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Concentration dependent phase transition of a small protein in synthetic lipid multilayers

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CONCENTRATION DEPENDANT PHASE TRANSITION OF A SMALL PROTEIN IN SYNTHETIC LIPID MULTILAYERS

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE MASTER OF ARTS

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Abstract

Concentration Dependant Phase Transition of a Small Protein in Synthetic Lipid Multilayers

by

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We present evidence that magainin I, a 23 residue antibiotic peptide, undergoes a concentration dependent phase transition in synthetic lipid multilayers. Using oriented circular dichroism (OCD) we show that in low concentrations, magainin forms $\alpha$-helices lying parallel to the membrane surface. When the concentration becomes greater than a lipid specific value, the magainin undergoes a conformational change to a yet unidentified state. The state of the system at concentrations above the critical concentration shows that this transition cannot be explained by the micellar effect. Correlations between our results and liposome leakage experiments indicate that this transition is responsible for magainin's antibiotic action.
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1 Introduction

Ion channels are among the most important structures in any living cell. They are responsible for maintaining the potential gradient across the cell membrane and generating the neural signals in the brain as well as many other important functions. Channels are also of interest because of the variety of interesting properties they exhibit. For example, many channels are gated, opening and closing in response to an electrical potential shift or the presence of a molecular key of some sort. Also, most channels exhibit some form of ion selectivity, that is, they allow only certain ions to pass through the cell membrane.

While a great deal is understood about what ion channels do, very little is known about how they operate on a molecular level. Most of the ion channels used in living cells are quite large, each one consisting of hundreds or thousands of amino acids. Naturally, studying the behavior of such large proteins is extremely difficult. Since these studies must be conducted in an environment similar to the one in which they normally operate, i.e. - in a membrane, the available experimental techniques are quite limited.

To simplify the problem somewhat, we have been studying another class of channel forming molecules. These molecules are much smaller than natural ion channels, most containing less than 50 amino acids. A variety of molecules fall into this classification most of which are antibiotics, venoms, or fusion peptides. Despite their small size, these proteins still exhibit many of the features of the larger channels such as ion selectivity, gating properties, and the formation of individual channels. By understanding the operation of these small channel-forming peptides, we hope to gain insight into how all ionophores operate.

Even studying such relatively simple peptides in membranes has proven difficult. This is chiefly due to the difficulty of obtaining structural information on proteins when they are imbedded in membranes. X-ray crystallography cannot be
used because proteins generally don’t crystallize in membranes. NMR also fails to provide much information. Since the proteins aren’t free to tumble as they would be in solution, solid state NMR techniques must be used. Not only is solid state NMR quite difficult and time consuming, but it requires preparation of a large number of modified proteins with $^2\text{H}$, $^{13}\text{C}$, $^{14}\text{N}$, or $^{31}\text{P}$ substitutions at the atomic sites to be studied. It also requires fairly high protein concentrations which may (as we will show) affect the structure being studied. Even under ideal circumstances, only approximate distance and orientation measurements can be made with NMR.

Since these methods are of limited usefulness, we have taken several novel approaches to this problem. Our methods include lamellar x-ray diffraction, in-plane x-ray scattering, and oriented circular dichroism (OCD). All 3 methods use synthetic lipid multilayer samples with imbedded proteins (Fig.1).

The most difficult part of any of these experiments is the preparation of well-aligned samples which yield repeatable results. In general, lipids (the membrane constituents) form structures where the tails (hydrocarbon chains) are isolated from water and the heads are near water. Several of these structures are shown in Fig.1. If a large amount of water is present, vesicles or micelles are generally formed. If most of the water is removed, smectic liquid crystals (multilayers) can be formed; However, obtaining multilayers which are well aligned is a non-trivial process which will be discussed further in section 3.1.

Once we have samples in the correct multilayer form, we can use the 3 methods mentioned above. Lamellar x-ray diffraction can be used to find a one-dimensional electron density profile of the multilayers. In-plane scattering gives correlation distances between proteins and between lipid molecules in the plane of the membrane. Finally, OCD (the primary method used in this thesis) provides information about the orientation and conformation of the imbedded proteins.
Figure 1: Cross-sections of various lipid phases (not to scale). Individual lipid molecules vary in size but are generally 20-30Å long. Multilayer samples usually have several thousand bilayers at ~50Å per layer including water.

Unfortunately, the theory of OCD is still quite primitive. Useful interpretations of OCD spectra still rely primarily on comparisons with other, well established, experimental results rather than theoretical calculations. The structures responsible for the OCD of proteins are the asymmetry of the peptide bond connecting the amino acids in the protein, and neighboring dipole transitions which interact with each other. The asymmetry of the peptide bond causes one of the absorption peaks to shift and gives it a slight helicity. Interaction between
neighboring degenerate dipole transitions causes a split in their absorption band with differing helicities.

Conformational changes in proteins cause changes in the helicities and positions of these peaks. By recording the difference in absorption between left and right circularly polarized light passing through the sample over a range of wavelengths, a spectrum is recorded which can be decomposed in terms of well established spectra for each of the known protein conformations. This process yields only rough information on overall protein conformation, but it is an excellent way to monitor conditions which cause a change in protein conformation and orientation. It is also particularly useful for small peptides (<50 amino acids) which tend to have simple secondary structures.

This study concerns an antibiotic called magainin I. This is one of a family of proteins (magainins) originally discovered in the african clawed frog (Xenopus laevis)\textsuperscript{18}. While relatively harmless to ordinary human cells, these peptides have been shown to be effective antibiotics against a variety of gram-negative and gram-positive bacteria\textsuperscript{18,19} as well as various types of cancerous cells\textsuperscript{5,13}. Since magainins show such promise as therapeutic agents, much of the research being done is concentrated on understanding and improving its antibiotic properties by selective mutation and testing against a variety of cellular targets. Our research, however, is concentrated on development of a basic understanding of the molecular structure of magainin in its active form, and of the interaction between magainin molecules and between magainin and the cell membrane. Understanding at this level should provide insight into the mechanism of larger ion channels as well as provide useful information for those who are trying to improve magainin's therapeutic properties by modifying its structure.
2 Magainin

Two varieties of magainin have been isolated from frog skin and thousands of other synthetic varieties have been designed with small substitutions to the natural protein sequences\(^1,9^{,4}\). This investigation concerns magainin I, one of the 2 natural varieties of magainin, primarily because the natural varieties have been studied in more detail, and are available in fairly large quantities.

Magainin I is a 23 residue protein with the sequence: Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Gly-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Lys-Ser. Published research gives some good background for the problem: 1) It has been shown conclusively that magainin acts through the cell membrane directly rather than interacting with receptor proteins imbedded in the membrane\(^2,14\). This means magainin's cellular selectivity must be strictly a function of membrane composition and intercellular environment rather than which protein receptors are present in the cell. Since most drugs act by interacting in some way with receptors, this behavior is considered quite unusual. 2) In polar solvents, such as water, magainin adopts a random coil conformation, while in nonpolar solvents, such as TFE (trifluoroethanol), a majority of the magainin adopts an \(\alpha\)-helical conformation. It has also been shown that it adopts a primarily \(\alpha\)-helical conformation when imbedded in lipid vesicles\(^11,8^{,6,10}\). This shows that the conformation of magainin is strongly dependant on its environment. 3) Magainin has been shown to form ion channels in various synthetic and cellular membranes. Cells that are not lysed by magainin show a ionic potential shift in its presence\(^15\). This means magainin can form channels without lysing the cell. This could mean that channel formation is not responsible for magainin's cell lysing ability, or simply that a very large number of channels must be opened before the cell is lysed. It has also been postulated that in high concentrations, magainin solubilizes the cell membrane in a fashion similar to detergents (which are also
small amphipathic molecules).

From a simple inspection of the size and structure of magainin, it is clear that several magainin molecules must be involved in the formation of each ion channel. Magainin is an amphipathic protein. When it is folded into an α-helix, one side of the molecule is primarily hydrophobic, and the other side is primarily hydrophilic. Fig. 3 contains a helical wheel diagram of magainin showing this property. This arrangement has lead to speculation that magainin might form channels consisting of several α-helices oriented perpendicular to the membrane, held together by the hydrophobic effect. That is, the hydrophilic side of the helices would line the water filled channel, and the hydrophobic side of the helices would face the hydrocarbon chains of the lipid molecules in the membrane which are also hydrophobic (Fig. 2). Thus the magainin would form two dimensional versions of the micelles formed by lipid molecules in water.

![Diagram of water filled pore and hydrophilic/hydrophobic regions in a membrane](Diagram.png)

**Figure 2**: One proposed geometry of the magainin ion channel. Several (6 in this case) magainin molecules are joined by the hydrophobic effect forming a water filled pore across the membrane. Once the pore has been formed, additional magainin molecules could be added or removed, increasing or decreasing the size of the channel.
Figure 3: Wheel diagrams showing magainin's amphipathic nature. In an α-helical conformation proteins have 3.6 residues/turn giving it an overall cylindrical shape with the sidechains radiating outward. The above diagrams are looking down on the top of this cylinder. Each letter represents a single amino acid. Amino acids are divided into 3 categories: hydrophobic, hydrophilic, and ambivalent. The distance each letter is from the center in the wheel diagram represents this classification. Residues on the inner ring are hydrophilic, those on the the middle ring are ambivalent, and those on the outer ring are hydrophobic. The left diagram shows all 23 residues in magainin 1. The ambivalent residues have been removed from the right diagram to more clearly show magainin's amphipathic nature.

This, however, is only speculation. A conclusive determination of magainin's open channel form has not yet been accomplished. Most researchers still believe that magainin adopts only one conformation when imbedded in the membrane, and when a sufficient amount is present, channels are formed. We will present evidence that this model is too simplistic. Using OCD, we have discovered that magainin actually undergoes a concentration dependant phase transition when imbedded in artificial membrane systems, and that each phase represents a distinct conformation. We also present evidence that this change in conformation represents the change between the membrane active and inactive forms of magainin and that the transition is not simply a result of the micellar effect.
3 Experimental Methods

3.1 Sample Preparation

In experiments involving synthetic membranes, sample preparation is the key to repeatable and useful results. A majority of the time put into research is spent making samples and developing new methods for sample preparation. Two methods were used for sample preparation in these experiments. The first preparation method, described in section 3.1.2, is based on the method developed by Yili Wu which is in turn based on the method developed by Glen Olah. While there are similarities to other methods used in the field, the second method, described in section 3.1.3, is principally my own.

Both methods began with a standard DPhPC solution in chloroform and a standard magainin I solution in TFE (trifluoroethanol) or distilled water. The DPhPC was shipped in chloroform at 20mg/ml, so no further preparation was necessary.

3.1.1 Preparation of Magainin Standard Solution

The magainin was shipped as a lyophilized powder. Due to it's high solubility in water, it rapidly absorbs water when exposed to air. This makes weight a highly unreliable method for measuring the amount of magainin to be added to the solvent. To get around this problem, a small (visually estimated) quantity of magainin was added to a few ml of solvent (magainin is also highly soluble in trifluoroethanol), then the magainin concentration in the resulting solution was measured using the phenylalanine absorption peak at 257nm (UV). 3 of magainin's 23 residues are phenylalanine, providing a small, but adequate signal for this measurement.

A sample absorption spectrum is shown in Fig.4. Its spectral lineshape qualitatively matches a published reference spectrum for Phenylalanine. The spectropolarimeter records %T, (% light transmitted). Absorbance is defined as
\[ A = \log_{10}\left(\frac{1}{T}\right) , \] where \( A = 3\varepsilon c_{\text{mag}} l \) which allows us to calculate the magainin concentration in solution. \( c_{\text{mag}} \) is the molar concentration of magainin and \( l \) is the pathlength in cm. The literature value of 220M\(^{-1}\)cm\(^{-1}\) for \( \varepsilon \) (the molar extinction coefficient) was used since an insufficient quantity of magainin was available for a direct measurement. The factor of 3 accounts for 3 phenylalanine residues in each magainin molecule. The absorbance was measured at the point shown in Fig.4. Measurements were made using a 1cm quartz cuvette in a Jasco J-500 spectropolarimeter. Calibration of the J-500 was verified by measuring the same solution in a dual beam spectrophotometer.

**Figure 4:** Absorbance of magainin standard solution. The peak absorbance, cell path length, and molar extinction coefficient are used to measure the concentration of magainin in solution.
3.1.2 Sample Preparation from a Large Batch

The first sample preparation technique begins with the preparation of a large batch (~30-50mg) of magainin in DPhPC with a known P/L (peptide/lipid) ratio. Individual samples are then prepared from this batch.

The process begins by mixing magainin and DPhPC standard solutions in a clean glass bottle (cleaned as described below). The solvent is evaporated under dry N₂, then the mixture is placed in vacuum for several hours to remove any remaining traces of solvent. A few ml of ultrapure (.5 micron filtered) water is then added, and the mixture is homogenized (and sometimes heated to ~50°C). The batch is then lyophilized, producing a fluffy mixture occupying several ml. Finally, the batch is hydrated for several days in a jar kept at 100% RH (relative humidity) at room temperature. The final result is a transparent sample with the consistency of soft butter.

An individual sample is prepared by taking a small amount (~1mg) of this batch and depositing it between 2 clean quartz slides. The sample is then manually perturbed until it seems well aligned by observing oily streaks with a polarizing microscope. Before measurement, samples are allowed to sit for several days in a jar at 100% RH. This gives them time to relieve any strain caused by the perturbation, and hopefully come to equilibrium with the RH in the chamber.

Unfortunately, due to poor mixing of the magainin/DPhPC or possibly due to magainin aggregation, samples prepared from different areas of the batch can have widely varying P/L ratios, especially when the concentration of magainin is high. This problem did not occur with other channel forming peptides studied by the group, so aggregation after mixing is the most likely cause of this problem. The second sample preparation method corrects this problem.
3.1.3 Sample Preparation from Standard Solution

This method involves preparing individual samples directly from magainin and DPhPC solutions. Since only a single sample is being prepared, the P/L ratio can be controlled much more accurately. The light source in the spectropolarimeter covers roughly 1 cm² of the sample. This is a large enough area to average out any local P/L ratio variations.

This method of sample preparation begins by mixing small quantities (~1mg total mass) of magainin and DPhPC solutions in a clean glass bottle. Solvent is evaporated under dry N₂, then 1-2 drops of chloroform are added. The sample in chloroform is vortexed to mix it well, then it is transferred to a clean quartz slide with a glass syringe. The chloroform is evaporated in air, then in vacuum for several hours to remove remaining traces of solvent. Next, the sample is hydrated at 100% RH at room temperature for ~1 hour. Finally, the sample is covered with a second quartz slide, and perturbed as in section 3.1.2. Other than the problem with P/L variations, samples prepared by both methods produce similar results.

3.2 Cleaning of Fused Silica Substrate

A clean substrate is extremely important for good alignment of the sample. The fused silica slides are cleaned in 5 steps. 1) The slide is sprayed with ethanol and abrasively cleaned with a kim-wipe or cotton swab. This process is repeated until the slide appears clean when held up to a light. 2) The slide is placed in a heated sulfuric/dichroic acid bath for 20-30 minutes. 3) The slide is removed from the bath and placed in a small ceramic crucible with draining holes in the base. The size of the crucible is chosen such that both surfaces of the slide are exposed to the air, the slide contacts the crucible only at 4 points. 4) The slide is rinsed repeatedly with ethanol and distilled water. 5) The slide is allowed to dry in the crucible under a dust-free hood. After cleaning, the slide is handled only with
forceps. Any dust or impurities on the slide can generally be seen under the microscope while the sample is being manually perturbed, in which case the cleaning process must be repeated. This is rarely necessary.

Glass bottles used for mixing samples are cleaned in a similar fashion.

3.3 OCD Spectra

OCD (oriented circular dichroism) spectra\textsuperscript{17} were recorded on a Jasco J-500 (located in our lab) and a Jasco J-720 spectropolarimeter (courtesy of the Nutrition Research Center). The J-500 was modified so samples could be rotated in the plane of the quartz slide, to remove linear dichroism artifacts. We found this effect to be negligible in all but the driest samples, so the lack of this modification on the J-720 was not a significant problem. Samples measured in the J-720 were manually rotated to at least 2 different angles to insure that this effect was not present.

Due to a design problem in the J-500 at low wavelengths, it was operated in fixed slit width mode which causes the bandwidth to vary as a function of wavelength. When attempting linear decomposition, this was a small effect, as long as all of the involved spectra (including the basis spectra) were recorded on the J-500. However, since the spectra on the J-720 were recorded in a fixed bandwidth mode, they could be decomposed much more accurately.

Due to the strong UV absorption of the samples, the J-500 is incapable of measuring CD spectra of most samples below 200nm. Data below 200nm provides a significant improvement in the uncertainty of the spectral decomposition. The J-720 has improved optics which allow scanning down to \(~180\) nm for most samples. The best quality results on the J-720 were obtained with a \(1\) nm bw. This represents a trade-off between wider bandwidths which cause the features of the spectrum to become smeared, and narrower bandwidths
which reduces the available light, reducing the range of wavelengths which can be measured. On both machines, a 4 second time constant with a 20 nm/min scanning rate were used.

3.4 Supplies

The magainin I used in all experiments was obtained from Multiple Peptide Systems (San Diego, CA), certified >97% pure by HPLC. DPhPC (diphytanoylphosphatidylcholine) was purchased from Avanti Polar Lipids (Alabaster, AL) and was >99% pure. Fused silica slides were purchased from Optical Instruments Inc. (Houston, TX).
4 Results

A large number of preliminary samples were prepared at various concentrations, allowing us to perfect the sample preparation techniques. This process also allowed us to determine which concentrations would provide interesting results. Once we were satisfied that samples could be reliably produced at a given concentration, eight samples at various P/L ratios were prepared for measurement of the final, quantitative results. These samples were prepared using the method described in section 3.1.3 with the exception of the 30/1 sample which was prepared using the method described in section 3.1.2. All of the samples were allowed to hydrate in a jar at 100% RH for several days before scanning.

The OCD spectra of all of these samples appears in Fig.5. Note that the spectra have two distinct overall shapes, one for high concentrations (HC) and one for low concentrations (LC). As described in section 5.1, 2 basis spectra representative of these two extremes, HC and LC, were extracted and put on an absolute scale (Fig.6a).

For comparison, Fig.6b shows the HC and LC basis spectra for alamethicin, a similar antibiotic peptide which also shows a concentration dependent phase transition. As in alamethicin, magainin is in an $\alpha$-helical conformation oriented parallel to the membrane surface in the LC state. While both peptides undergo a phase transition, alamethicin remains $\alpha$-helical, but rotates so it is oriented perpendicular to the membrane. Magainin, on the other hand, adopts a new conformation which can't be decomposed in terms of any of the known protein conformations. We hope to identify this conformation as part of a further study.
Figure 5: OCD spectra for several samples at various P/L ratios. All of the spectra can be linearly decomposed in terms of HC and LC basis spectra representative of the two
Figure 6: HC and LC basis spectra for Alamethicin (b) (shown for comparison only) and magainin (a). The LC spectrum of magainin I is that of an alpha-helix oriented parallel to the membrane surface, as is the LC spectrum of alamethicin. See section 5.1 for information on how these spectra were extracted.
The spectra of all of these samples could be linearly decomposed in terms of the two basis spectra with only slight mismatches. Using the procedure outlined in section 5.2, all of the spectra in Fig.5 were decomposed in this way, giving a phase diagram showing change of state as P/L ratio is varied (Fig.7).

![Figure 7: Phase diagram showing % in HC state at various magainin concentrations for well hydrated samples.](image)

From earlier experiments done on alamethicin, we knew that other variables such as temperature and sample hydration might also have an effect on the state of the samples. For magainin at most concentrations these effects proved to be fairly small. Also, results were extremely difficult to reproduce, making the effect of these variables difficult to quantify. The mixed region of the phase diagram is where these other variables tend to dominate. At concentrations in this region, it is possible to obtain a sample with any mixture of states by varying hydration and temperature. However, the equilibrium of these samples is very unstable, so repeatable results are nearly impossible to obtain. We did find qualitatively that an
increase in sample hydration would cause an increase in the amount of magainin in the HC state. Temperature was not controlled enough to note any systematic patterns.

Hydration and temperature are also responsible to a large extent for the smaller variations of state in the LC and HC regions of the phase diagram. As discussed earlier, the samples were allowed to sit in a 100% RH jar for several days before measuring to minimize this effect. Unfortunately, samples can take up to several weeks to come to full equilibrium, and the rate at which this occurs depends strongly on the concentration and thickness of the sample. Once obtained, this equilibrium can easily be disturbed when the samples are removed for measurement. Several days proved to be a sufficient amount of time for reasonably accurate results in the LC region of the diagram. The uncertainty due to the decomposition process is also responsible for some of the deviation. Samples in the HC region of the diagram tend to be more stable and easier to hydrate, so less variation is seen in this region.
5 Data Analysis

OCD data was acquired on IBM compatible computers connected to the J-500 and J-720 spectropolarimeters. This data was transferred to a NeXT where the data was analyzed. The software used in the analysis was custom written in C and Objective C.

5.1 Scaling and Extraction of Basis Spectra

Putting an OCD spectrum on an absolute scale requires a corresponding absorption spectrum. Unfortunately, due to the small amount of magainin present in samples in the LC state, accurate absorption measurement is not possible. Samples that tend to be in the HC state, however, have just enough magainin for absorption measurements with ~15% uncertainty.

The first step in scaling the basis spectra is to record 3 spectra from the same (1/15) sample in different hydration states (Fig.8a). The “wet” spectrum was taken after soaking the sample in distilled water. This spectrum is almost completely in the HC state. The 2 “dry” spectra were taken after the sample was removed from the water and allowed to dry at ~45% RH for several days. Since the state of the magainin depends not only on the concentration, but also the hydration of the sample, these 3 spectra all have different proportions of magainin in the HC and LC states (see chapter 4).

For each OCD spectrum, a corresponding absorption spectrum was recorded while the sample was still in place in the spectropolarimeter (Fig.9a). There is some overlap between the phenylalanine absorption peak and the nearby peptide bond absorptions, so a lorentzian centered at 224nm was subtracted from these curves to make the lineshape more closely match the reference spectrum(Fig.9b).

Using the peak value of the corrected phenylalanine absorption, the OCD spectra were put on an absolute scale by multiplying the ellipticity in mdeg by
\( \frac{\varepsilon \cdot n_{\text{phe}}}{10} \cdot \frac{1}{n_{\text{aa}}} \cdot \frac{1}{A} = \frac{22}{10} \cdot \frac{3}{23} \cdot \frac{1}{A} \) where \( n_{\text{phe}} \) is the number of phenylalanines per molecule, \( n_{\text{aa}} \) is the number of amino acids/molecule, \( \varepsilon \) is the extinction coefficient of phenylalanine, and \( A \) is the absorbance of the sample (section 3.1.1). This produces spectra in the usual CD units used in publication, \( \text{deg} \ \text{cm}^2/\text{dmol} \) (Fig.8b). Note that these units are independent of sample thickness and concentration, permitting direct comparison of protein conformations in any environment.

Next, the two dry spectra were decomposed (see section 5.2) in terms of the wet spectrum and an \( \alpha \)-helix spectrum, yielding coefficients a-d in the following:

Define:

\( S_{\text{HC}} \) ≡ HC basis spectrum
\( S_{\text{LC}} \) ≡ LC basis spectrum
\( S_{\text{dry}} \) ≡ spectrum being decomposed
a-d ≡ coef. from decomposition
f-g ≡ scaling coef.

Define our data as a linear combination of the 2 basis sets:
\[
f \cdot a \cdot S_{\text{HC}}(\lambda) + g \cdot b \cdot S_{\text{LC}}(\lambda) = S_{\text{dry1}}(\lambda)
\]
\[
f \cdot c \cdot S_{\text{HC}}(\lambda) + g \cdot d \cdot S_{\text{LC}}(\lambda) = S_{\text{dry2}}(\lambda)
\]

We then require:
\[
fa + gb = 1
\]
\[
f c + gd = 1
\]
Figure 8: a) Raw spectra of 15/1 sample in various hydration states done on the J-500. b) The same 3 spectra after using the absorption spectra (Fig.9) to put the ellipticities on an absolute scale.
Figure 9: a) Smoothed absorbance spectra for the 3 spectra in Fig.8. b) The same spectra after subtracting a Lorentzian centered at 224 nm until the spectra more closely matched the reference spectrum.
solving for \( f \) and \( g \):

\[
f = \frac{b - d}{bc - ad}
\]

\[
g = \frac{c - a}{bc - ad}
\]

\( S_{HC} \) can then be divided by \( f \) and \( S_{L} \) can be divided by \( g \), giving basis spectra on an absolute scale which decompose both \( S_{dry1} \) and \( S_{dry2} \).

Next, the best two spectra most representative of the HC and LC states were selected as final basis spectra. That is, the spectrum with the strongest \( \alpha \)-helical character was chosen as the basis spectrum for the LC state and the spectrum with the smallest amount of \( \alpha \)-helical content (judged by line flatness between 190 and 210nm) was chosen as the basis spectrum for the HC state. The sample used for the HC basis spectrum was taken from a 1/30 batch sample and was very well hydrated before measurement (by keeping it in a 100% RH jar). The sample used for the LC basis spectrum was a 1/150 sample prepared using the second method discussed in section 3.1.

Finally, these basis spectra were scaled to match the amplitudes of the scaled spectra found above, yielding 2 scaled, high quality basis spectra. Unfortunately, due to large uncertainties in the absorption spectra, the overall scaling of the basis spectra has roughly a 15% uncertainty. If more mixed spectra were available, this process could be repeated, and the absolute scaling of the basis spectra could be improved.
5.2 Linear Decomposition of Spectra

Data was acquired at 5 points/nm, the subscript \( \lambda \) denotes wavelength.

\( D_{\lambda} \) = the spectrum being decomposed and scaled.

\( H_{\lambda} \) = the high concentration basis spectrum. (see Fig.6 top)

\( L_{\lambda} \) = the low concentration basis spectrum.

Define:

\[
\chi^2 = \sum_{\lambda} \left(D_{\lambda} - (AH_{\lambda} + BL_{\lambda})\right)^2
\]

\( \chi \) is then minimized by adjusting the coefficients A and B. This is done by repeatedly varying A and B by a random amount, accepting a change only if \( \chi \) is improved. While very simple, this algorithm is quite sufficient to produce an optimal fit in this case.

Since using an absorption spectrum to scale the data is quite time consuming both in data acquisition and analysis, \( D_{\lambda} \) is not initially on an absolute scale. \( \chi \) is minimized without the condition \( A + B = 1 \), then \( D_{\lambda} \) is scaled such that this condition is satisfied. This produces a scaled data set as well as the coefficients A and B which indicate the relative proportion of the sample in each of the two states.
6 Discussion

6.1 Is this a micellar effect?

Since magainin is an amphipathic protein, it seems possible that the observed change of state might simply be due to the micellar effect. This is the effect that causes amphipathic molecules, such as detergents to cluster together and form micelles in water. Generally, however, this effect is not really a phase transition, but is a simple chemical equilibrium between the monomeric state, $X$, and the N-meric state, $X_N$: $NX \rightleftharpoons X_N$. In general $N$ can take a range of values; That is, amphiphiles form micelles and vesicles with a variety of sizes. However, we can gain understanding of the effect by considering aggregates of one fixed size. We can then determine if magainin is simply experiencing a micellar effect:

Define:

$[M] \equiv$ total magainin conc. (P/L)

$[M_C] \equiv$ critical concentration

$[m_1] \equiv$ magainin conc. in LC state

$[m_N] \equiv$ magainin conc. in HC state

$\beta \equiv 1/K_BT$

$N \equiv$ number of molecules in aggregate form.

$K \equiv$ chemical equilibrium constant

$\mu_1 \equiv$ chemical potential of LC

$\mu_2 \equiv$ chemical potential of HC
For an ideal solution:

\[ \mu_1 = \frac{1}{\beta} \log [m_1] + \mu_{01} \]

\[ \mu_N = \frac{1}{\beta} \log [m_N] + \mu_{0N} \]

Since the monomers and N-mer are in equilibrium, the chemical potentials must be equal, so:

\[ \mu_N = N \mu_1 \]

Hence we have:

\[ N \log [m_1] + N \beta \mu_{01} = \log [m_N] + \beta \mu_{0N} \]

\[ [m_1]^N = [m_N] e^{\beta (\mu_{0N} - \mu_{01})} = \frac{[m_N]}{K} \]

The total magainin concentration is:

\[ [M] = [m_1] + N [m_N], \]

or

\[ [M] = [m_1] + NK [m_1]^N \]

This allows us to define a critical concentration, \([M_C]\):

\[ \frac{[M]}{[M_C]} = \frac{[m_1]}{[M_C]} + N \left( \frac{[m_1]}{[M_C]} \right)^N \]

where:

\[ [M_C]^{N-1} = \frac{1}{K} = e^{\beta (\mu_{0N} - \mu_{01})} \]
Figure 10: The points on the plots above are from the phase diagram after conversion to concentration units. The solid lines represent the theoretical concentrations given by the micellar effect. A critical concentration, $[M_C]$, of 1/50 was used and $N$ was set at 10. Variation of these parameters does not provide a significant improvement in the HC region.
This equation can be solved numerically for \([m_1]\) and \([m_N]\) vs. \([M]\) for a fixed \([M_C]\). Fig.10 shows a plot of this solution along with the points from the phase diagram (Fig.7) which have been converted to concentration units. It is clearly seen that in the LC state there is a fairly good fit between the two, but at concentrations above 1/50, far too much magainin is in the HC state for this simple theory to be satisfied. That means, rather than the LC state becoming saturated, and the excess going into the HC state; Once the critical concentration is passed, much of the LC magainin shifts to the HC state. The amount of magainin the LC state actually decreases. Clearly the magainin is experiencing some sort of cooperative phase transition which cannot be explained by a simple chemical equilibrium.

6.2 Significance of the Peptide Phase Transition

While it seems reasonable that high concentrations of magainin will disrupt membranes, our measurements cannot measure this effect directly. However, other research has shown that at high concentrations, magainin spontaneously solubilizes phosphatidylcholine liposomes\(^{16}\). Also, Matsuzaki et al.\(^{11,12}\) performed liposome leakage experiments and found that magainin induced leakage was significant only when the concentration of the magainin bound to the liposome exceeded a critical value. This value varied with the lipid composition of the membrane, but was generally between 1/30 and 1/100. The critical concentration we estimate for our phase transition is 1/60, which falls near the middle of this region. The correspondence between these two effects is strong evidence that they are linked, implying that the HC state of magainin is responsible for vesicle lysis.

Amphiphilic helical structure is a common motif in membrane active proteins. They are found in channels, antibiotics, virus protein sheaths, and membrane
fusion peptides. We have now seen concentration dependent transitions in 3 different proteins of this class. Clearly this phase transition must be involved in the function of these proteins.

The next planned step in our study is to use lamellar diffraction and in-plane x-ray scattering to determine more about the structure of magainin in the HC state. The OCD spectrum we observed in the HC state cannot be decomposed in terms of the currently known protein structures, so any structural information we can obtain would be extremely useful. Since this state is observed only when magainin is dissolved in a liquid crystalline membrane, x-ray diffraction can only provide a limited amount of information. Still, hopefully these two experiments will yield enough clues to identify the mysterious HC structure, and provide more information on the effects of the HC/LC phase transition.
Appendix A:
Glossary and list of abbreviations

α-helix - One of the basic protein conformations. The protein backbone is coiled into a helical shape with 3.6 residues/turn and a length of roughly 1.5Å/residue. The structure is maintained by hydrogen bonds between H and O in the protein backbone.

conformation - The secondary structure of a protein. Primary structure is the amino acid sequence. Secondary structure is the local organization of this linear array of amino acids, i.e. - α-helix, β-sheet, random coil, etc.... Tertiary structure is the overall shape formed when the secondary structure is folded into superstructures.

gram-negative bacteria - Bacteria with a double cell membrane, inner and outer.

gram-positive bacteria - Bacteria with a single, thicker cell membrane.

HC - Refers to the high concentration state of magainin.

LC - Refers to the low concentration state of magainin.

liposome - generally synonymous with vesicle.

lyse - To destroy a cell generally involving disruption of the membrane.

lyophilize - A process of turning a sample in water solution into a low density powder. The sample is quick-frozen, then freeze-dried until the ice has evaporated.

micelle - A roughly spherical structure formed by lipid molecules in water. The hydrophobic tails are imbedded in the center, and the hydrophilic heads are exposed to the water. see Fig.1

OCD - oriented circular dichroism. This is the primary experimental technique used in this thesis.

P/L - Peptide/Lipid ratio. A measure of magainin concentration in an artificial membrane environment.

phenylalanine - One of 3 amino acids with significant UV absorptions which may be used to measure solution concentrations of proteins.
RH - Relative humidity.

random coil - One of the basic protein conformations. Rather than having a fixed structure, this conformation refers to the state where no backbone hydrogen bonds have formed, so the protein chain is changing shape continuously.

residue - A single amino acid within a protein.

TFE - Trifluoroethanol, used as a solvent.

vesicle - a closed, bilayer surrounded structure as shown in Fig.1
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