flow of nitrogen gas, using the tip of a micropipet to improve homogeneous spreading of the sample over approximately 20 mm^2. The quartz plate was dried overnight in a vacuum desiccator at <0.05 Torr over diphosphorus pentoxide. Next the quartz plate, now containing oriented dried membranes (Fringeli & Günthard, 1981), was assembled into a closed cell by placing it at one end of a Teflon tube with a 10 mm bore and a length of 12 mm, with a second quartz plate at the other end. A sample measured under these conditions is referred to be in the low hydration state. Halfway down the Teflon tube a circular slot was present, which could be used for the addition of a salt solution before sealing the cell. This enabled us to control the humidity within the cell without direct contact between the protein−lipid film and the salt solution. The high hydration state was obtained by equilibration of the sample overnight at room temperature in a closed cell with 50 μL of a 1 M sodium chloride solution. In a similar way, control samples were made of lipids in the absence of protein. For all data shown, the corresponding protein-free spectra were subtracted. Because no significant absorbance of the protein−lipid films could be detected (data not shown), scattering and therefore differential scattering artifacts are excluded from these samples.

In order to be able to eliminate linear dichroism artifacts arising from inhomogeneities of the sample or the quartz plates, the cell was positioned in a special holder that could be rotated in the plane perpendicular to the incident light beam. CD spectra, as averages of four scans, were recorded every 22.5 deg of rotation of the cell, as described by Vogel (1987). The 16 spectra subsequently are averaged. Accumulation of spectra recorded every 11.25 deg did not alter the final spectrum.

CD Spectral Analysis. (A) Principle. First the reference spectra of polylysine in the α-helix, β-strand, and random-coiled conformations, as described by Greenfield and Fasman (1969) and Chang and co-workers (1978), were compared with our recorded CD spectra of polylysine under conditions identical to those described by these authors and were found to be very similar (data not shown). Because each reference spectrum of a secondary structure element is a superposition of several Gaussian absorption bands (Urry, 1968), it was our strategy to deconvolute the reference spectra described in the literature in terms of Gaussian absorption bands. The absorption bands obtained were then used to reconstruct a new spectrum for each secondary structure element, now allowing us to correlate the direction of absorption for each band with the molecular axis system of the secondary structure element.

(B) Theory. Fitting of the spectra was performed by a nonlinear regression procedure, making use of the Gauss−Newton algorithm on a 20 MHz Laser-386 computer. The reference spectra of polylysine in the α-helix, β-strand, and random-coiled conformations and the spectrum of the β-turn, obtained as an average of 24 spectra of proteins with known X-ray structure [data from Chang et al. (1978)], were fitted independently from 190 to 240 nm with 1 nm resolution by three Gaussian absorption lines [and an additional Gaussian and a derivative Gaussian line (the so-called helix band) for the α-helix spectrum (Tinoco, 1964)]. Every Gaussian line is defined by three parameters: the amplitude, the line width, and the wavelength of maximal absorption. No constraints were used in the fit procedure, except that the helix band was restricted to be positive at long wavelengths, as described by Tinoco (1964). The quality of the fit was expressed using the definition of the normalized root-mean-square (RMS) error as described by Brahms and Brahms (1980). The values for the various parameters obtained after deconvolution of the reference spectra in terms of Gaussian bands are presented in Table 1.

Table 1: Parameters Describing the Reference Spectra for the Different Secondary Structure Types by Gaussian Absorption Bands ($Ae^{(-(\lambda-\lambda_o)^2/\Delta^2)}$)

	band	A ($\times 10^{-3}$ mdeg·cm^2 dmol^{-1})	Δ (nm)	λ_o (nm)	RMS
α-helix	1	−29.55 ± 0.75	7.16 ± 0.33	220.48 ± 0.31	
	2	−63.58 ± 0.51	8.99 ± 0.27	207.70 ± 0.09	
	3	95.65 ± 2.05	7.63 ± 0.11	190.44 ± 0.30	
	4	−69.40 ± 1.01	8.99 ± 0.35	227.70 ± 0.31	
	5[a]	6.09 ± 0.09	9.02 ± 0.43	188.50 ± 0.33	2.30
β-strand	1	−18.55 ± 0.04	14.29 ± 0.38	216.45 ± 0.11	
	2	29.89 ± 0.08	8.85 ± 0.17	197.77 ± 0.08	
	3	12.78 ± 0.17	5.29 ± 0.18	191.42 ± 0.09	1.78
β-turn	1	19.28 ± 0.24	8.92 ± 0.24	223.17 ± 0.08	
	2	35.90 ± 1.65	6.68 ± 0.12	199.37 ± 0.13	
	3	−83.58 ± 0.73	8.13 ± 0.33	190.64 ± 0.06	3.00
random coil	1	3.88 ± 0.04	9.12 ± 0.17	217.85 ± 0.13	
	2	−40.15 ± 0.08	8.22 ± 0.07	197.54 ± 0.03	
	3	−8.91 ± 0.18	4.22 ± 0.08	189.55 ± 0.13	0.96

[a] Helix band as described by Tinoco (1964): $A(2(\lambda - \lambda_o)(\lambda_o/\Delta^2) + 1)e^{(-(\lambda-\lambda_o)^2/\Delta^2)}$.

All of the CD absorptions of proteins in the 180−260 nm region are n−π^* or $\pi-\pi^*$ transitions [for details, see Urry (1968)] and have a transition moment in a defined direction in their molecular axis system. On the basis of the data presented by Urry (1968) and Wu et al. (1990), we have come to the following identification for the bands obtained for an α-helix in relation to the molecular axis of an α-helix. These fall in two categories: (1) three bands absorbing light traveling perpendicular to the helix axis, with maxima at 220.5, 207.7, and 190.4 nm (bands 1, 2, and 3, respectively, in Table 1), and (2) two bands absorbing light propagating parallel to the helix axis, with absorbance maxima at 227.7 and 188.5 nm (bands 4 and 5). For all absorptions by a β-strand, the transitions for both n−π^* and $\pi-\pi^*$ lie in the plane parallel to the molecular axis of the strand (Kelly et al., 1977; Rosenheck & Sommer, 1967). Therefore, the intensities for all transitions will be reduced when the strand changes from a parallel to a perpendicular orientation with respect to the incident light beam. This has also been demonstrated for polypeptide monolayers by Cornell (1979). Although in this work no attention will be paid to the preferred orientation of β-structures, a large extent of the preferred orientation of a β-strand can be identified by comparison of the absolute intensities of the secondary structure elements in the fit of an oriented and a nonoriented sample. The orientation dependency of the absorption by β-turns is not known, whereas an ensemble-averaged preferred orientation (from now on simply preferred orientation) of random coil is unlikely.

From the parameters presented in Table 1, new reference spectra can be generated for the β-strand, β-turn, and random coil by summation of the three bands, whereas for the α-helix two spectra now are obtained: (1) the summation of bands 1−3, absorbing light perpendicular to the helix axis, and (2) the summation of bands 4 and 5, absorbing the light propagating parallel to this axis. Figure 1 shows these two newly generated spectra, which are denoted $\theta_{||}$ and $\theta_\perp$ for the parallel and perpendicular components of the α-helix spectrum, respectively. Summation of these two spectra according to

$$\theta_v = (\theta_{||} + 2\theta_\perp)/3 \quad (1)$$

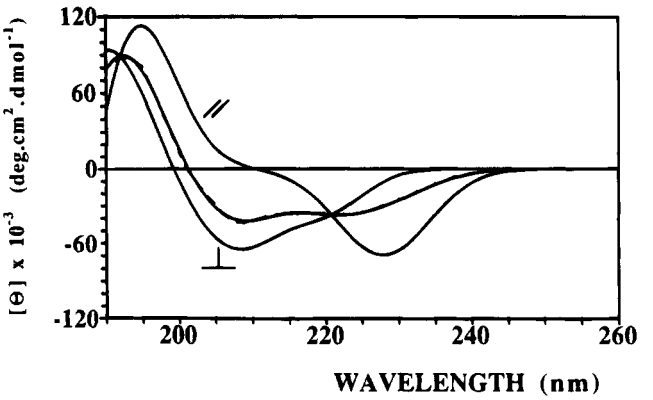

FIGURE 1: Reconstructed spectra of the parallel and perpendicular components of the CD spectrum of the α-helix as described in the text, together with the summation representing isotropic distribution of helices. The dashed line is the spectrum of the reference spectrum of polylysine as given by Greenfield and Fasman (1969).

where θ_v represents the ellipticity in an isotropically distributed sample, results in a spectrum that is, as expected, very similar to the original spectrum of α-helical polylysine (Figure 1). The intensity at 207 nm of a helical polypeptide oriented on a quartz plate can serve as a fingerprint of its orientation. A reduced absolute intensity at 207 nm compared to the situation of an isotropically distributed sample is indicative of a preferred orientation of the helix axis parallel to the incident light beam. Enhanced intensity at 207 nm is suggestive of a more perpendicular orientation of the helix axis.

Fitting of an experimental CD spectrum is performed in the range 190−260 nm with 0.2 nm resolution, using a free nonlinear regression method as described earlier. Besides the reconstructed spectra for the β-strand, β-turn, and random coil, $\theta_{||}$ and $\theta_\perp$ are used to fit the recorded spectrum. The ratio between the parallel and the perpendicular contributions of the helix is directly related to the orientation of the helix with respect to the incident light beam. We therefore define the orientation parameter, ξ, for a helix as

$$\xi = a_\perp/a_{||} \quad (2)$$

where $a_\perp$ and $a_{||}$ are the parameters for the perpendicular and parallel contributions of the helix, respectively. For an isotropic distribution of a helix, ξ will be equal to 2, because the perpendicular contribution is, according to eq 1, twice as large as the parallel component. The orientation parameter, ξ, can be related to the ensemble-averaged angle, φ, between the helix axis and the incident light by to the relationship

$$\theta(\lambda) = a_\perp\theta_\perp(\lambda) + a_{||}\theta_{||}(\lambda) = (\cos^2\varphi)\theta_\perp(\lambda) + (\sin^2\varphi)\theta_{||}(\lambda) \quad (3)$$

as described by Wu et al. (1990), resulting in the simple relation

$$\xi = \tan^2\varphi \quad (4)$$

Characterization of the Hydration State. In order to determine the hydration state of the lipid films, we prepared films, in experiments separate from the CD measurements, using ^2H_2O instead of H_2O in all steps of sample

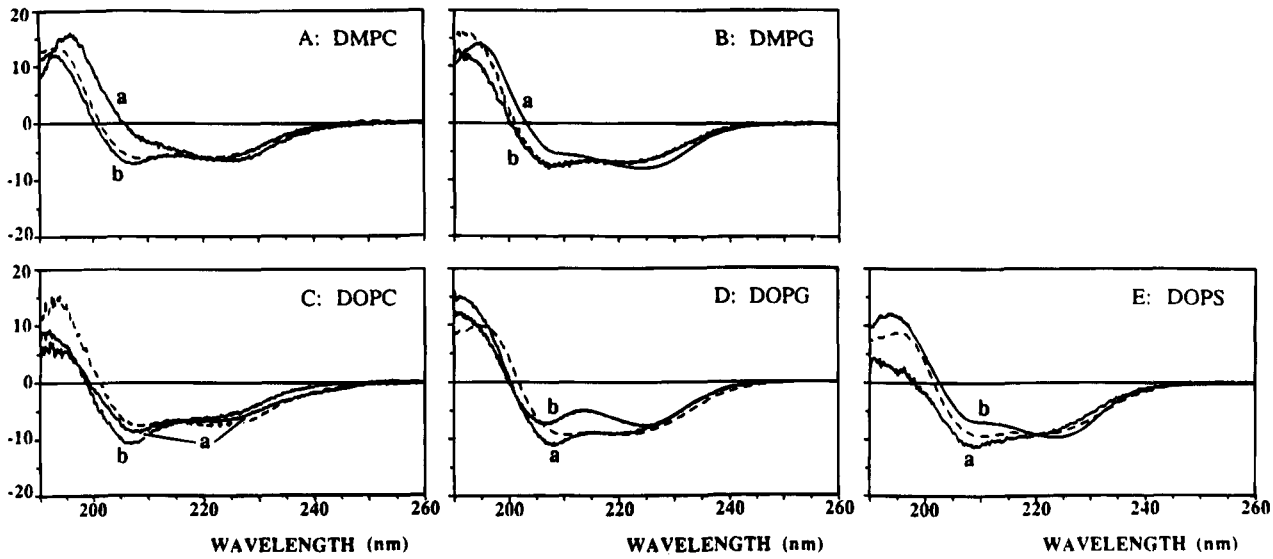

FIGURE 2: CD spectra of melittin in the presence of DMPC (A), DMPG (B), DOPC (C), DOPG (D), and DOPS (E) in solution (dashed) and oriented on quartz plates in two different hydration states: low hydration (spectra a) and high hydration (spectra b).

preparation. The (hydrated) lipid films were next recollected by resuspension of the film in a total volume of 0.5 mL of deuterium-depleted water, which was then transferred to an NMR tube. The deuterium content was determined using ^{2}H NMR on a Bruker MSL-300 machine (at 46.14 MHz using a single 90° pulse of 11 μs, an interpulse time of 2.5 s, and a sweep width of 10 kHz) by comparing the integral of the H^{2}HO resonance of a spectrum of 2500 accumulated scans with that of a standard of 5 μmol of ^2H_2O in 0.5 mL of deuterium-depleted water. The numbers presented are averages and standard deviations from five separate experiments.

Fourier Transform Attenuated Total Reflection Infrared Measurements. Germanium crystals (50 × 20 × 2 mm, Harrick EJ2121) were cleaned by washing with destilled water, methanol, and chloroform, respectively, and subsequently placed for 5 min in a Plasma Cleaner PDC-23G (Harrick Scientific, Ossining, NY) in order to obtain a hydrophilic surface. Samples (10 μg of melittin; lipid to melittin molar ratio of 60), identical to those spread on quartz plates described earlier, were spread on the crystals, revealing films with hydration expected to be comparable to the low hydration state defined previously. The spectra were recorded at room temperature on a Perkin-Elmer 1720X FT-IR spectrophotometer equipped with a mercury cadmium telluride detector at a nominal resolution of 4 cm^{-1}, encoding for every 1 cm^{-1} in the range 0−4000 cm^{-1}. The crystals were placed under an aperture angle of 45°, yielding 25 internal reflections. Every spectrum is an average of 64 scans, simultaneously corrected for the background using a clean germanium plate. Perkin-Elmer gold wire grid polarizers were placed in front of the sample and reference holder, enabling us to record spectra at 0° and 90° polarization of the incident light beam.

RESULTS

The large abundance of literature on the conformation and orientation of bilayer-associated melittin makes this a good system to test the validity of our CD approach for obtaining the content and orientation of secondary structure elements of bilayer-associated proteins. First we will present CD data on melittin and compare the obtained results with (ATR-FT)IR measurements on the same systems. Next, we will briefly describe the application of CD to some other protein−lipid complexes.

Circular Dichroism of Melittin. In Figure 2A−E, the CD spectra are shown of melittin in the presence of the phospholipids DMPC, DMPG, DOPC, DOPG, and DOPS. Because it has been described in the literature that the orientation of secondary structure elements might depend strongly on the degree of hydration of the system (Vogel, 1987; Wu et al., 1990), we investigated the samples under three different conditions: in solution at maximal hydration and in oriented systems at low and high hydration.

For all systems, the solution spectra (Figure 2, dashed lines) exhibit three extremes around 196, 207, and 222 nm, which are typical features of polypeptides with a high degree of helicity (Chang et al., 1978). Analysis of the CD spectra reveals that the α-helix is indeed the most dominant secondary structure type present in these samples (Table 2). Remarkable for these solution spectra is the lower helix content and the higher contributions of β-strand and random conformations in the presence of DOPC, DOPS, and DOPG compared to the saturated lipids DMPC and DMPG. No clear influence of the headgroup on the secondary structure can be observed. The orientation parameter, ξ, determined as the ratio of the perpendicular and parallel contributions to the helix, is close to 2 for all of these systems (Table 3, first column), indicating that no preferred orientation of the helices is present, as expected for vesicle-associated polypeptides that can tumble freely in solution.

When these samples are spread on quartz plates and subsequently dried, optically clear films are obtained. Characterization of this low hydration state reveals that about 2 water molecules per lipid are present for DMPC and DMPG (Table 3). The hydration numbers for the unsaturated lipids DOPC, DOPG, and DOPS are slightly higher, around 3 or 4 water molecules per lipid.

For all systems studied, the CD spectra of the low hydration samples (Figure 2, spectra a) differ from the corresponding solution spectra. For both DMPC and DMPG, a decrease in absolute intensity at 207 nm and a slight

Table 2: Percentages of the Secondary Structure of the CD Spectra of Melittin with Various Phospholipids

	solution	low hydration	high hydration
DMPC			
α-helix	85.4	56.2	61.6
β-strand	9.4	30.4	11.7
β-turn	5.0	13.5	0.2
random	0.2	0	26.5
RMS	7.95	7.40	4.40
DMPG			
α-helix	91.6	34.8	39.3
β-strand	4.2	38.6	25.9
β-turn	0.6	7.1	2.2
random	3.6	19.5	32.6
RMS	4.75	4.47	5.14
DOPC			
α-helix	63.0	27.3	78.4
β-strand	13.5	23.6	1.1
β-turn	1.5	13.2	0.5
random	21.9	36.0	20.0
RMS	6.89	6.45	8.50
DOPG			
α-helix	51.3	52.1	62.5
β-strand	32.0	17.0	8.7
β-turn	12.0	5.4	1.2
random	4.7	25.5	27.6
RMS	8.42	4.42	6.55
DOPS			
α-helix	65.7	48.6	72.0
β-strand	10.4	21.7	6.9
β-turn	3.1	8.4	3.2
random	20.8	21.3	17.9
RMS	7.64	5.21	4.74

Table 3: Orientation Parameter, ξ, of the α-Helix and Its Derived Angle, φ, with Respect to the Normal of the Plate As Obtained from Analysis of CD Spectra of Melittin with Various Phospholipids at Different Hydration States

	solution		low hydration			high hydration		
	ξ	φ (deg)	ξ	φ (deg)	n_w[a]	ξ	φ (deg)	n_w[a]
DMPC	1.92	54.2	0.37	31.3	1.8 ± 0.6	1.85	53.7	18.6 ± 1.9
DMPG	1.89	54.0	0.79	41.6	2.7 ± 0.6	1.85	53.7	24.1 ± 1.6
DOPC	2.04	55.0	4.55	64.9	3.2 ± 1.1	5.88	67.6	19.7 ± 5.1
DOPG	1.98	54.6	4.02	63.5	4.2 ± 1.0	1.94	54.3	20.8 ± 2.2
DOPS	2.18	55.9	4.19	64.0	3.5 ± 0.9	0.56	36.8	19.3 ± 1.5

[a] n_w is the experimentally determined number of water molecules per lipid.

increase at 228 nm can be observed, suggesting that the parallel contribution of the helix is more dominant than the perpendicular one (compare Figure 1). From the small value obtained for the orientation parameter ξ (Table 3), it can be concluded that for both DMPC and DMPG the helix is oriented with the molecular axis preferentially parallel to the incident light beam. The calculated angle obtained in the presence of DMPC of about 30° with respect to the incident light could correspond to (1) the situation where all helices are aligned with this angle with respect to the normal of the quartz plate, (2) the situation in which a population of 82% of the helices is aligned parallel to the light beam, whereas the remaining percentage is distributed isotropically, or (3) a combination of these two extremes. An angle of 41.6°, as found for DMPG, could be interpreted as if 61% of the helices are aligned exactly parallel to the incident light. Analysis of the spectra shows that, in the presence of both DMPC and DMPG at low hydration, the percentage of β-structures is increased at the expense of the α-helix as compared to the solution sample (Table 2). Also, for melittin associated to DOPC, DOPG, and DOPS, changes in the secondary structure of the protein are induced by the preparation of the lipid film, as revealed upon analysis of the CD spectra (Table 2). The CD spectra in the presence of these unsaturated lipids in the low hydration state (Figure 2C–E) show the opposite effect upon orienting the samples compared to the saturated lipids: an increased absolute intensity around 207 nm and a decreased intensity around 228 nm can be observed, suggestive of a preferred orientation of the helices perpendicular to the incident light beam. This suggested orientation is apparent from the orientation parameters deduced from the analysis of the spectra (Table 3), which are all found to be larger than 2. The calculated angles of 64.9° (DOPC), 63.5° (DOPG), and 64.0° (DOPS) could correspond to populations of helices aligned perpendicular to the incident light beam (i.e., flat on the membrane) of 56%, 50%, and 52%, respectively.

When the film is hydrated via equilibration with controlled atmospheric humidity, the number of water molecules per lipid is increased to 18–24 for all lipids (Table 3). In the absence of melittin this number was found to be lower: 14–18 water molecules per lipid (data not shown).

Hydration of the samples results in changed CD spectra, as shown in Figure 2 (spectra b). From the analysis of these spectra, it can be found that the amount of β-strand is greatly reduced and the α-helicity is increased at high hydration (except for DMPG) compared to the low hydration state. The orientation parameters obtained for the systems at high hydration (Table 3) show that barely any preferred orientation of the helices is observed for DMPC, DMPG, and DOPG (Table 3). For DOPC, the preferred perpendicular orientation is more extreme compared to the low hydration state (66% of the helices would be aligned perpendicular to the light beam). In the presence of DOPS, melittin shows the most unexpected behavior: whereas at low hydration the helices were preferentially aligned perpendicular to the light, at higher hydration the protein becomes oriented more parallel (72% of the helices are oriented parallel).

Upon dehydration of the systems in the high hydration state by reduced pressure, CD spectra could be obtained that were identical to those of the corresponding samples at low hydration. Also, upon subsequent hydration, the spectral changes were found to be reversible for all samples (data not shown).

When melittin was spread on the quartz plates in the absence of phospholipids, no α-helix induction could be observed in the CD spectrum (data not shown). Analysis of the spectrum shows that melittin has, under these conditions, 24% β-strand and 72% random coil, irrespective of the hydration state.

Infrared Spectroscopy of Melittin. As an independent control to check the preferred orientation of the helices, we performed (ATR-FT)IR measurements on samples identical to those used for the CD measurements, but spread now on germanium crystals. This technique also allows us to obtain information on the orientation of the phospholipids relative to the crystal. In Figure 3, IR difference spectra are shown for melittin associated to DMPC and DOPC. These difference spectra are obtained by subtracting the 0° polarization from the 90° polarization spectrum, normalized to each other by zeroing the net integral of the intensities of the ester C=O stretching bands of the sn-1 and sn-2 chains in the 1710–

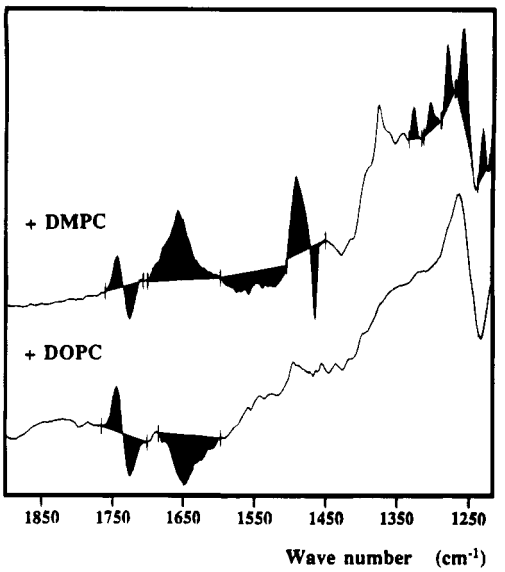

FIGURE 3: IR difference spectra (90° − 0° polarization) of melittin in the presence of DMPC (upper trace) and DOPC (lower trace).

Table 4: Dichroic Ratios of Various Bands in the IR Spectra for Melittin with Various Systems

	R^{iso}	R^{AmI}	$R^{AmI} - R^{iso}$	angle (deg)
DMPC	1.34	2.47	+1.13	25
DMPG	1.32	2.41	+1.09	25
DOPC	1.53	1.45	−0.08	60
DOPG	1.53	1.36	−0.17	65
DOPS	1.63	1.55	−0.08	58

1760 cm^{-1} region of the difference spectrum. The rationale behind this lies in the fact that both the sn-1 and sn-2 carbonyl groups are found to make angles with respect to the bilayer normal that are close to the value for an isotropic orientation (Hübner & Mantsch, 1991; Smith et al., 1992), and their IR intensities are therefore expected to be independent of the polarization. These normalization factors (R^{iso}) are given in the first column of Table 4. In agreement with the literature for DMPG and DMPC, systems R^{iso} is found to be 1.3−1.4 (Goormaghtigh & Ruysschaert, 1990). Higher R^{iso} values are obtained for the unsaturated lipids.

The orientation of the phospholipid acyl chains parallel to the normal of the plate is demonstrated by the progression of the positive bands in the difference spectrum of DMPC (Figure 3, upper trace) in the region 1350−1250 cm^{-1}, arising from the γ_w vibrations of the CH_2 groups. This progression appears only when the acyl chains are in the *all-trans* conformation (Fringeli & Günthard, 1981), indicating that under these conditions DMPC was in the gel state. This progression could also be observed for DMPG (data not shown). The positive peak at 1230 cm^{-1} (phosphate) and the negative band at 1467 cm^{-1} (acyl chain) demonstrate the orientation of DMPC with the bilayer normal parallel to the plate normal (Goormaghtigh & Ruysschaert, 1990). Interestingly, the choline headgroup is also oriented parallel with respect to the germanium crystal, as demonstrated by the positive peak at 1493 cm^{-1} (Mendelsohn & Mantsch, 1986). The absence of the progression of the CH_2 γ_w vibrations for DOPC (Figure 3, lower trace) indicates high mobility for the acyl chains (and for DOPG and DOPS, data not shown), suggestive of a fluid crystalline state for these lipids. As a consequence, no information on the orientation of the lipids with respect to the crystal is available for these systems.

In the amide I region (1700−1600 cm^{-1}), a positive peak can be observed in the difference spectrum of melittin in the presence of DMPC (Figure 3). The main contribution in the amide I region is the C=O stretching vibration, which is oriented perpendicular to the helix axis, and therefore a net positive intensity can be interpreted as the helix axis having a preferred orientation parallel to the crystal normal. The net negative intensity in the amide II region (1600−1500 cm^{-1}) confirms the observed orientation, because the transition moments of the amide I and amide II bands are presumed to be perpendicular (Tsuboi, 1962). In the presence of DOPC, the amide I band in the difference spectrum is clearly opposite in sign compared to that found in the presence of DMPC (Figure 3). In the second column of Table 4, the dichroic ratios (i.e., the ratios obtained for the intensities at two polarizations) for amide I (R^{AmI}) are denoted for the various systems.

In the third column of Table 4, the differences between R^{iso} and R^{AmI} for the various systems are presented. The sign of this difference can be used as a qualitative measure for the orientation of the protein with respect to the lipids. Positive values are found for DMPC and DMPG, indicating that the molecular axis of melittin is preferentially oriented parallel to the germanium crystal normal. For all unsaturated lipids negative values are obtained, showing that in these lipids melittin is oriented perpendicular to the normal of the plate. The degree of orientation can be estimated by translating $R^{iso} - R^{AmI}$ to the approximate angles of the polypeptide with respect to the lipids, as described in detail by Goormaghtigh and Ruysschaert (1990). Angles of 25° are found for DMPC and DMPG (Table 4, fourth column), which is slightly smaller than those obtained from CD for the low hydration state. For DOPC, DOPS, and DOPG, angles of 58−65° are calculated from the IR data, which are comparable to the 63−65° obtained by CD for these lipids at low hydration. It should be noted that we assumed that the bilayer normal is perpendicular to the germanium crystal. Deviations from this ideal situation would result in larger angles for the saturated lipids and smaller values for the unsaturated lipids.

Other Oriented Protein−Lipid Complexes. In order to test the general applicability of the approach to determining the orientation of secondary structure elements as described in this work, we also investigated some other protein−lipid films using CD.

Bacteriorhodopsin mainly consists of seven α-helical membrane-spanning domains, which all have an approximately parallel orientation with respect to the membrane normal (Henderson et al., 1990). Compared to the CD spectrum of patches of native purple membrane in solution (Figure 4A, dashed line), the oriented sample shows, as expected, a reduced intensity at 207 nm (solid line), suggestive of a preferred parallel orientation of the helices with respect to the incident light. The fits of the solution and oriented spectrum, however, reveal large RMS values of 48 and 37, respectively (data not shown). This is attributed to absorption flattening. This phenomenon has been described in the literature for bacteriorhodopsin in solution, arising from an inhomogeneous distribution of

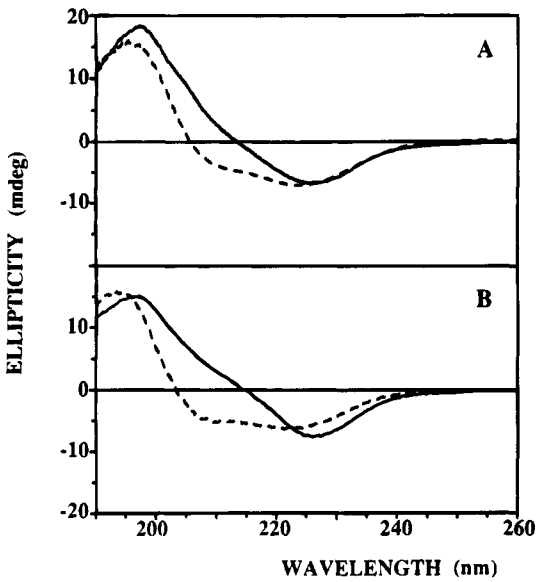

FIGURE 4: CD spectra of patches of native purple membrane containing bacteriorhodopsin in solution (dashed lines) and oriented on quartz plates (solid lines) at low hydration before (A) and after (B) sonication for 15 min in a sonicator water bath.

chromophores in the sample due to the existence of large hexagonal aggregates of proteins in the patches of native purple membrane (Henderson, 1975), and it is most apparent from the reduced intensity around 210 nm. As a consequence, it has not been possible to obtain proper fits of the spectra of this sample in solution (Wallace & Teeters, 1987; Swords & Wallace, 1993). Apparently this absorption flattening also perturbs the CD in films.

As described by Mao and Wallace (1984), absorption flattening can be reduced in the CD spectra by reduction of the particle size. The results of sonication of patches of native purple membrane are shown in Figure 4B, where a clear increased absolute intensity around 210 nm can be observed in the solution spectrum. Also, the CD spectrum of the oriented sample is changed by sonication. Analysis of these spectra reveals fits with RMS values of 18 and 13 ($\xi = 0.03$), respectively (data not shown). Comparison of the absorbance spectra of both the sonicated and nonsonicated samples in the 190−240 nm region shows that, in the latter case, the intensity in the 200−220 nm region is reduced (data not shown), supporting the suggested absorption flattening apparent in this region.

Similar spectral distortions due to absorption flattening were found for the membrane-spanning helical peptide alamethicin in the presence of DOPS vesicles (data not shown). It has been reported in the literature that alamethicin is able to form aggregates in membranes (Schwarz et al., 1986; Rizzo et al., 1987). From the CD spectra of the oriented film, it was suggested that, upon hydration of the film, the preferred orientation of the helix became parallel to the incident light, in a way similar to that described by Wu and co-workers for films of diphytanoylphosphatidylcholine (DPhPC) and alamethicin. After extensive sonication using a microtip (30 min, 30 W, 0°C), the spectra of both the solution and the oriented system could be fit with RMS values smaller than 8 (data not shown). Incubation of the sonicated sample in solution for 1 h at room temperature again resulted in considerable distortion of the spectrum by absorption flattening.

No such artifacts were found in the case of films of various fragments of apocytochrome *c* complexed to different membranes [see the accompanying paper: de Jongh et al. (1994)]. As was described for the melittin−lipid samples in this work, the obtained spectra could be well analyzed with relatively low RMS values for the fits, strongly suggesting the absence of absorption flattening effects from the spectra. These examples indicate that our approach has a general applicability to the analysis of orientation of secondary structure elements in protein−lipid films.

DISCUSSION

In this work, we present a CD method to investigate the secondary structure and preferred orientation of membrane-associated polypeptides. We chose the strategy of deconvolution of the in literature described reference spectra for the four different secondary structure types in terms of Gaussian absorption bands; parameters describing these bands for each secondary structure are presented in Table 1. After assignment of these bands to the direction of absorption within the molecular axis system for the secondary structure elements, we are able to calculate the secondary structure and the preferred orientation of polypeptides in oriented samples. Our work differs from the two previously published approaches (Vogel, 1987; Wu et al., 1990) to deducing spectra related to the direction of absorption within the molecular axis system by the fact that we employ commonly used reference spectra instead of experimentally recorded spectra of the protein of interest. We therefore do not need to make assumptions on the secondary structure to deduce the spectra related to the direction of absorption and to analyze the experimental data. The advantage of this is illustrated clearly in this work, in that the secondary structure of melittin is strongly affected upon preparation of the protein−lipid film. For all systems investigated, dehydration of the lipids causes an increase in β-strand formation in melittin. Using this approach, we also can detect possible errors due to absorption flattening or side-chain contributions in the spectra to analyze experimental data. However, it should be mentioned here that peptide-specific deviations, or environmentally induced shifts of the absorption maxima and intensities as described recently by Park and co-workers (1992) or Woolley and Wallace (1993), cannot be taken into account.

Despite the large number of reports in the literature, the orientation of the helix of melittin in membranes is still controversial. A reason for this might be the large variety of lipids used with different fatty acid compositions or the strong dependency of the orientation on the hydration state of the lipids, both demonstrated to be of importance in this work. For the α-helical part of melittin, a significant preferential orientation has been observed, depending on the phospholipid and the hydration state (Table 3). In general, it can be stated that the IR data agree very well with the CD results for the low hydration state (Table 4). At low hydration, melittin is preferentially oriented parallel to the acyl chains of the lipids in the presence of DMPC and DMPG (Table 3), both in the gel state (Figure 3). This is in agreement with earlier reports of Vogel et al. (1983) and comparable to the observations of melittin in the presence of DTPC (Vogel et al., 1983; Vogel, 1987) or DPPC (Brauner et al., 1987; Frey & Tamm, 1991) in the gel state. No clear headgroup specificity was observed for DMPC and

DMPG, in agreement with the data of Frey and Tamm (1991). At high hydration the preferred orientation of the α-helix of melittin is lost (Table 3), which correlates well with the situation described for DTPC where no preferred orientation of melittin was observed at high humidity in the gel state of the lipids (Vogel, 1987). In the presence of the unsaturated lipids DOPC, DOPG, and DOPS in the fluid crystalline phase (Figure 3), melittin preferentially orients perpendicular to the membrane normal of these lipids in a low hydration state (Table 3). Comparable behavior in the presence of DOPC and DMPC is also found for the helix of the amphiphilic carboxy-terminal fragment (residues 81-104) of apocytochrome *c* [see the accompanying paper: de Jongh et al. (1994)]. The dependency of the orientation of the helix of melittin on the nature of the lipid headgroup and hydration is remarkable, but at present not understood.

Our observation that the degree of hydration of the protein−lipid film is important implies that the use of a closed cell, as described in this work, is essential. Unfortunately, in the present setup it is not possible to tilt the sample in the plane of the light as described by Wu and co-workers (1990), which could serve as a test to determine the orientation of secondary structure at different angles of incident light. However, we believe that the CD method to determine the secondary structure and orientation of polypeptides as described in this work can be applied to a variety of oriented protein−lipid complexes.

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