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Biochimica et Biophysica Acta 1467 (2000) 124–130

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Oriented circular dichroism of a class A amphipathic helix in aligned phospholipid multilayers

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Received 11 January 2000; accepted 28 March 2000

Abstract

The effect of lipid phase state on the orientation and conformation of a class A α-helical peptide on aligned lipid multilayers was examined using oriented circular dichroism spectroscopy. A comparison of oriented spectra in aligned peptide–lipid multilayers with CD spectra of unaligned peptide–lipid vesicle complexes is consistent with a preferential alignment of helices parallel to the membrane surface at temperatures above and below the main acyl-chain melting transition temperature of the phospholipid. Changes are observed in the oriented CD spectra with lipid phase state which are attributed to a subtle conformational change of the peptide on the lipid surface. The results are compared with available experimental data on membrane-active lytic and antimicrobial helical peptides.

Keywords: Amphipathic helix; Lipid–protein interaction; Circular dichroism spectroscopy; Helix orientation; Order parameter

1. Introduction

Approximately 30–40% of the proteins coded in the human genome are associated with membranes. Their membrane domains are made up of transmembrane α-helices, amphipathic α-helices, or occasionally antiparallel β-strands. The sequence characteristics of these conformational motifs, and the development of algorithms for their prediction, have been subjects of intense study. Experimentally, phospholipid monolayer and bilayer systems have frequently been used as model systems to study the interaction of small peptides with lipid surfaces. A fundamental question that arises in such studies concerns the orientation of the α-helix - whether it lies parallel to the membrane surface as in the case of an amphipathic helix, or normal to the membrane surface as in the case of a transmembrane α-helix. Experimental approaches to this question should be capable of providing an unambiguous answer, and should also be capable of examining cases where the peptide undergoes a change in orientation in response to changes in solution conditions, the phase state of the phospholipid bilayer, or a change in membrane charge or polarisation. In this connection, 'fluorescence footprinting', in which a tryptophan residue is moved progressively along a peptide sequence [1–4] is a useful, but somewhat cumbersome technique. Circular dichroism spectroscopy of helical peptides bound to oriented planar phospholipid multilayers is somewhat simpler in its application and

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0005-2736/00/$ – see front matter
PII: S0005-2736(00)00208-X

can provide a direct measure of the orientational distribution of helices with respect to the incident light beam. The technique is particularly useful for tracking changes in peptide orientation which can occur in response to changes in peptide surface density, bilayer hydration, acyl-chain saturation, or lipid phase state [5–8].

The present work was undertaken to verify the orientation of an amphipathic helix on a phospholipid bilayer surface and to examine the effect of the phase state of the lipid on the orientational distribution and conformation of the peptide. The peptide under investigation (18A, H_2N-DWLKAFYDK-VAEKLKEAF-COOH) has been studied previously as a putative lipid-binding peptide mimetic of the amphipathic helical regions in apolipoprotein A-I. The peptide is referred to as a class A amphipathic helix because when represented as a helical wheel, the positively charged residues are clustered near the peptide polar/non-polar interface, the negatively charged residues are near the centre of the peptide polar face, and the hydrophobic and hydrophilic amino acids are distributed on opposing faces along the helical axis. Oriented phospholipid multilayers, formed from a mixture of lipid and peptide, were examined using oriented circular dichroism spectroscopy and used to determine the helix orientation above and below the crystalline (L_β)–liquid-crystalline (L_α) phase transition of hydrated dimyristoylphosphatidylcholine (DMPC). The results are compared with available experimental data on class A helices and other membrane associating peptides.

2. Materials and methods

DMPC was purchased from Sigma (St. Louis, USA) and used without further purification. The peptide 18A (DWLKAFYDKVAEKLKEAF) was synthesised using Fmoc chemistry and purified by HPLC as described previously [4]. Peptide–DMPC complexes were prepared by mixing appropriate amounts of methanol solutions of peptide and DMPC and applying the solution dropwise to the window of a quartz cuvette and removing the methanol under vacuum. The lipid:peptide mole ratio was 20:1. The samples were oriented using the single-substrate solvent evaporation method as described by Heller et al. [9]. To hydrate the sample, a drop of 1 M aqueous NaCl was added to the bottom of the cuvette which was then sealed [6]. Melittin-multilayer complexes prepared, aligned, and hydrated in this manner had negligible spectral artefacts as judged by comparison with the published oriented circular dichroism spectra (data not shown) [8]. Unoriented peptide–phospholipid multilayers were prepared by two methods. In the first, a drop of ethanol was added to the aligned multilayers and allowed to evaporate [10,11]. The second method involved the use of sonicated suspensions of aqueous peptide–vesicle complexes (lipid:peptide ratio 200:1, 0.15 M NaCl, 10 mM Tris buffer, pH 7.4) to provide an isotropically averaged reference to compare with the ethanol treated film spectrum. A high lipid-to-peptide ratio was used to ensure that all the peptide was bound to the vesicles (as determined by fluorescence titration experiments). The high turbidity of these samples prevented the measurement of ellipticities below 203 nm, and thus only a semi-quantitative comparison of the two methods is possible. Another check of the validity of using the ethanol treatment to produce isotropically averaged CD spectrum was made by comparison of the mean residue ellipticity values near 210 and 222 nm of the ethanol-treated sample with those reported for peptide–DMPC discoidal bilayer complexes [12]. The mean residue ellipticities reported for DMPC–peptide discoidal complexes at 210 nm ($-15\,500$ deg cm^2 dmol^{-1}) and 222 nm ($-14\,300$ deg cm^2 dmol^{-1}) agree within 7% with the corresponding values for the ethanol treated DMPC–peptide multilayer sample of ($-16\,500$ deg cm^2 dmol^{-1} and $-15\,100$ deg cm^2 dmol^{-1}, respectively).

Circular dichroism spectra were recorded with the light beam at normal incidence to the quartz cuvette using an Aviv Model 62DS spectrometer with spectral widths of 2 nm, an integration time of 4 s per point, and a step size of 1 nm. The sample temperature was controlled electronically with a Peltier device. The raw CD spectra of 18A were converted to mean residue ellipticity units (deg cm^2 dmol^{-1}). Peptide concentrations were measured with a Cary 5 UV-vis spectrophotometer with spectral band widths and integration times as for the CD measurements and an extinction coefficient of 6970 M^{-1} cm^{-1} at 280 nm.

The rotational strength of the 207 nm α-helix band in the CD spectra was estimated from the area under the resolved Gaussian component near 207 nm. A single spectrum could be resolved into Gaussian components using a non-linear least squares fitting program (SigmaPlot, Jandel Scientific). Such a decomposition was not unique. Therefore, the wavelength maxima and bandwidths (widths at half peak height) were fixed to the expected values for polypeptides possessing both α-helix and β-sheet [13] and the amplitudes of the component Gaussian spectra were allowed to vary. The quality of the fit was assessed by the value of the global reduced χ^2, and by inspection of the residuals.

For an axially symmetric molecule with an axially symmetric distribution of the molecular director around the membrane normal, characterised by an angle θ, an orientational order parameter S ($=\langle 3\cos^2\theta\text{-}1\rangle/2$) describes the space and time-averaged fluctuations of the molecular director around the membrane normal. A quantitative estimate of the helix orientational order parameter, S_h, with respect to the optical propagation axis, is given by the ratio of the rotational strength of the resolved 207 nm transition in the aligned bilayers (R_o) to that for the unaligned sample (R_d) [13].

$$\langle \sin^2\theta \rangle = 2R_o/3R_d \quad (1)$$

$$S_h = (1-(R_0/R_d)) \quad (2)$$

The helix orientational order parameter is particularly useful for distinguishing between transmembrane and surface helices [5,6,8,13]. The sign of S_h is positive if the helix is aligned parallel to the optical propagation axis and transmembrane, and negative if the helix is perpendicular to the optical propagation axis and parallel to the lipid surface. The proportionality between the rotational strength of the 207 nm band and the angular distribution given in Eq. 1 has been proved [14].

A second method of assessing the orientation of the peptide, which does not require spectral deconvolution, was made by analysis of the CD difference spectrum, obtained by subtraction of the unaligned peptide-multilayer spectrum from that of the aligned multilayer sample spectrum. The rationale behind this method is that only structures with CD transitions that are ordered with respect to the membrane plane carry rotational strength in the CD difference spectrum. This eliminates CD transitions from disordered or unstructured regions. The order parameter was determined from the difference CD spectrum between the aligned multilayer sample ($[\theta]^\circ$) and the unaligned sample ($[\theta]^v$) [6].

$$S_h = S^{ref} \times [f_{ref}/f_h] \times ([\theta]^v - [\theta]^\circ)/([\theta]^v - [\theta]^\circ)^{ref} \quad (3)$$

where f_h is the fractional helicity of the peptide. The published CD data from melittin was used to give estimates for the reference helicity ($f_{ref} = 0.74$), reference order parameter ($S^{ref} = 0.62$), and reference CD difference spectrum ($([\theta]^v - [\theta]^\circ)^{ref} = 1.9 \times 10^4$ deg cm^2 dmol^{-1} at 210 nm) since both its helicy and order parameter in membranes are known independently from infrared spectroscopy measurements [6]. Eq. 3 assumes that non-helical segments have no preferred orientation with respect to the membrane plane. The ellipticity values at 210 nm were deemed to be most reliable for the order parameter calculation since the polarisation of this band is parallel to the helix axis.

3. Results and discussion

The far-ultraviolet CD spectrum of the class A peptide 18A in unaligned DMPC multilayers in the liquid-crystalline (L_α) phase (35°C) is shown in Fig. 1 (*square symbols*). The spectrum is characteristic of a peptide with a significant α-helical content, with minima at ca. 210 and ca. 220 nm, and a maximum near 195 nm. The solution CD spectrum of 18A–DMPC vesicle complexes is shown for comparison (Fig. 1, diamond symbols) and is similar in amplitude (to within 10%) in the region 215–250 nm, but undergoes an increase in ellipticity near 210 nm compared with the ethanol-treated 18A–DMPC multilayer spectrum. Similar effects have been noted when comparing ethanol-treated film and membrane suspension CD spectra of bacteriorhodopsin and have been attributed to differences in light scattering between membrane suspensions and membrane films due to the greater refractive index mismatch in the former samples [10,11]. The corresponding CD spectrum of 18A in aligned multilayers in the liquid-crystalline phase (35°C) is also shown in Fig. 1 (circles), whereupon it is noted that the ellipticity at 210 and 220 nm be-

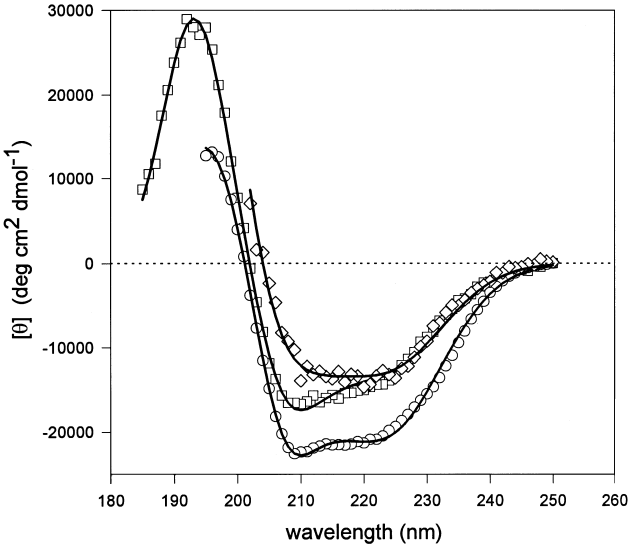

Fig. 1. CD spectra of 18A in non-aligned (squares) and aligned (circles) multilayers of DMPC at a molar lipid/peptide ratio of 20:1 at 35°C. The continuous solid lines are the fits to a sum of four Gaussian components (Table 1). CD spectrum of 18A–DMPC vesicle complexes at a molar lipid/peptide ratio of 200:1 at 35°C (diamonds). The solid line has no theoretical significance.

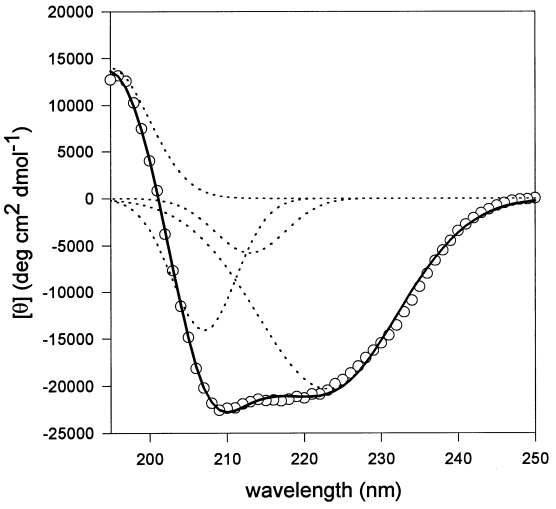

Fig. 2. CD spectrum of 18A in aligned (circles) multilayers of DMPC at a molar lipid/peptide ratio of 20:1 at 35°C. The resultant fit to the sum of four Gaussian components is indicated by the solid line and the Gaussian components by the dotted lines.

comes more negative than for both the unoriented multilayer sample and the vesicle suspension.

The 210- and the 220-nm bands are assigned to the π–π* transition and the *n*–π* transition of the α-helix whose transition moments are polarised parallel to the helix axis [14]. The decreased ellipticity of these bands in the aligned film as compared to the unaligned samples is consistent with a helix oriented perpendicular to the light propagation direction and parallel to the membrane surface. To more precisely define the contribution of the 210-nm band to the CD spectrum, we resolved the spectrum into its various Gaussian components by globally fitting the spectra as described in Section 2. Gaussian deconvolution was not attempted for the 18A–DMPC vesicle suspension spectra because the poor signal-to-noise of the data at wavelengths below 200 nm. However, the unaligned 18A–DMPC multilayer film spectrum should provide an upper estimate of the contribution of the 210 nm helix band for the isotropically-averaged helices. An example of this deconvolution into four components is shown in Fig. 2 for the aligned 18A–DMPC sample in the liquid-crystalline phase.

The data in Table 1 show that all four resolved components are sensitive to the alignment of the multilayers and to the phase transition of the DMPC. However, we focus on the 210-nm transition because its main contributor is the π–π* transition of

Table 1
Band parameters from the Gaussian deconvolution of 18A CD spectra assuming four Gaussian components

Fixed parameters		Floated amplitudes, (deg cm^2 dmol^{-1})		
λ^a (nm)	Δ^b (nm)	$[\theta]^{unaligned}$	$[\theta]^{aligned-gel}$	$[\theta]^{aligned-fluid}$
195.0[c]	7.0	+29 200	+12 200	+14 070
207.0	5.8	−10 700	−12 600	−14 147
213.0	6.4	−5 700	−3 220	−5 785
223.0	12.9	−12 900	−23 620	−20 340

[a]Wavelength maximum of Gaussian component spectrum.
[b]Half of the band width at e^{-1} times the peak height.
[c]Unaligned sample wavelength maximum was 193.3 nm.

the α-helix whose transition moment lies parallel to the helix axis [14]. The rotational strength and ellipticity of this transition provides the most accurate estimates of S_h, calculated according to Eqs. 2 and 3.

Fig. 3 shows the CD spectra for the aligned film 18A–lipid complex and the vesicle suspension 18A–lipid complex, above (35°C) and below (15°C) the phase transition temperature of DMPC. The corresponding values of the helix order parameter are listed in Table 2. First, there is good agreement between the order parameters calculated from deconvolved rotational strengths (Eq. 2) and the difference ellipticities at 210 nm (Eq. 3) when a literature value for the fractional helicity of 18A in 18A–DMPC discoidal bilayer complexes is used (0.49, [12]).

Second, we note that the values of S_h are negative, both above and below the transition temperature of the lipid, indicating that the helix lies predominantly parallel to the lipid surface, rather than in a transmembrane orientation. This conclusion is in accordance with the spectral features of the aligned samples as compared the unaligned samples which show that the helices are aligned perpendicular to the light propagation direction above and below the transition temperature of the lipid (Figs. 1 and 3). This result is in agreement with fluorescence footprinting experiments on tryptophan analogues of 18A bound to vesicles of egg phosphatidylcholine in the fluid phase [4]. According to these measurements, the tryptophan residues on the non-polar face of the peptide helix experience the non-polar lipid phase of the bilayer while tryptophan residues on the polar face of the peptide are exposed to the aqueous phase, as expected for a helix that is parallel to the lipid surface. Recently, White and co-workers [15] reported the results of X-ray diffraction experiments on fluid multilayers of dioleoylphosphocholine containing a N- and C-terminally blocked version of the 18A peptide and also conclude that the peptide is located predominantly at the lipid interface. Taken together, these experiments are consistent with a parallel orientation of the 18A peptide on the lipid surface of fluid membranes.

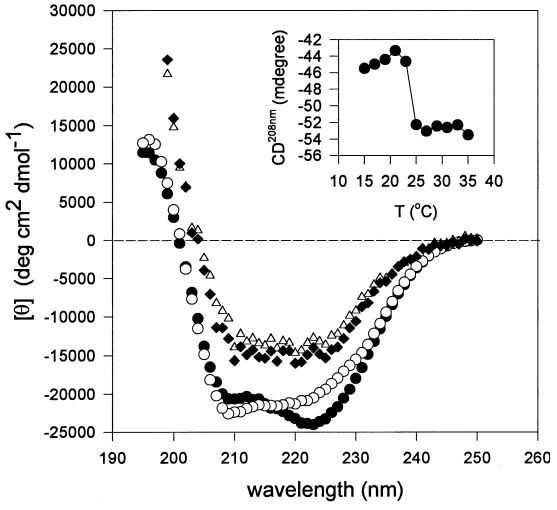

Fig. 3. Effect of lipid phase state on the CD spectrum of 18A in aligned DMPC multilayers (circles) and in suspensions of vesicle complexes (diamonds and squares). Open symbols, 35°C; filled symbols, 15°C. Inset: temperature dependence of θ_{208}, the ellipticity at 208 nm of the 18A peptide in DMPC aligned multilayers.

Table 2
Helix order parameters (S_h) of 18A in lipid multilayers of DMPC as determined by the rotational strengths ratio (Eq. 2) or difference ellipticity (Eq. 3)

Method[a]	15°C ($T < T_m$)	35°C ($T > T_m$)
Ratio[b]	−0.18	−0.32
Difference[c]	−0.20	−0.28

[a]Values determined using the ethanol-treated multilayer spectrum at 35°C as an isotropically averaged helix reference spectrum. The order parameters calculated at 15°C are therefore approximate.
[b]Determined using Eq. 2 after deconvolution into four Gaussian bands as shown in Table 1.
[c]Determined using Eq. 3.

Thirdly, conformational changes in the peptide resulting from changes in the lipid-phase state are observed by comparison of the peptide–vesicle CD spectra recorded at 15 and 35°C. The ellipticity of the gel-phase peptide–vesicle spectrum is decreased in magnitude in the 210–250-nm region relative to that of the fluid-phase spectrum possibly owing to a slightly increased helicity (ca 6%; estimated from the ellipticity decrease at 222 nm). Further support for this observation is based on examination of the oriented CD spectra. Cooling the sample from 35 to 15°C results in an increase in ellipticity of the 210-nm band and a concomitant decrease in ellipticity of the 220-nm band. These results cannot be reconciled with changes in peptide orientation alone since for a constant helix geometry, the 210- and 220-nm

bands are polarised in the same direction and would, therefore, be expected to undergo the same ellipticity changes in response to changes in orientation. This suggests that changing the lipid phase state has altered the conformation of the peptide. The order parameter derived for the peptide in the lipid gel-state assuming constant conformation between fluid and gel states is therefore approximate. However, the changes in the CD spectra with temperature are relatively minor and the main conclusion, that of a peptide aligned parallel to the lipid surface in the gel-phase lipid, is unaffected.

It is instructive to compare both the sign and the changes in the order parameter reported above with those for other peptides reported in the literature [6]. Both alamethicin and melittin have positive order parameters (0.88 and 0.62, respectively) in the liquid-crystalline phase of ditetradecylphosphatidylcholine, indicating that they are oriented predominantly perpendicular to the membrane surface. The order parameters for the peptides become negative (−0.03 and −0.29, respectively) upon cooling to below the phase transition temperature. Thus, helices which are parallel to the acyl-chains of the phospholipid in the fluid phase are ejected from the bilayer structure in the gel phase and adopt a more parallel orientation relative to the bilayer surface. We have observed similar behaviour for mellitin in oriented DMPC multilayers (results not shown). Thus, the orientation of these lytic/antimicrobial peptides is strongly coupled to the phase state of the phospholipid, but their behaviour is quite different from that of the class A 18A amphipathic helix. It would appear that 18A does not insert into the bilayer to adopt a transmembrane orientation, but remains on the lipid surface.

The orientation of some membrane-bound peptides is determined by their surface density. At high lipid-to-peptide ratios (150–200:1) in hydrated bilayers, alamethicin and magainin are oriented largely parallel to the lipid surface, but undergo a cooperative reorientational transition to a transmembrane state at a critical peptide surface density, corresponding to lipid-to-peptide ratios in the range 30:1 to 100:1, depending on the peptide and lipid [7,16]. The low lipid-to-peptide ratio (20:1) employed in the present work raises the question of whether the 18A peptides exist in an associated state on the membrane surface as has been suggested on the basis of binding isotherms [17]. If helix–helix interactions are present in the 18A–DMPC multilayer system, they do not give rise to peptide insertion, as observed for magainin and alamethicin, but would occur between helices on the membrane surface.

The order parameters of 18A–DMPC complex do not approach the limit −0.5 which describes a rigid helix oriented parallel to the membrane surface. Although CD spectra do not provide direct information on the dynamics of peptides on the membrane surface, it is reasonable to expect that in a hydrated bilayer, peptide helices undergo certain internal structural fluctuations surperimposed on more global orientational fluctuations within the membrane. Fluorescence anisotropy decay experiments and molecular dynamics simulations of peptides in bilayers confirm the possibility of flexible helical structures [18]. Moreover, the estimated thickness of bilayer interfaces, according to diffraction experiments, is large enough to easily accommodate a range of α-helix orientations on the lipid surface [19]. In this respect, the absolute values for the order parameters of 18A in DMPC could reflect dynamic and well as static disorder.

In summary, oriented CD measurements show that the 18A class A amphipathic helix adopts a predominantly parallel orientation on the lipid surface, but shows subtle changes in conformation with a change in the phase state of the lipid. Further work is in progress to understand factors which affect the structure and dynamics of surface-associated helices and linked helical domains in phospholipid membranes.

Acknowledgements

We thank Professor Horst Vogel for helpful discussions concerning the oriented circular dichroism technique. This research has been partially supported by the Australian Research Council. A.H.A.C. is a Postdoctoral Research Fellow of the Australian Research Council.

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