Abstract  Circular dichroism (CD) spectroscopy is an essential tool for determining the conformation of proteins and peptides in membranes. It can be particularly useful for measuring the free energy of partitioning of peptides into lipid vesicles. The belief is broadly held that such CD measurements can only be made using sonicated small unilamellar vesicles (SUVs) because light scattering associated with extruded large unilamellar vesicles (LUVs) is unacceptably high. We have examined this issue using several experimental approaches in which a chiral object (i.e., peptide or protein) is placed both on the membrane and outside the membrane. We show that accurate CD spectra can be collected in the presence of LUVs. This is important because SUVs, unlike LUVs, are metastable and consequently unsuitable for equilibrium thermodynamic measurements. Our data reveal that undistorted CD spectra of peptides can be measured at wavelengths above 200 nm in the presence of up to 3 mM LUVs and above 215 nm in the presence of up to 7 mM LUVs. We introduce a simple way of characterizing the effect on CD spectra of light scattering and absorption arising from suspensions of vesicles of any diameter. Using melittin as an example, we show that CD spectroscopy can be used to determine the fractional helical content of peptides in LUVs and to measure their free energy of partitioning into LUVs.

Keywords  Large unilamellar vesicle · Small unilamellar vesicle · Peptide conformation · Secondary structure · Membrane binding

Introduction

Circular dichroism (CD) spectroscopy is the most widely used method in structural biology for determining the secondary structure of peptides and proteins. It has proven to be invaluable for studies of the binding and folding of membrane active peptides. With only a few exceptions (Wimley et al. 1998; Ladokhin and White 1999, 2001, 2004; Ladokhin et al. 1999), small unilamellar vesicles (mean diameter ~30 nm, SUVs) made by sonication (Huang 1969) are used nearly exclusively for such studies in order to minimize the effects of light scattering on the observed CD spectra. The difficulty with SUVs is that they are metastable (Schmidt et al. 1981; Wong et al. 1982) and produce anomalous peptide partitioning as a result of high vesicle curvature (Greenhut et al. 1986; Seelig and Ganz 1991; Plager and Nelsestuen 1994; Ladokhin et al. 2000). This is confirmed by high-sensitivity isothermal titration calorimetry, which reveals that binding enthalpies observed with SUVs are generally more exothermic than those observed with extruded large unilamellar vesicles (mean diameter ~100 nm, LUVs) (Seelig and Ganz 1991; Beschiaschvili and Seelig 1992; Gazzara et al. 1997; Wieprecht et al. 2000). SUVs are therefore not generally
suitable for thermodynamic measurements. In contrast, LUVs produced by extrusion (Enoch and Strittmatter 1979) are suitable for thermodynamic measurements because they are equilibrium structures. An important methodological question thus concerns the conditions under which accurate CD measurements can be carried out in the presence of LUVs.

We described earlier the procedures for making artifact-free fluorescence measurements (Ladokhin et al. 2000) using LUVs or SUVs. Here, we introduce a simple method for determining the conditions under which accurate CD spectra can be obtained for a given vesicle preparation, regardless of diameter. We demonstrate for LUVs that useful CD spectra of peptides can be obtained in the range of 215–260 nm in the presence of up to 7 mM LUVs. Using melittin as an example, we show that it is possible by means of CD spectroscopy to determine accurately the free energy of peptide partitioning ($\Delta G$) into LUVs as well as the helical fraction of the LUV-bound peptide.

Materials and Methods

Materials

1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids (Alabaster, AL) and melittin (sequencing grade) from Sigma (St. Louis, MO). The peptide Ac-Y(AEAAKA)$_4$F-NH$_2$ was a gift from Drs. Martin Scholtz (Texas A & M) and Robert Baldwin (Stanford). The buffer, a 10 mM potassium phosphate solution (pH 7.0), was used to reduce the UV absorbance in CD experiments.

Preparation of Vesicles

A defined amount of lipid in chloroform was dried first under nitrogen and then overnight under high vacuum. Typically, 1–2 ml of buffer (10 mM potassium phosphate solution, pH 7.0) was added to the lipid and the dispersion was extensively vortexed. For preparation of SUVs, the lipid dispersion was sonicated under nitrogen gas using a titanium tip ultrasonicator until the solution became transparent (Huang 1969). An ice-water bath was used to reduce further the possibility of lipid oxidation during sonication. Metal debris from the titanium tip was removed by centrifugation (Eppendorf table-top centrifuge, 25 min at 10,000 rev/min). LUVs of the lipids with an approximate diameter of 0.1 μm were formed by extrusion under nitrogen through Nucleopore polycarbonate membranes (10 times through two stacked 0.1-μm filters), using the method of Mayer et al. (1986).

Circular Dichroism

CD measurements were performed using a Jasco-720 spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan). Normally, 10–30 scans were recorded at ambient temperature ($\sim 25^\circ$C) between 190 and 260 nm, using a 1-mm optical path. Spectra were corrected for background scattering by subtracting a vesicle-only spectrum measured with an appropriate concentration of vesicles in buffer, without the peptide. Measured values of ellipticity ($\Theta$) were converted into the ellipticity per amino acid residue ($[\Theta]$). CD and absorbance were measured in the same cuvette in order to minimize errors in determination of molar ellipticity.

Calculated CD Spectra

Calculated CD spectra for melittin bound 100% to LUVs and SUVs were obtained from the spectra for 0 and 5 mM lipid concentrations. The fraction of melittin bound at 5 mM was estimated to be 70% for LUVs and 95% for SUVs, based upon the measured partitioning free energy, $\Delta G$. The contribution of the free peptide was subtracted (30% for LUVs, 5% for SUVs) from the spectra measured in the presence of 5 mM lipid in order to estimate the spectra for 100% binding. Nonlinear least-squares analysis was performed using the Origin 7.0 software package (OriginLab, Northampton, MA).

Absorbance Spectroscopy

UV absorbance was measured with a Cary 3E spectrophotometer (Varian Analytical Instruments, Sugar Land, TX). Molar concentrations of the peptides were determined using a molar extinction coefficient of $\varepsilon_{274\text{nm}} = 1,440$ M$^{-1}$ cm$^{-1}$ for tyrosine and $\varepsilon_{280\text{nm}} = 5,600$ M$^{-1}$ cm$^{-1}$ for tryptophan. For measurements of apparent absorbance of vesicle preparations caused by light scattering and by buffer absorbance, the Jasco-720 was used in order to assure self-consistent correction of CD spectra.

Results and Discussion

Most studies of peptide–bilayer interactions by CD spectroscopy involve experiments in which peptides or proteins are present in both bound and unbound forms. There are two potential artifacts in such measurements. For the unbound peptide, the scattering of light by vesicles can distort the UV absorbance of the chiral peptide. This is also true for peptides bound to the vesicles, but an additional distortion of the CD signal from the bound peptide might
exist because the bound peptide plus vesicle represent together a chiral scattering object. It is thus important to be sure that the CD spectrum of the bound peptide is not distorted by being part of a chiral scattering object.

Experiments with Peptides Outside the Membrane

SUVs are widely used because of their reduced light scatter in spectroscopic measurements. Figure 1 shows the dramatic increases in light scattering that accompany increasing concentrations of LUVs and SUVs from 0.25 to 7.0 mM as a function of wavelength. The optical density, which was recorded using the Jasco-720 spectropolarimeter, varies as $\frac{1}{k^4}$ down to 210 nm because of light scattering and then increases much more rapidly due to absorbance by the buffer. LUVs scattered more strongly than SUVs, as expected; but the scattering from SUVs was nevertheless significant.

The spectra of membrane-active peptides change as they are adsorbed into vesicles because binding generally induces secondary structure formation (Wimley et al. 1998; Ladokhin and White 1999). This complicates the determination of the effect of light scattering on the CD spectra of peptides in the presence of vesicles. A simple way to determine the effect of scattered light on spectra is to place the peptide and vesicles in separate cuvettes, as shown schematically in Fig. 2. For the data presented in Fig. 3, a fixed concentration of the peptide melittin was placed in a cuvette containing methanol (cell 1), which induced maximal secondary structure and yielded CD spectra similar to those of peptide bound to vesicles. Vesicle suspensions of increasing lipid concentration were placed in the other cuvette (cell 2). Although cell 2 is closer to the detector in

![Fig. 2 Schematic representation of the two-cuvette experimental scheme for studying the influence on CD spectra of peptides of light scattering caused by LUVs. Drawing is not to scale. A standard solution of melittin in methanol was placed in cell 1, while LUV suspensions of different concentrations were placed in cell 2. The results presented here did not depend on whether cell 1 or cell 2 was closer to the light source.](image)

Fig. 1 Optical densities as a function of wavelength for suspensions of LUVs (solid lines) and SUVs (dashed lines). Optical densities of POPC vesicles at lipid concentrations of 0.25, 3 and 7 mM were determined using a Jasco-720 spectropolarimeter (see text)

![Fig. 3 CD spectra of melittin in methanol (40 μM, cell 1) recorded in the two-cuvette experiment shown in Fig. 2. a Cell 2 containing SUV suspensions at concentrations up to 7 mM. Undistorted CD spectra of melittin with up to 7 mM SUVs can be obtained for the spectral region above 200 nm. b Cell 2 containing LUV suspensions at concentrations up to 7 mM. Undistorted CD spectra of melittin can be obtained with up to 5 mM LUVs for the spectral region above 200 nm and with up to 7 mM for the spectral region above 215 nm (see values of molar ellipticity in Table 1).](image)
The melittin in methanol and the vesicles in buffer are in separate cuvettes. The peptide (in buffer) and the LUVs are in different cuvettes. The peptide (which does not bind to vesicles) and the LUVs are in the same cuvette.

Table 1
Changes of molar ellipticity of Ac-Y(AEAAKA)4F-NH2 and melittin under different conditions

| POPC vesicle concentration | Ac-Y(AEAAKA)4F-NH2 (same cell)a | Ac-Y(AEAAKA)4F-NH2 (two cells)b | Melittin (methanol) SUVd | Melittin (methanol) LUVd |
|---------------------------|----------------------------------|----------------------------------|------------------------|------------------------|
| No lipid                  | Θ222b = -20,100                  | Θ222b = -20,300                  | Θ222b = -22,900        | Θ222b = -22,100        |
| +0.25 mM                  | -20,100                          | -20,400                          | -23,200                | -22,000                |
| +3 mM                     | -19,800                          | -20,100                          | -22,900                | -21,900                |
| +5 mM                     | -19,700                          | -20,100                          | -22,700                | -21,600                |
| +7 mM                     | -19,700                          | -19,900                          | -22,600                | -21,500                |

a The peptide (which does not bind to vesicles) and the LUVs are in the same cuvette
b Units: deg cm$^2$ dmol$^{-1}$. Errors of determination of Θ in the presence of lipids are estimated to be 10%
c The peptide (in buffer) and the LUVs are in different cuvettes
d The melittin in methanol and the vesicles in buffer are in separate cuvettes

Fig. 2, the results were the same when the cell positions were switched.

Figure 3 shows the CD spectrum of melittin in methanol, recorded from cell 1 while increasing the concentration of SUVs or LUVs in cell 2 (panels a and b, respectively). For a concentration of 7 mM SUVs, the CD spectra were affected only slightly by light scattering. With LUVs in cell 2, the CD spectra of melittin in methanol were very similar to those with SUVs with up to 5 mM lipid; the spectra coincided almost completely with those measured in the absence of lipid (see molar ellipticity values at 220 nm in Table 1). At 7 mM LUVs, only a slight decrease of the signal could be observed at wavelengths below 215 nm. The optical densities of samples in this wavelength region are quite high, meaning that this region should be discarded in accordance with general recommendations for CD samples (optical density should not exceed 1 unit). However, by reducing the optical path, the entire spectral region can be used, which is necessary for deconvolution of a CD spectrum into components associated with various elements of secondary structure (data not shown); but if one is interested in detecting only changes in helical content at 222 nm, the spectral region above 215 nm is sufficient.

How can one be certain that this two-cuvette approach provides a valid means for assessing the effect of light scattering on the CD spectra? This question can be answered only by recording spectra from peptides that are in the same cuvette as the vesicles. However, this cannot be done using a membrane-active peptide, such as melittin, because of the induction of secondary structure caused by binding to vesicles. We circumvented this problem by using the synthetic peptide Ac-Y(AEAAKA)4F-NH2 (Scholtz et al. 1991), which is strongly z-helical in buffer but does not bind to vesicle membranes. The peptide was found by Scholtz et al. (1991) to be monomolecular and ~60% z-helical in buffer. The blocking groups on the termini eliminate unfavorable charge–helix dipole interactions, and the salt-bridged side-chain interactions are optimized by the i, i + 3 spacing of the Glu and Lys residues. Using the experiment-based whole-residue interfacial hydrophobicity scale of Wimley and White (1996), the free energy of partitioning in POPC is estimated to be +4 kcal mol$^{-1}$, meaning that the peptide cannot partition significantly into neutral POPC vesicles.

Figure 4a shows the CD spectra of Ac-Y(AEAAKA)4F-NH2 in buffer containing LUVs at concentrations of up to 7 mM. The value of the molar ellipticity of the peptide at 25°C was found to be −20(±0.5) × 10$^5$ deg cm$^2$ dmol$^{-1}$, which is in agreement with the value measured by Scholtz et al. (1991). The spectra have features that are characteristic of a z-helical structure: minima at 222 and 208 nm and a maximum at around 190 nm. The CD spectra of the peptide shown in Fig. 4a are practically indistinguishable (see molar ellipticity values in Table 1). Figure 4b shows the CD spectra obtained with peptide in buffer in cell 1 and increasing concentrations of LUVs in cell 2. The spectra obtained in this two-cuvette experiment are almost identical to the spectra obtained in Fig. 4a. This validates the two-cuvette experiment and confirms the assumption that Ac-Y(AEAAKA)4F-NH2 does not bind to POPC vesicles.

Experiments with Peptides and Proteins Bound to the Membrane

In the previous section we demonstrated that unpolarized scattering from LUVs does not affect CD measurements of the peptides that reside outside the scattering object. In order to demonstrate that we can accurately measure CD of membrane-bound proteins and peptides, we need to demonstrate the lack of distortion due to differential scattering between the left- and right-polarized light by placing the...
chiral object (polypeptide chain) into the scattering object (vesicle). This is not an easy experiment to construct as most peptides undergo conformational change upon interaction with the lipid bilayer due to partitioning–folding coupling (Wimley et al. 1998; Ladokhin and White 1999; White et al. 1998). Thus, we used a large protein, Annexin B12, which has been shown to associate interfacially with the membranes in the presence of Ca\(^{2+}\) without rearrangements of the secondary structure (Isas et al. 2003). As expected, our measurements indicate that aqueous and LUV-associated Annexin B12 have essentially the same CD spectrum (Fig. 5), thus confirming the absence of specific artifacts originating from the placement of the chiral object on the scattering vesicle.

To further demonstrate the use of LUVs for determining structural and thermodynamic properties of peptide binding in a real experiment, we used melittin, which changes its conformation upon partitioning into lipid bilayers (Ladokhin and White 1999; Vogel 1981; Kuchinka and Seelig 1989; Beschiaschvili and Baeuerle 1991). Melittin in solution has a random coil structure, whereas upon partitioning into lipid membranes it adopts a characteristic \(\alpha\)-helix ellipticity at 222 nm that shows a progressive increase with increasing lipid concentration for both LUVs and SUVs (Fig. 6). These results show that changes in the CD spectra that depend on lipid concentration can be used to determine quantitatively the binding isotherm and, consequently, the free energy of partitioning. Using the formula \(\Delta G = -RT \ln K_x\), where \(K_x\) is the partition coefficient (White et al. 1998), the data of Fig. 6 yield \(\Delta G = -6.0 \pm 0.4 \text{ kcal mol}^{-1}\) for melittin partitioning into POPC LUVs and \(\Delta G = -7.2 \pm 0.3 \text{ kcal mol}^{-1}\) for SUVs. Previously, we reported a similar difference between SUV and LUV titration of melittin using intrinsic fluorescence (Ladokhin et al. 2000). The difference is not surprising because SUVs are nonequilibrium systems known to produce artificially high binding affinity (as discussed in Ladokhin et al. 2000).

An important question is whether the spectra of melittin bound to SUVs and LUVs are inherently the same. Comparisons are complicated for several reasons: (1) the light scattering of LUVs at high lipid concentrations, (2) possible structural differences between SUVs and LUVs that might affect the structure of bound melittin and (3) differences in binding affinity (which we know from Fig. 6). An approach to this problem is to reconstruct spectra for
100% binding to LUVs and SUVs, using spectra obtained for <100% binding, as described in “Materials and Methods”. Figure 7 shows the calculated CD spectra of melittin for 100% binding to SUVs and LUVs. The value of the molar ellipticity at 222 nm \( \Theta_{222} \) is estimated to be \(-23.7 \times 10^3\) deg cm\(^2\) dmol\(^{-1}\), which is the same for both spectra and corresponds to values reported earlier (Vogel 1981). The spectra coincide down to 217 nm, with only slight differences at shorter wavelengths, which could be due to the different CD signatures of the two systems. However, the overall agreement of the two spectra is good enough to assume that the structures of melittin are very similar in the two systems.

The mean residue ellipticity of \(-23.7 \times 10^3\) deg cm\(^2\) dmol\(^{-1}\) at 222 nm agrees with results obtained in earlier studies with SUVs and membrane-mimicking solvents (Ladokhin and White 1999; Vogel 1981; Kuchinka and Seelig 1989; Beschiaschvili and Baeuerle 1991). The fractional helical content \( f_H \) of melittin can be estimated using the formula

\[
f_H = \frac{\Theta - \Theta_{RC}}{\Theta_H - \Theta_{RC}}
\]

where \( \Theta \) is the observed ellipticity and \( \Theta_{RC} \) and \( \Theta_H \) are the limiting values for a completely random coil and a completely helical conformation, respectively. Although this formula is simple and well accepted, there is a certain ambiguity in the result, due to the uncertainty in prediction of what the actual values for \( \Theta_{RC} \) and \( \Theta_H \) should be (Greenfield and Fasman 1969; Luo and Baldwin 1997; Rohl and Baldwin 1997; Shalongo and Stellwagen 1997). Here, we used the following values (222 nm): \( \Theta_{RC} = -1.5 \times 10^3\) deg cm\(^2\) dmol\(^{-1}\) and \( \Theta_H = -33.4 \times 10^3\) deg cm\(^2\) dmol\(^{-1}\). The \( \Theta_H \) number is calculated at 25°C for a peptide the size of melittin according to Luo and Baldwin (1997). The CD spectra of melittin bound to POPC LUVs (25°C) reveal a conformation with \sim 70% \( \alpha \)-helical content, corresponding to 18–19 amino acids, which is in good agreement with results obtained in various membrane and membrane-mimicking systems by different methods (Vogel 1981; Lam et al. 2001; Brown et al. 1982).

Our results demonstrate the ease with which LUVs can be used in studies of peptide–bilayer interactions. As for fluorescence studies of peptide partitioning into lipid bilayers (Ladokhin et al. 2000), there is no apparent need to use SUVs in preference to LUVs for optical reasons. Indeed, because they are free of curvature-stress and are equilibrium structures (a necessary condition for thermodynamic measurements), LUVs should be used in preference to SUVs. LUVs are perfectly suitable for both fluorescence and CD measurements that require lipid bilayers. We can thus think of no compelling reason for using SUVs in studies of peptide–bilayer interactions.

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