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Solution Conformation of De Novo Antimicrobial Peptides

Maryam Javadpour
Louisiana State University and Agricultural and Mechanical College

SOLUTION CONFORMATION OF *DE NOVO* ANTIMICROBIAL PEPTIDES

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In the name of God, Most Gracious, Most Merciful.

Praise be to God, Lord of the universe. Most Gracious, Most Merciful.

Master of the Day of Judgement. You alone we worship. You alone we ask for help. Guide us in the right path; the path of those whom You blessed;

not of those who have deserved wrath, nor of the strayers.

Dedication

To my parents

Mahin and Hossein

and

my sisters

Roya and Lailee

For being my source of inspiration, encouragement and support

and for believing in me. I love you all very much.

In Memory

I also wish to dedicate this work to the memory of my grandmothers.
Their sincere prayers have helped me throughout my life.
I miss you both very much and wish you could
have seen this. I am sure you would have been very proud.

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Abstract

De novo antimicrobial peptides were designed to help study the necessary conformational properties of biologically active peptides. One set of peptides are: $(KLAKKLA)_n$, $(KLAKLAK)_n$, (n=1, 2, 3), $(KALKALK)_3$, $(KLGKKLG)_n$, and $(KAAKKAA)_n$ (n=2, 3), while the second set have phenylalanine substituted for leucine, $(KFAKFAK)_3$ and $(KFAKKFA)_n$, (n=3, 4). These peptides were designed to be perfectly amphipathic in helical conformations.

Circular dichroism was used to study their secondary structure in aqueous solution, sodium dodecylsulfate micelles, and phospholipid vesicles. Peptide antibacterial activity was tested against *E. coli*, *P. aeruginosa*, and *S. aureus*. Peptide cytotoxicity was tested against a mammalian cell line, 3T3 mouse fibroblasts. Our results demonstrate that the propensity to α-helical conformation of the peptides in amphipathic media is proportional to their cytotoxicity.

Sedimentation equilibrium was used to determine the aggregation state and equilibrium constant of the self-association of the peptides in aqueous solution. The thermodynamics of these associations were calculated from the temperature dependence of the association constants. With the exception of $(KLGKKLG)_3$ which does not self-associate, the other peptides are in a monomer-tetramer equilibrium. The peptides aggregate through the interaction of their hydrophobic residues. This was detected using 1-Anilio-naphthalene-8-sulfonate fluorescence. The phenylalanine peptides are 1.0-1.5 kcal/mol less stable than the equivalent leucine peptides.

To correlate the aggregation and biological activity, carboxyfluorescein labeled peptides were used to determine the aggregation state of CF-$(KLAKKLA)_3$ and CF-$(KLGKKLG)_3$ in DPC micelles. CF-$(KLAKKLA)_3$ forms dimers while CF-

$(KLGKKLG)_3$ is a monomer. These findings suggest that aggregation may contribute to the selective cytotoxicity of the peptides.

Chapter 1

Introduction

1.1 Overview

Peptides have emerged as useful tools in biomedical research as well as effective therapeutics. The main classes of "natural" amphipathic peptides include the cecropins (Brown & Wuthrich, 1981), magainins (Zasloff, 1987), mellitin (Blondelle & Houghten, 1991) and defensins (Ganz et al., 1990). Studies show that the amphipathic α-helical secondary structure is important for the biological activity of these naturally occurring peptides (Kaiser & Kezdy, 1987). Investigators have designed and synthesized amphipathic α-helical peptide analogs to these natural peptides in order to understand the mechanism of action and conformational requirements that are necessary for biological activity.

Boman et al. were the first to investigate the humoral defense system used by the North American silk moth *Hyalophora cecropia* (Hultmark et al., 1980; Hultmark et al., 1982). Infection of the insect induces the synthesis of specialized peptides capable of disrupting the bacterial cell membranes resulting in the lysis and cell death. These peptides are collectively known as cecropins. The principle cecropins: A, B and D, are small basic peptides made up of 37, 35, and 36 amino acid residues respectively. Insect cecropins are highly homologous, but there is only a 33% sequence homology between insect and mammalian cecropins (Lee et al., 1989). The amino-terminal half of the different cecropins contains a sequence that forms an amphipathic α-helix, while the carboxy-terminal half comprises a hydrophobic tail (Andreu et al., 1985). Cecropins A and B exhibit strong antibacterial activity against several Gram-positive and Gram-negative bacteria while the D form shows high activity only against *E. coli* (Boman et al., 1991). The decreased activity of cecropin D compared to cecropin A and B is attributed to the

lower basicity of its N-terminus. Cecropins do not have biological activity against eukaryotes (Steiner et al., 1988). In addition, cecropins induce membrane leakage in liposomes (Steiner et al., 1988), and form voltage-dependent ion channels in planer bilayers (Christensen et al., 1988).

Circular dichroism studies show that cecropins are highly unordered in solution and are in a random coil conformation. However in aqueous hexafluoroisopropyl alcohol cecropins form helices (Steiner, 1982; Andreu et al., 1985). NMR studies in the same solvent system shows that the secondary structure of cecropin A is comprised of two α-helical regions connected by a hinge (Holak et al., 1988). The hinge region between the helices is formed by Gly 23 and Pro 24 residues, which are conserved in all of the cecropin sequences. Cecropins induce a rapid lysis of bacterial cells, indicating a direct action on their membranes. The mechanism of action of cecropins has been studied through the use of model peptides (Christensen et al., 1988; Fink et al., 1989). These results are consistent with a proposed mechanism of action which involves initial electrostatic interactions between the positively charged N-terminus helix and the negatively charged head groups on the membrane surface, followed by insertion of the hydrophobic C-terminus into the membrane core which results in the formation of positively charged channels through the membrane. The channels are formed by the association of multiple transmembrane NH_2-terminal helices, such that the hydrophilic residues form the aqueous pore and the hydrophobic residues are in contact with the aliphatic phase of the membrane (Durell et al., 1992). Mchaourab et al. (1993) have shown that cecropins self-associate in the presence of hexafluoroisopropyl alcohol and that the aggregation process is promoted by hydrophobic interactions and ion-pair formation. However the aggregation state of cecropins have not yet been determined.

Magainins consist of a family of basic antimicrobial peptides produced in the granular glands present in amphibian skins and are released as part of the immune-defense mechanism in response to challenges by foreign invaders . They have been isolated from the skin of the clawed African toad *Xenopus laevis*. The peptides exhibit a broad spectrum of antimicrobial activity against various microorganisms including Gram-positive and Gram-negative bacteria as well as tumor cells by permeabilizing the cell membranes (Zasloff, 1987; Zasloff et al., 1988; Juretic et al., 1989; Westerhoff et al., 1989; Cruciani et al., 1991) . Magainins are not hemolytic, thus being a candidate compound for therapeutic use. Magainins consist of 23 amino acid residues and have a net 4 positive charge at physiological pH.

Circular dichroisim studies by Chen et al. (1988) indicate that magainins exhibit little α-helical structure in aqueous solution but helix content is increased in the presence of hydrophobic solvents. NMR studies of magainins in structure inducing solvents demonstrate an α-helical structure with amphipathic character (Marion et al., 1988). With the aid of electrostatic interactions magainins bind to negatively charged phospholipid membranes, forming an amphipathic helix and permeabilizing the bilayers (Matsuzaki et al., 1991; Vaz Gomes et al., 1993). Magainins forms a voltage-dependent channel in artificial membranes (Cruciani et al., 1988). The disruption of membrane caused by magainins has been studied using the leakage of fluorescent dyes from vesicles (Matsuzaki et al., 1991). In general, negatively charged vesicles exhibit leakage to a greater extent, emphasizing the importance of electrostatic interaction between the peptide and membrane. Using oriented circular dichroism, Ludtke et al. (1994) have studied the secondary structure of magainins in lipid bilayers. These findings show that magainins adopt an α-helical conformation with two distinct orientations when interacting with a lipid bilayer. At low concentrations, the helix axis of the peptide is parallel to the

membrane surface, however, at high concentrations the peptide is inserted into the membrane. This transition occurs at roughly the same critical concentration required for cytolytic activity, implying that the membrane insertion is responsible for cell-lysing activity. Matsuzaki et al. (1994) have used fluorescence to study the orientation and aggregation states of magainins in phospholipid bilayers. Based on the binding isotherms of magainin analogs to membranes they have shown that the bound peptides self associate and form dimers.

Mellitin, a 26 amino acid residue peptide with a net charge of +6, is the main toxic component from the bee venom of *Apis mellifera* (Habermann, 1972; Dempsey, 1990). Unlike cecropins and magainins, this peptide has a powerful hemolytic activity. In addition to hemolytic activity, melittin induces voltage-dependent ion-conductance across planar lipid bilayers and causes selective micellization of bilayers as well as membrane fusion (Dempsey, 1990). Melittin has been extensively studied as a substance capable of causing profound membrane changes in eukaryotic cells (Boman et al., 1989). The essential requirement for the antibacterial activity of mellitin appears to be the N-terminal part of the peptide which is hydrophobic as a whole and is capable of forming an amphipathic helix, followed by a highly basic C-terminal hexapeptide which is hydrophilic . As a result of electrostatic forces, the basic amino acid residues at the C-terminus are thought to mediate the lytic process through interactions with the cell surface (DeGrado et al., 1981).

In aqueous solution melittin adopts different conformations and aggregation states depending on the peptide concentration, pH and ionic strength. Melittin is either monomeric in water or associated as a tetrameric aggregate (Gauldie et al., 1976; Faucon et al., 1979; Talbot et al., 1979; Brown et al., 1980; Lauterwin et al., 1980; Quay & Condie, 1983; Schubert et al., 1985). As determined by circular

dichroism and NMR the aqueous monomer has no detectable secondary structure (Dawson et al., 1978; Knoppel et al., 1979; Talbot et al., 1979; Lauterwin et al., 1980). The secondary structure of the tetramer peptide predicted by Dawson et al. (1978) and subsequently established by X-ray crystallography (Anderson et al., 1980) and NMR spectroscopy (Brown et al., 1980; Brown & Wuthrich, 1981) possesses two regions of α-helix separated by a proline 'hinge'. In methanol solution and micelles, melittin is a monomeric α-helix with a bend at the central proline (Bazzo et al., 1988; Dempsey, 1988; Inagaki et al., 1989). In micelles, slower amide hydrogen exchange rates on the hydrophobic face compared to the hydrophilic face of the peptide suggests that the peptide lies on the micelle surface with the nonpolar side of the helix facing the interior (Inagaki et al., 1989). Circular dichroism studies show that melittin has a helical conformation in membrane bilayers (Vogal, 1987). The exact orientation and aggregation state of melittin in bilayers remains unclear (Hermetter & Lakowicz, 1986; Vogel & Jaehnig, 1986; Altenbach & Hubbell, 1988).

Defensins are small cationic peptides originally found in mammals (Ganz et al., 1990). Defensin peptides have also been found in insects (Trenczek & Faye, 1988) and higher plants (Dimarcq et al., 1990). Although variable within and between species, defensin sequences all possess six to eight cysteines connected by disulfide bonds. In contrast to the cecropins and magainins these peptides do not assume an α-helical structure but an anti parallel β-sheet conformation. However similar to cecropins and magainins these peptides do form voltage-dependent ion channels in lipid bilayer membranes at concentrations comparable to those required for *in vitro* antimicrobial activity (Kagan et al., 1990).

Work with natural antimicrobial peptides has led to different hypotheses of the mechanisms of peptide-membrane interaction that are responsible for cell lysis. The raft model suggests that these peptides aggregate on the surface of the

membrane in sufficient numbers to disrupt the cell membrane causing a loss of osmotic integrity and cell killing (Opella et al., 1993). The channel model starts as the raft model and suggests that an aggregate of four to six peptides form transmembrane channels wherein the nonpolar faces of the aggregated peptides interact with the nonpolar region of the lipid bilayer and the polar faces of the peptides line the channel allowing the passage of ions through the cell membrane. If these channels are sufficient in number this could cause a loss of cell osmotic integrity and cell lysis (Lear et al., 1988).

Natural or synthetic model peptides represent a fertile source of novel chemotherapeutic agents. Although few of these compounds will be effectively used as drugs, they provide new classes of lead compounds as well as useful tools for studying the mechanism of action against bacterial cell membranes. Over the past decade research has focused on the characterization of new natural antimicrobial peptides and the *de novo* design and synthesis of peptides containing the sequences of amphipathic α-helices. These studies have greatly increased our knowledge of the structure/function properties of such helices (Kanellis et al., 1980; Segrest et al., 1983; Anantharamaiah et al., 1985; Chung et al., 1985; Anantharamaiah, 1986; Epand et al., 1987; Epand et al., 1989) and have provided insight into the design of totally synthetic amphipathic peptides which exhibit significant antimicrobial activity. It has been our aim to design, synthesize, and characterize small model peptides of defined structure with the capabilities of mimicking the function of natural antibacterial peptides. The crucial step toward this goal was to design α-helical model peptides with the appropriate amphipathicity. To be the ideal immune substance, the designed peptide should act against a very wide range of foreign cells without causing any destruction to the host.

1.2 Circular Dichroism

The optical activity characteristic of organic molecules is a result of the absorption of light as electrons are promoted to higher molecular orbitals. In UV-visible spectroscopy, the transition process is described by the Beer-Lambert Law, $A = \varepsilon cd$, where A is the experimentally measured absorbance, ε is the extinction coefficient, c is the molar concentration of solute, and d is the pathlength of the light. Absorbance, A, is plotted vs. energy or wavelength of the light.

Plane-polarized light can be resolved into its two circularly polarized components: left circularly polarized light, whose electric vector rotates counterclockwise about the axis perpendicular to the direction of travel of the light beam, and right circularly polarized light, whose rotation is clockwise. A chiral compound exhibits optical activity because its absorption of left circularly polarized light is not equal to its absorption of right circularly polarized light. After passing through a chiral medium, the electric vectors describe an ellipse whose major axis lies along a new angle of rotation. The measured eccentricity of the ellipse represents the unequal absorption of left and right circularly polarized light referred to as circular dichroism, CD. Written as analogous to Beer's Law, the experimentally measured ellipticity = $\theta\ cd$ where θ is the molar value in radians. A CD spectrum plots the differential absorbance of left and right circularly polarized light vs. wavelength.

CD is useful in determining the secondary structure of peptides because we can reasonably assume that in the absence of aromatic amino acid residues only the peptide backbone contributes significantly in the far UV region and because the spectrum reflects the spatial arrangement of chiral units in the peptide chain. The presence of aromatic amino acids induces a positive CD signal, causing significant errors in ellipticity values (Manning & Woody, 1989; Chakrabartty et al., 1993).

There are three main classes of secondary structure (Figure 1.1): the alpha helix, the beta sheet, and the random coil. The alpha helix produces the most distinctive CD spectrum: a very strong positive band near 192 nm which corresponds to $\pi \rightarrow \pi^*_{\perp}$ transitions, and two negative maxima of approximately equal intensity near 222 nm ($n \rightarrow \pi^*$) and 208 nm ($\pi \rightarrow \pi^*_{||}$). The beta sheet exhibits a single negative band near 217 nm, representing $n \rightarrow \pi^*$. The random coil exhibits a strong negative band at 197 nm ($\pi \rightarrow \pi^*_{||}$) and a small positive band at 217 nm ($n \rightarrow \pi^*$).

Peptide conformation is highly sensitive to solution variables; a peptide may be insoluble in one condition, but well behaved under other conditions. In characterizing the secondary structure of peptides by CD, the effect of pH, ionic strength of the buffer, and peptide concentration should be examined. As a general rule, aggregation increases with peptide concentration, ionic strength, or pH. Concentration dependence reveals whether the peptide is monomeric or self-associating: for example, a shift from random coil to α-helix as the peptide concentration increases indicates an association equilibrium. Self-association may be obtained by changing the pH (generally increasing) to titrate the amino groups present in the peptide sequence, or by changing the ionic strength of the buffer to optimize charge-screening. Variations in these parameters are examined systematically in an effort to determine the number and type of species present in the solution. Stable equilibria between only two states are identified by the presence of an isodichroic point.

The concentration dependence of possible transitions should be studied by CD prior to undertaking sedimentation equilibrium studies in the analytical ultracentrifuge. The choice of solution conditions which yield an isodichroic point will result in a well-behaved system for further study.

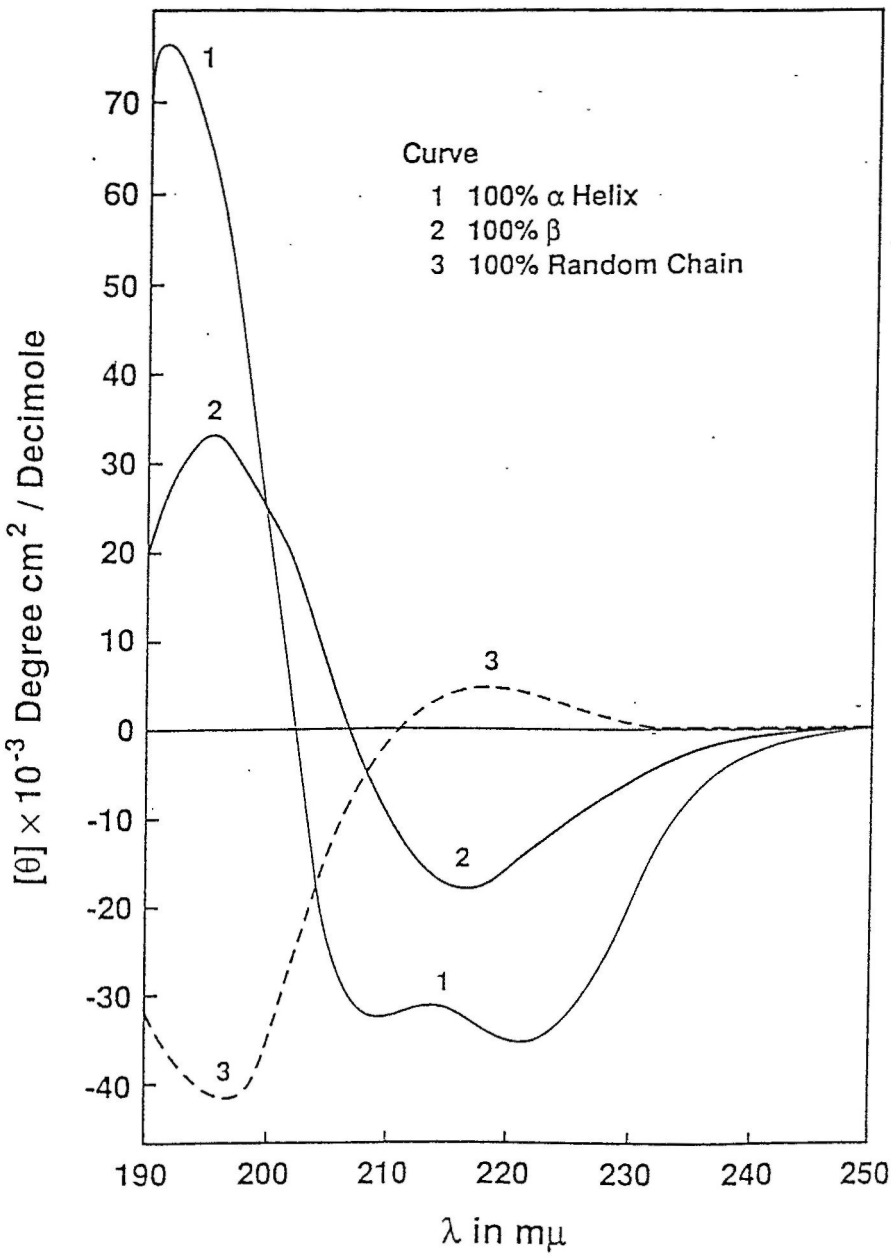

Figure 1.1. Circular dichroism spectra of poly-L-lysine in the α-helical, β, and random conformations. Reprinted from Biochemistry (1969), 8, 4108-4116, by permission of the American Chemical Society.

1.3 Sedimentation Equilibrium

One of the most influential developments in the study of macromolecules was that of the analytical ultracentrifuge by Svedberg and his colleagues in the 1920s. Sedimentation analysis is very valuable in studies of changes in molecular weight when molecules associate to form more complex structures. It also allows the study of a wide range of interactions, including the binding of small molecules and ions to macromolecules, the self-association of macromolecules and heterogeneous macromolecular interactions (Minton, 1983).. Sedimentation equilibrium in the analytical ultracentrifuge is the only technique presently capable of analyzing such interactions over a wide range of solute concentrations, without perturbing the chemical equilibrium (Kim et al., 1977). While electrophoresis in gels containing SDS can provide information on the components and their relative stoichiometry in a complex, sedimentation equilibrium provides the means of determining the molecular weight of the complex as it exists in solution, and independent of the shape of the particle. The method of sedimentation analysis is firmly based on thermodynamics and all terms in the equations describing sedimentation behavior are experimentally determined.

In sedimentation equilibrium experiments, a small volume of an initially uniform solution is centrifuged. As the solute begins to sediment toward the cell bottom and the concentration at the bottom increase, the process of diffusion opposes the process of sedimentation. Because of the sedimentation process, within the sample cell there will be a range of concentrations from very low at the meniscus to much higher at the cell bottom. After an appropriate period of time, the two opposing processes approach equilibrium. At equilibrium there is no net movement of molecules, so diffusional flow exactly balances sedimentation flow everywhere in the cell. The equilibrium concentration distribution formed over a period of several

hours is analyzed using known monomeric molecular weights to determine the aggregation number and thermodynamic parameters such as equilibrium constants, ΔG°, ΔH°, ΔS° and ΔC_P.

1.4 References to Chapter 1

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Chapter 2

De Novo Antimicrobial Peptides with Low Mammalian Cell Cytotoxicity

2.1 Introduction

The amphipathic α-helix is a common structural motif of many proteins and biologically active peptides (Epand et al., 1995). Amphipathic helical domains are found in membrane recognition sites, such as specific ion channel proteins, signal peptides, and antimicrobial and venom peptides (Stewart, 1993). The interaction of amphipathic peptides with membranes depends at least in part on the relative sizes of the hydrophobic and hydrophilic faces and the charge of the hydrophilic face. Antimicrobial peptides generally have an equivalent number of polar and nonpolar residues within the amphipathic domains and enough basic residues to give the peptide an overall positive charge at neutral pH (Saberwal & Nagaraj, 1994). Peptide venoms are also positively charged but generally have more extensive hydrophobic surfaces with narrower polar faces. Melittin is a 26-residue peptide found in the venom of the honey bee, *Apis mellifera* (Habermann & Jentsch, 1967). It is cytotoxic and has broad spectrum antimicrobial activity at micromolar concentrations (Dempsey, 1990). There are a number of other natural amphipathic peptides that are much less cytotoxic than melittin but have comparable broad spectrum antimicrobial activity. Magainins and cecropins exhibit bacteriostatic and bactericidal activity at concentrations that are not cytotoxic toward normal mammalian cells (Zasloff, 1987; Hultmark, et al., 1980). These peptides are unstructured in dilute aqueous solution, but become helical in amphiphathic media such as micelles, synthetic bilayers, and cell membranes (Kaiser & Kezdy, 1987).

It has been found that sequence homology is not a prerequisite for biological activity. Scores of natural antimicrobial peptides of widely varying sequence have been isolated with amphipathic helical domains as a consistent structural feature

(Saberwal & Nagaraj, 1994). Numerous analogs of native peptides with amino acid substitutions expected to enhance amphipathicity and helicity have shown increased biological activity. Most analogs with increased antimicrobial activity also have increased cytotoxicity (Maloy & Kari, 1995). A notable exception are the melittin-cecropin hybrids which are more bacteriostatic than cecropins and less hemolytic than melittin (Andreu et al., 1989). One of the earliest designed peptides was a melittin analog with a simplified N-terminus and the native C-terminal segment which had hemolytic activity comparable to melittin (DeGrado et al., 1981). A designed amphiphipathic α-helical peptide composed of 2:1 Leu and Lys residues, has a narrow polar face and over 10-fold higher hemolytic activity than melittin (Cornut et al., 1994). D-Melittin, D-magainin, and D-cecropin derivatives were found to have biological activities that were essentially the same as the native peptides (Wade et al., 1990). De novo designed peptides that use the amphipathic helix as the only starting point have been synthesized and have bacteriostatic and cytotoxic activity similar to the native peptides (Lee et al., 1986; Blondelle & Houghten, 1992; Bessalle et al., 1993).

Antimicrobial peptides selectively inhibit and kill bacteria while maintaining low mammalian cell cytotoxicity. The selectivity has been attributed to membrane differences between bacteria and mammalian cells. The exterior membranes of bacteria are negatively charged, whereas mammalian cell exterior membranes are generally neutral (Op den Kamp, 1979). Antimicrobial peptides are positively charged and therefore might preferentially bind to bacteria over mammalian cells (Matsuzaki et al., 1989). The cholesterol in mammalian cell membranes has also been suggested as the basis of the selectivity of antimicrobial peptides (Tytler et al., 1995). Membrane disruption by the antimicrobial peptides could be inhibited by the cholesterol in mammalian cells. Finally the lower membrane potential across

mammalian cells or some combination of the above factors could be responsible for the observed selectivity of the antimicrobial peptides (Matsuzaki et al., 1995).

Whatever the selectivity basis of antimicrobial peptides, the structural differences between cytotoxic and antimicrobial peptides are subtle. This chapter describes our efforts to design, synthesize, and characterize small model peptides that retain or enhance the selectivity of natural antimicrobial peptides. We have used the repetitive heptad approach pioneered by Hodges (Zhou et al., 1992) and DeGrado (Åkerfeldt et al., 1993) which places i and i + 7 residues of the heptads in close proximity when viewed along the helical axis. The heptad building block scheme has been simplified so that repetitive heptads are composed of repetitive trimers plus an additional residue. Peptides with the general sequences $[(PNN)(PNN)P]_n$ and $[(PNN)P(PNN)]_n$ (P= polar residue, N= nonpolar residue, and n=1, 2, 3) were prepared as the C-terminus amides (Figure 2.1).

This design allows global substitution of several amino acids by other amino acids of similar polarity with minimal change in the amphipathy of the peptide. We have examined length effects on biological activity and secondary structure by comparing heptad, 14-mer, and 21-mer peptides. The peptide bacteriostatic and bactericidal activity were tested against Gram negative and Gram positive bacteria and cytotoxicity was tested against a mammalian cell line. The CD spectra of these peptides were measured in buffer, SDS micelles, and phospholipid vesicles. The secondary structure of the peptides in model membranes was correlated with their biological activity.

2.2 Materials and Methods

Peptide Synthesis. Peptides were synthesized by the solid-phase method using a MilliGen 9050 Pep Synthesizer. The peptides were cleaved and deprotected with a trifluoroacetic acid based reagent (88% TFA, 5% water, 5% phenol, 2%

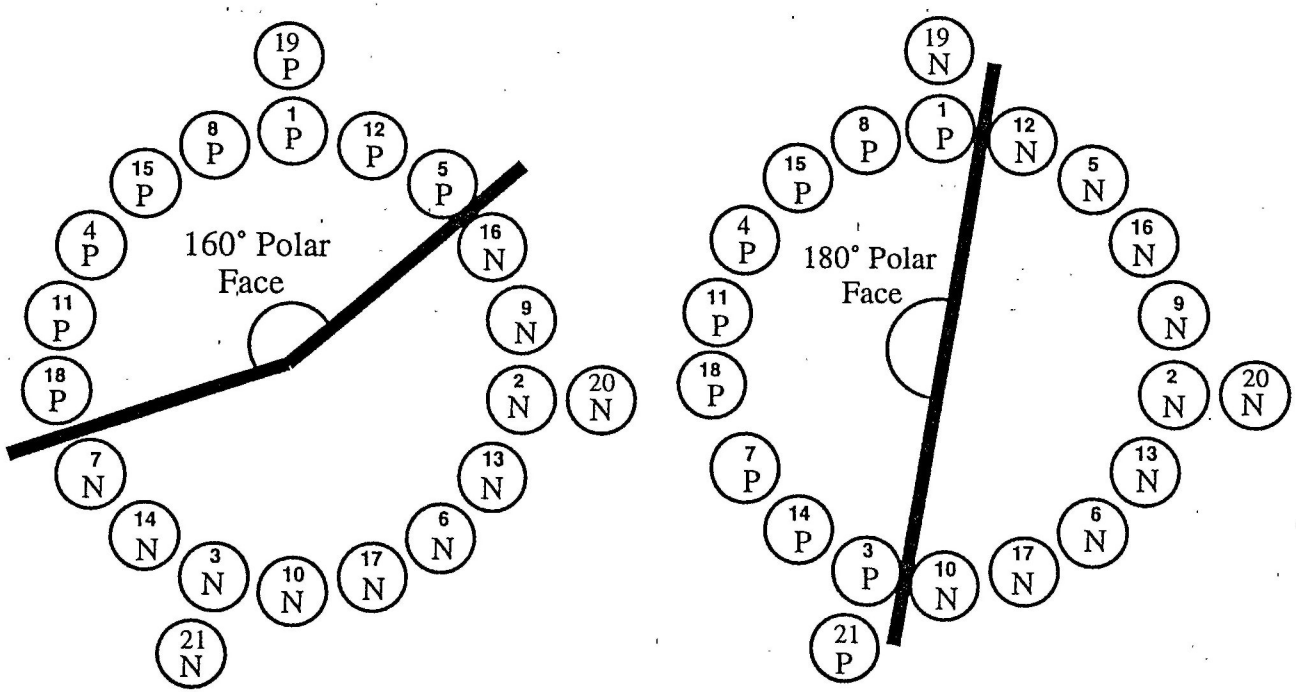


Figure 2.1: Wheel diagram of peptides with the general sequences $[(PNN)P(PNN)]_n$ and $[(PNN)(PNN)P]_n$.

triisopropylsilane) for 2-4 h, dried, taken up in cold 20% acetic acid, extracted with diethyl ether, and lyophilized. Peptides were purified by reverse phase preparative HPLC on a Waters 15-μ Deltapak C_4 column, 200 x 25 mm, using a mobile phase of acetonitrile (0.05% v/v TFA) and water (0.05% v/v TFA) running a gradient of 10-50% of the organic phase over one h. Purity was checked on an analytical Vydac 5-μ C_{18} column running a similar mobile phase gradient and monitoring at 220 nm. The molecular weight of purified peptides was verified by plasma desorption mass spectrometry on a BioIon 20. Peptides were stored as lyophilized powders at -20 °C. Melittin was purchased from Sigma Chemical Company and purified as above.

Amino Acid Analysis. Peptide concentrations are based on quantitative amino acid analysis. Peptide samples containing a norleucine standard were hydrolyzed in 6N HCl, 0.1% phenol, for 24 h at 110 °C, followed by sodium cation exchange chromatography on a Pickering 3 x 250 mm column at 65 °C and post-column derivatization with ninhydrin at 130 °C. Lyophilized peptide samples contain solvent and trifluoroacetate as counterion. The peptide content of lyophilized powders are between 50-80% peptide by weight depending on the charge of the peptide and the efficiency of the solvent removal.

Minimum Inhibitory Concentration Assays. The MIC of peptides were determined against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25723. Peptide 1:2 serial dilutions were prepared from 512 μg/mL stock solutions to give a range of 256 - 2 μg/mL in the culture media. Bacterial cultures were grown to mid-log phase in nutrient broth and were standardized to a 0.5 McFarland turbidity tube before dilution. To each sterile well containing 5 x 10^4 cells in 50 μL, an equal volume of peptide solution was added. The MIC is the lowest concentration that inhibits cell growth as evidenced by absence of turbidity after 4 h (Waitz et al., 1990). The median assay values are

reported for 3-7 separate tests. Assay results vary by no more than one dilution from the median value.

Minimum Bactericidal Concentration Assays. Peptide lysis of *E. coli* and *S. aureus* were tested in a concentration range of 100 μM - 0.1 μM. Bacteria were grown to mid-log phase in nutrient broth, washed in 10 mM phosphate buffer, pH 7, and standardized to a McFarland tube so that the number of cells approximated 10^5 in each test tube. Tubes containing the buffer, cells, and peptide were incubated at 37 °C for 1 h. Serial 1:10 dilutions were made and aliquots were spread on duplicate tryptic soy agar plates. Surviving cells were counted after 18 h of incubation. A set of control plates containing the same number of cells but no peptide were also counted to determine the actual number of cells present and the survival rate.

Mammalian Cell Assays. The efficacy of peptides to induce cell death of 3T3 mouse fibroblasts was determined by 2-fold serial dilution assay. 50 μL of a stock peptide solution was diluted with an equal volume of MEM and 1:2 serial dilutions in MEM were prepared. Each dilution was applied to a 1-day old monolayer of 3T3 cells (approximately 1 x 10^4 cells per well) maintained in a 96-well plate with fresh MEM (50 μl per well). Peptide-treated and control (no peptide) cells were incubated at 37 °C for 30 min. The supernatant was removed and the cells were gently treated with 0.2% trypan blue stain and viewed in an inverted light microscope. Inclusion of trypan blue dye within a cell is indicative of cell death. A sub-lethal dose is defined as the highest dilution in which only 1-10 adherent cells are not stained. In Table 2.1, not lethal means that cell population survival was unchanged from controls when greater than 400 μM peptide was used and the values in Table 2.1 listed as greater than means that at the highest concentration tested, there was some cell lysis but it was less than 99% lysis based on controls defined as sub-lethal.

Circular Dichroism Measurements. CD measurements were made using an Aviv 60DS spectropolarimeter. The instrumental outputs were calibrated with (+)-10-camphorsulfonic acid (Chen & Yang, 1977). Measurements were made over a 250 - 190 nm range in quartz cells of 0.1 - 0.001 cm path length. All CD spectra were recorded at room temperature and were obtained with a 1-nm bandwidth, scan speed of 10 nm/min, and time constant of 5 s. Two scans were obtained to improve the signal to noise ratio. A baseline was recorded and subtracted after each spectrum. Ellipticity is reported as the mean residue ellipticity [θ] in deg cm^2 $dmole^{-1}$; $[\theta] = [\theta]_{obs}$ (MRW/10*lc*), where $[\theta]_{obs}$ is the ellipticity measured in millidegrees, MRW is the mean residue molecular weight of the peptide (molecular weight divided by the number of peptide bonds), *c* is the concentration of the sample in mg/mL, and *l* is the optical pathlength of the cell in cm. Since all the peptides were synthesized using a peptide amide linker, the C-terminus acts as an extra residue (Greenfield & Fasman, 1969).

Vesicle Preparation. Phospholipids were purchased from Avanti Polar Lipids (Alabaster, Alabama). Neutral vesicles were prepared from DLPC and negatively charged vesicles from a mixture of DLPC and DLPG in a molar ratio of 4:1. Unilamellar vesicles of 50-60 nm diameter were prepared from a lipid dispersion in 2.5 mM sodium phosphate, pH 7.4, via extrusion 19 times through a polycarbonate filter (0.05 μm pore size) using a Lipofast extruder device (Avestin, Ottawa, Canada) (New, 1990). Vesicle size was determined using dynamic light scattering as previously described (Kim et al., 1993).

2.3 Results

Several multimers of heptads $[(PNN)(PNN)P]_n$ and $[(PNN)P(PNN)]_n$ with 7, 14, and 21 residues with P equal to lysine and N equal to leucine, alanine, or glycine were synthesized. The sequences are shown Table 2.1. Alanine was replaced by

Table 2.1: Biological activity of peptides[a].

Peptide	*E. coli*	*P. aeruginosa*	*S. aureus*	3T3
(KLAKKLA)	>100	>100	>100	Not Lethal
$(KLAKKLA)_2$	6	3	6	>272
$(KLAKKLA)_3$	4	4	4	11
(KLAKLAK)	>95	>95	>95	Not Lethal
$(KLAKLAK)_2$	6	6	6	>517
$(KLAKLAK)_3$	4	4	4	9
$(KALKALK)_3$	4	4	8	11
$(KLGKKLG)_2$	22	-	22	Not Lethal
$(KLGKKLG)_3$	4	4	4	>393
$(KAAKKAA)_2$	47	-	>256	>525
$(KAAKKAA)_3$	8	-	8	>440
Melittin	3	6	3	1
Magainin 2 amide	10	5	19	60
Cecropin B amide	1	-	12	102

[a]: Bacterial lysis is measured as MICs (μM) and 3T3 mouse fibroblast lysis is measured as sublethal concentration (μM), as described in Materials and Methods section.

glycine in two peptides, $(KLAKKLA)_{2,3}$ vs. $(KLGKKLG)_{2,3}$, to determine if peptide amphipathy would overwhelm the helix destabilization of several glycine residues (O'Neil & DeGrado, 1990; Lyu et al., 1990; Lyu et al., 1991). In another variation, the leucine was replaced with alanine in two peptides, $(KLAKKLA)_{2,3}$ vs. $(KAAKKAA)_{2,3}$, to ascertain whether the reduced hydrophobicity of the alanine peptide would reduce its biological activity.

Bioactivity of Designed Peptides. The MICs for these peptides tested against *E. coli*, *P. aeruginosa*, and *S. aureus* are summarized in Table 2.1. *E. coli* and *P. aeruginosa* are Gram negative bacteria and *S. aureus* is a Gram positive bacterium. The MIC values for cecropin B amide, magainin 2 amide, and melittin are reported for comparison. The 7-mer peptides are inactive. The leucine/alanine containing 14-mers inhibit all three bacteria at approximately 6 μM. $(KLGKKLG)_2$ is about three times less active than the alanine analog $(KLAKKLA)_2$. $(KAAKKAA)_2$ shows only partial inhibition of *S. aureus* at the highest concentration tested. The leucine containing 21-mers are active in the 3-4 μM range against the bacteria tested with the exception of $(KALKALK)_3$ which is less active against *S. aureus*. $(KAAKKAA)_3$ is less active than the leucine containing peptides. Cecropin B amide is the most inhibitory of the peptides tested against *E. coli*. Melittin has MIC values in the range of the most active 21-mers. Magainin 2 amide has activity similar to the moderately active 14-mer, $(KLGKKLG)_2$.

Figure 2.2 shows the minimum bactericidal concentrations for these peptides against *E. coli* (top panel) and *S. aureus* (bottom panel). MBC values should in principle have equal or greater concentrations than the corresponding MIC values, since dead bacteria are also inhibited. The values for the MBC of *E. coli* are within the range expected from the MIC assays for $(KLGKKLG)_2$, $(KLAKLAK)_2$, $(KLGKKLG)_3$, $(KLAKLAK)_3$, and magainin 2 amide, but lower than the

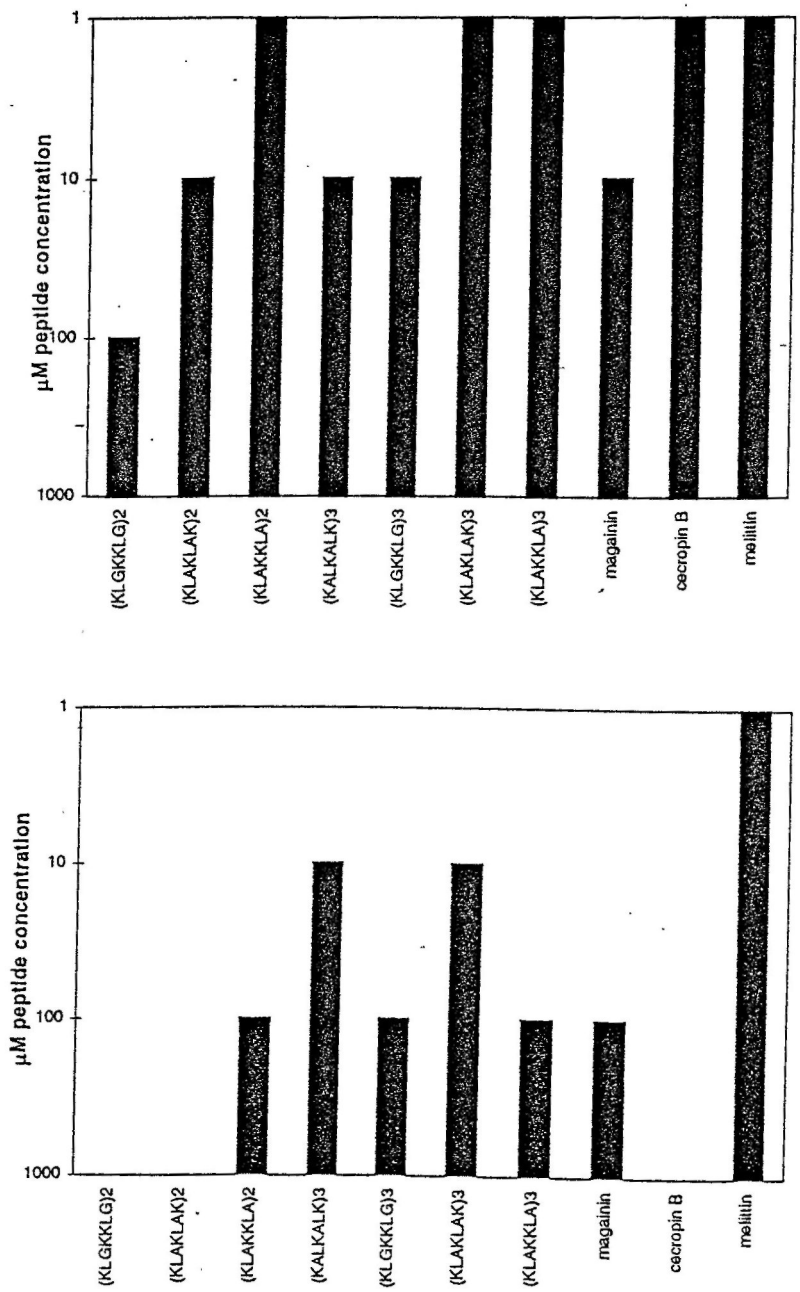


Figure 2.2. Minimum bactericidal concentrations of peptides against *E. coli* (top panel) and *S. aureus* (bottom panel).

corresponding MIC values for $(KLAKKLA)_2$, $(KLAKKLA)_3$, $(KLAKLAK)_3$, melittin, and cecropin B amide. For the latter peptides the *E. coli* MIC values are between 1-10 μM, whereas the MBC values are 1 μM. This difference is within a dilution between the two assays. The *S. aureus* MBC assays give concentrations that are equal to or greater than the corresponding MIC values except for melittin. All of the 21-mers were effective at 10-100 μM against *S. aureus*. None of the 14-mers were effective at killing 99.9% of the *S. aureus* population at the highest concentration tested (100 μM) except $(KLAKKLA)_2$. Melittin performed better in the *S. aureus* MBC assay than in the MIC assay, but the differences between the two assays are within a dilution.

The activity of these peptides towards lysis of mouse embryo fibroblast 3T3 cells is shown in Table 2.1. There is a definite length dependence on bioactivity, 7-mers are inactive, 14-mers have low cytotoxicity, and the 21-mers have greatly increased cytotoxicity except for $(KLGKKLG)_3$ and $(KAAKKAA)_3$.

Helicity of Peptides in Model Membranes. The CD spectra of all the designed peptides in 2.5 mM sodium phosphate buffer, pH 7.4, showed random coil structures at micromolar concentrations. Peptide concentrations as high as 12 mM had no effect on the secondary structure of the 7- and 14-mers. On the other hand, the molar ellipticity at 222 nm of $(KLAKKLA)_3$, $(KLAKLAK)_3$, and $(KLAKKLA)_3$ was concentration dependent, with an apparent increase in helicity with increasing peptide concentration in the range of 8-12 mM (not shown).

Table 2.2 gives the helical content of all the designed peptides in 25 mM SDS. These results clearly demonstrate that the molar helicity of the peptides in SDS is correlated to the chain length of the peptide. As the number of heptad repeats increases, the helical content of the peptide increases. The alanine containing peptides of the same length show a similar helical content, whereas the analogous

Table 2.2: α-Helicity of peptides in SDS micelles[a]

Peptide	$[\theta]_{222}$[b]	% α-Helix[c]
(KLAKKLA)	-4,848	0
$(KLAKKLA)_2$	-10,647	24
$(KLAKKLA)_3$	-29,049	79
(KLAKLAK)	-1,126	0
$(KLAKLAK)_2$	-15,334	37
$(KLAKLAK)_3$	-29,195	79
$(KALKALK)_3$	-25,053	67
$(KLGKKLG)_2$	-911	0
$(KLGKKLG)_3$	-13,856	33
Melittin	-33,600	93
Magainin 2 amide	-18,300	46

[a]: 10mM sodium phosphate buffer, pH 7.4, except $(KALKALK)_3$. 2.5mM sodium phosphate buffer pH 7.4, was used for $(KALKALK)_3$ which was insoluble at higher phosphate concentration.

[b]: CD spectra were recorded from 250 to 190 nm in 25 mM SDS.

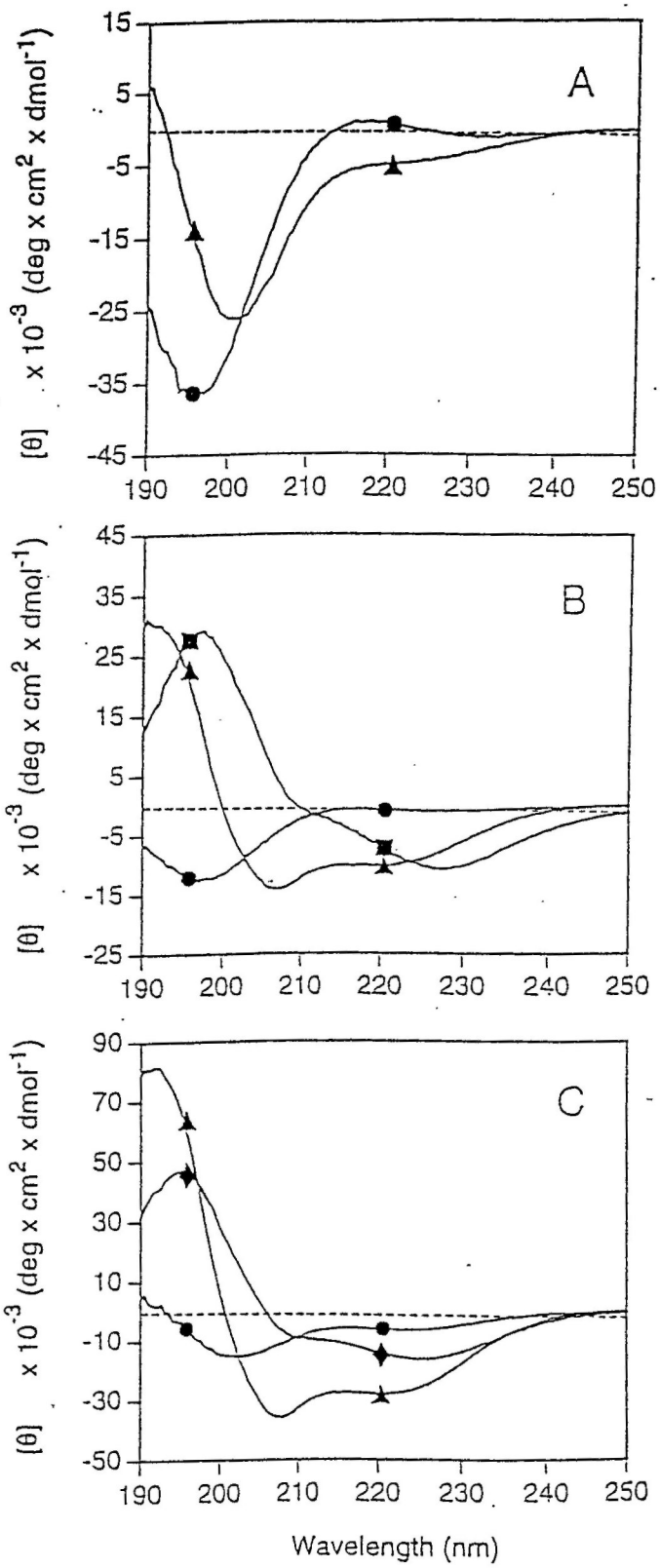
[c]: The % α Helix = $-100\ ([\theta]_{222} + 3000)/33000$ (Mc Lean 1991)

glycine containing peptides have significantly lower helical content. The helical content of $(KLGKKLG)_3$ and the alanine containing 14-mers are in the 24-37% range. Magainin 2 is moderately helical; the leucine/alanine containing 21-mers and mellitin are more helical.

Several CD spectra from a titration of $(KLAKKLA)_n$ (n=1, 2, 3) in SDS are shown in Figure 2.3. SDS titration of (KLAKKLA), (panel A), fails to induce any secondary structure. In the absence of SDS, the CD spectra of dilute aqueous solutions of the 14- and 21-mer indicate that $(KLAKKLA)_2$ (panel B) is in a random coil conformation, and $(KLAKKLA)_3$ (panel C) lacks well-defined structure but is not completely random. This is revealed by the weak negative band around 200 nm. In solutions of SDS below the cmc, the CD spectra of $(KLAKKLA)_2$ and $(KLAKKLA)_3$ show a type II β-turn secondary structure (Wu et al., 1981). At SDS concentrations of 8-12 mM, the onset of α-helicity occurs and helicity levels off at the maximum observed for each peptide at about 20 mM SDS (not shown). The CD spectra from the SDS titration of the remaining 14- and 21-mers follow the same trend, except for $(KLGKKLG)_2$ which fails to show helical structure and $(KAAKKAA)_{2,3}$ which precipitates at intermediate SDS concentrations. None of the peptides in the series show an isodichroic point during the SDS titration.

The CD spectra from the titration of a 30 μM aqueous solution of $(KLAKKLA)_3$ (panel A) and $(KLGKKLG)_3$ (panel B) in DLPC/DLPG vesicles are shown in Figure 2.4. In the absence of vesicles, the peptides display a mostly random coil CD spectrum. Upon vesicle addition, the peptides become α-helical. Table 2.3 shows a comparison of the helical content of $(KLAKKLA)_3$ and $(KLGKKLG)_3$ in DLPC and DLPC/DLPG vesicles. The peptides show maximum helicity in neutral and negatively charged vesicle preparations at a peptide/lipid ratio of at least 1:100. The amount of helix formed by $(KLAKKLA)_3$ in either vesicle

Figure 2.3. SDS titration of $(KLAKKLA)_n$ (n=1,2,3) CD spectra in 10 mM sodium phosphate buffer pH 7.4; (A). 240 μM (KLAKKLA), (B). 120 μM $(KLAKKLA)_2$, and (C). 32 μM $(KLAKKLA)_3$. The concentration of peptide is constant with varying, (●) no SDS, (■) 1.25 mM SDS, (◆) 2.5 mM SDS, (▲) 25 mM SDS micelle concentration.


A
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200
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230
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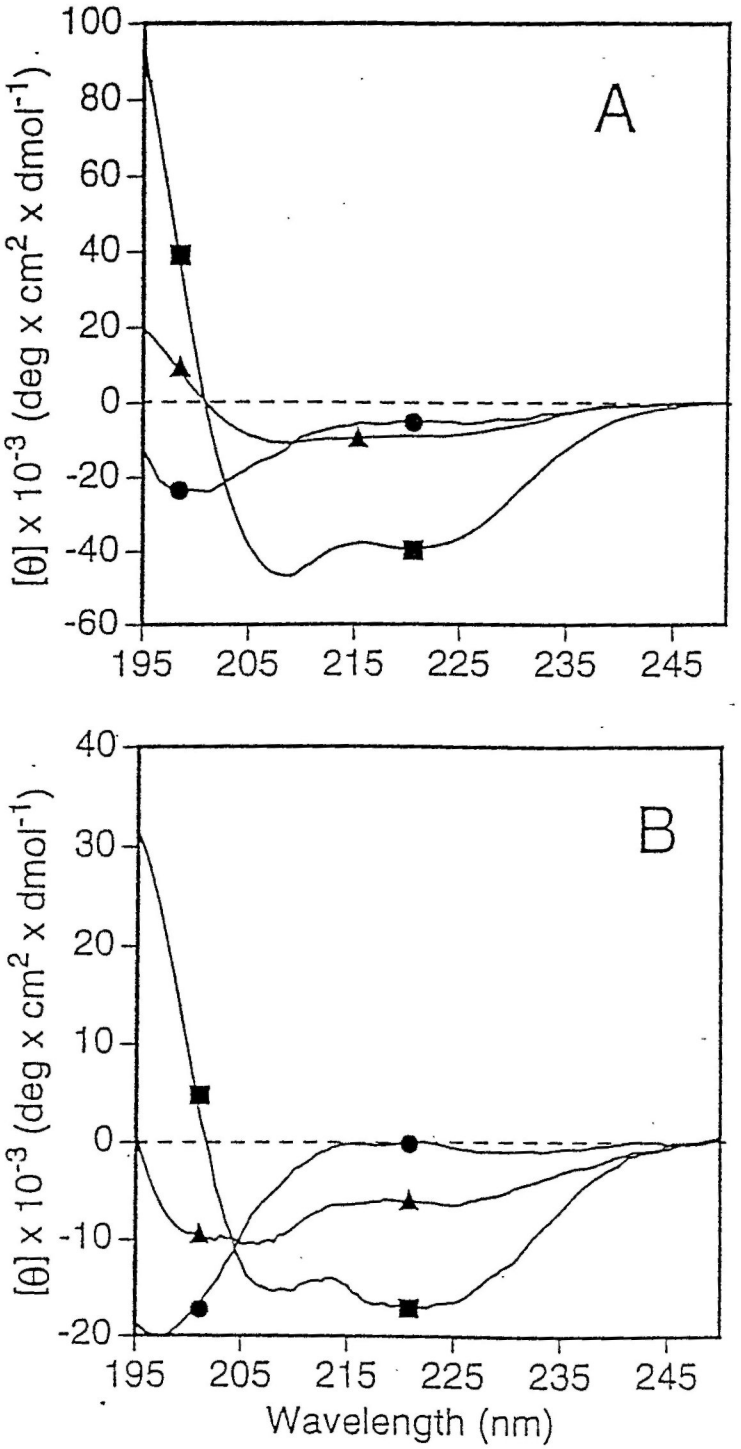
250

Figure 2.4. Circular dichroism spectra of A. $(KLAKKLA)_3$ and B. $(KLGKKLG)_3$ plus DLPC/DLPG (4:1) liposomes. The ratios are lipid to peptide concentrations, (●) 0, (▲) 1:30, (■) 1:100, with constant peptide concentration of 30 μM.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235
245
[θ] x 10-3 (deg x cm2 x dmol-1)
Wavelength (nm)

preparation is much greater than that for $(KLGKKLG)_3$. Both peptides have higher α-helicity in negatively charged vesicles than in neutral vesicles (Table 2.3).

2.4 Discussion

We have designed a series of highly amphipathic α-helical peptides of the general sequences $(PNNPPNN)_n$ and $(PNNPNNP)_n$ with P equal to lysine; N equal to leucine and alanine or glycine, or just alanine, and n equal to 1-3 or 2 and 3. Leucine and alanine were specifically chosen because of their high helical propensity (Chou & Fasman, 1978; Scheraga, 1978; Sueki et al., 1984). Most antimicrobial peptides have several basic residues. Lysine was chosen as the polar residue because of high helical propensity and ease of peptide synthesis compared to arginine (Bessalle et al., 1993). These peptides were designed to determine the effect of length, sequence, and structure on biological activity. All of the leucine and alanine containing peptides of the same length have similar MIC values. The difference in polar face angle predicted from the wheel diagram, 160 ° for $(KLAKKLA)_n$ and 180 ° for $(KLAKLAK)_n$, has no discernible effect on the biological activity of these peptides. The 7-mers were inactive. The leucine containing 21-mers were about twice as potent as the 14-mers. The $(KALKALK)_3$ 21-mer sequence was slightly less active. The MIC values for melittin, magainin 2 amide, and cecropin B amide provide a basis for comparison with assays performed in other laboratories. Most researchers reporting on the design of new natural or *de novo* antimicrobial peptides have reported MIC values. The MIC assay measures bacteriostatic activity. It is generally believed that these peptides inhibit bacterial growth by destroying most of the cell population. If this is the case, then MIC assays should agree with MBC assays which measure bactericidal activity. Our results with *E. coli* show only small or insignificant differences between the two assay procedures. But with assays run against *S. aureus*, MIC values can be several dilutions lower than the corresponding

Table 2.3: % α-Helicity[a] of peptides in DLPC and DLPC/DLPG (4:1) liposomes[b].

Peptide		DLPC		DLPC/DLPG (4:1)	
	Free Peptide	1:30	1:100	1:30	1:100
$(KLAKKLA)_3$	0	25	60	55	85
$(KLGKKLG)_3$	0	3	12	21	45
Melittin	0.5	43	58	48	50
Magainin 2 amide	0	13	40	35	38

[a]: % α-helix = -100 [([θ]222 + 3000)/33000] (Mc Lean, 1991)
[b]: 2.5 mM sodium phosphate, 10 mM NaCl, pH 7.4

MBC values, suggesting that *S. aureus* is inhibited but not killed. It is clear from this study using both assays, that bacteriostatic concentrations can be significantly lower than bactericidal concentrations at least for *S. aureus*.

The leucine/alanine containing peptides with the same number of heptad repeats, $[(KLAKKLA)_n$, $(KLAKLAK)_n$ (n=1, 2, 3), and $(KALKALK)_3]$, have nearly equivalent cytotoxicity against 3T3 cells The 21-mers are only about ten times less toxic than melittin. The 14-mer peptides and $(KLGKKLG)_3$ have low cytotoxicity against 3T3 cells. The leucine/alanine containing 14-mers and $(KLGKKLG)_3$ are potent antibacterial agents. These peptides are bacteriostatic at 100-200 times lower concentrations than the sub-lethal concentration against 3T3 cells. Moreover, they are 2-5 times less cytotoxic than the natural peptides, magainin 2 amide and cecropin B amide.

The natural antimicrobial peptides, magainin 2 amide and cecropin B amide, have no defined structure in dilute aqueous media but become helical upon membrane association. Several studies of idealized amphipathic peptides have demonstrated that peptide-peptide hydrophobic interactions can induce peptide secondary structure and contribute to the stability of α-helices in aqueous solution (Kanehisa & Tsang, 1980; Hodges et al., 1981; Eisenberg et al. 1984; Lau et al., 1984; DeGrado et al., 1989). Coiled-coil peptide dimers are only stable at micromolar concentrations with at least 28 residues (Landschulz et al., 1988; Hodges et al., 1990; Zhou, 1992; Alber, 1992). All of the designed peptides reported herein were unstructured in aqueous buffer at micromolar concentrations. The leucine/alanine containing 21-mer peptides showed apparent self-association induced helicity, but at concentrations approximately 100 times higher than the bacteriostatic concentrations. Therefore, potential peptide aggregation in solution is probably not playing a role at the concentrations used in the assays. As expected, we failed to

detect any concentration dependent helicity in aqueous buffer for the intrinsically less helical peptide, $(KLGKKLG)_3$. The greater conformational flexibility of glycine relative to alanine is the likely cause for this difference.

The CD spectra of peptides in micelles and of representative peptides in phospholipid vesicles suggests a structure/function relationship for these biologically active peptides. SDS forms micelles that partially mimic the amphipathic environment of membranes. SDS possesses an aliphatic tail and a negatively charged head group. The negatively charged head group provides the initial electrostatic interactions needed and enhances the binding affinity of peptides to the nonpolar interior (Wu et al., 1981; Wu & Yang, 1988; Gierasch, 1989). The percent helicity of the peptides in 25 mM SDS micelles, well above the cmc, correlates with their cytotoxicity to mammalian cells. The SDS titration experiments show that peptide helicity peaks and levels off at less than the 25 mM SDS used for peptide comparisons. Melittin has very high helicity (93%) and cytotoxicity, the leucine and alanine containing 21-mers have helicity of 66-79% and high cytotoxicity, magainin 2 amide has modest helicity and cytotoxicity, and $(KLGKKLG)_3$ and the leucine and alanine containing 14-mers have lower helicity values and lower cytotoxicity than magainin 2 amide. The 7-mer peptides lack any helicity in membrane mimetic environments and are devoid of biological activity. A plot of helicity versus log of the sub-lethal concentration gives a correlation coefficient of 0.92.

The SDS titrations indicate transitions in peptide secondary structure from mostly random coil in aqueous buffer to a type II β-turn in submicellar SDS concentrations and to an α-helix at 8-12 mM SDS. Low concentrations (2-4 mM) of SDS induce β-structure (Wu et al., 1981). At SDS concentrations less than 3.5 mM, the 14- and 21-mer peptides show varying amounts of β-structure. The induction of a β-structure in submicellar concentrations of SDS is possibly due to hydrophobic

interactions between monomeric SDS and the peptides. Presumably the hydrophobic tail of the SDS interacts with the hydrophobic regions of the peptides, and the hydrophilic head group of SDS keeps the β-structure in solution (Zhong & Johnson, 1992). At SDS concentrations above 8-12 mM, near the SDS cmc, the peptides tend to fold into an α-helical conformation (data not shown). At a ratio of about 1:1 peptide to micelle (micelle aggregation number of 62 SDS monomers), the peptides reach maximum helical content and no change is observed upon further addition of SDS.

The secondary structure of $(KLGKKLG)_3$ and $(KLAKKLA)_3$ in phospholipid vesicles was also determined using CD. The exterior phospholipid surface of bacteria are generally negatively charged whereas most mammalian cells are neutral (Op den Kamp, 1979). Vesicles were prepared from a mixture of DLPC/DLPG in a 4:1 molar ratio (20% negative charge on the surface) to mimic bacterial exterior cell membranes. Pure DLPC vesicles (neutral) were used as a model membrane for mammalian cells. The peptides show maximum helicity in neutral and negatively charged vesicle preparations at a peptide/lipid ratio of at least 1:100. The amount of helix formed by $(KLGKKLG)_3$ in either vesicle preparation is lower than that for $(KLAKKLA)_3$. Both 21-mer peptides show greater α-helicity in negatively charged vesicles than in neutral vesicles.

In correlating cytotoxicity with α-helicity, we have ignored the binding properties of the peptides. Differences in helical content could be due to the binding affinity of the peptide with membrane as well as the helical content of bound peptide. These considerations are being addressed for some of these peptides in phospholipid model membrane environments (Bishop et al., 1996). These results show that bound peptide is essentially 100% helical and that binding affinity differences account for the reduced of helicity of $(KLGKKLG)_3$ compared to $(KLAKKLA)_3$.

The alanine containing 14-mer peptides have similar bacteriostatic activity to magainin 2 amide and cecropin B amide with substantially lower cytotoxicity. The analogous $(KLGKKLG)_{2,3}$ peptides have slightly less antibacterial activity but are much less cytotoxic than magainin 2 amide or cecropin B amide. Magainin 2 amide, cecropin B amide, and several other selective natural lytic peptides contain one or more helix breaking residues and are less perfectly amphipathic than the idealized peptides described herein. The imperfect amphipathy and resulting lower binding affinity of the natural antimicrobial peptides were probably selected to minimize indiscriminate host organism cell lysis.

2.5 References to Chapter 2

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Chapter 3

Self-assembly of Designed Antimicrobial Peptides in Solution and Micelles

3.1 Introduction

Among the different types of noncovalent interactions in polypeptides and proteins, hydrophobic interactions between nonpolar residues are thought to be one of the most important in forming and stabilizing the native protein structure (DeGrado & Lear, 1985; Muller, 1991). Amphipathic α-helices have opposing polar and nonpolar faces oriented along the helix axis. These helices not only make up 50% of the α-helices found in soluble globular proteins (Cornette et al., 1987; Segrest et al., 1990), but they also occur in DNA-binding proteins (Landschulz et al., 1988; O'Shea et al., 1989), fibrous proteins such as myosin (Cohen & Parry, 1986) and tropomyosin (Sodek et al., 1972; Hodges et al., 1981), as well as smaller molecules such as polypeptide hormones (Kaiser & Kezdy, 1983; Taylor et al., 1984) and polypeptide venoms (Taylor et al., 1984; Argiolas & Pisano, 1985). Recently, it has become apparent that many organisms, ranging from prokaryotes to humans, use peptides that assume a unique amphipathic secondary structure upon interaction with the outer phospholipid bilayer of the bacteria, as part of their host defense system (Boman & Hultmark, 1987; Zasloff, 1987; Ganz et al., 1990).

The coiled-coil is a widespread structural motif consisting of two, three, or four α-helices characterized by a heptad repeat of seven amino acids designated a through g (McLachlan & Stewart, 1975). The most striking feature of the coiled-coil structure is the hydrophobic 3-4 or 4-3 repeat, in which hydrophobic residues fall on the same face of the helix, resulting in a hydrophobic interface between the helices of the coiled-coil. The term "leucine zipper" is used for coiled-coils if the hydrophobic a and d positions of the heptad repeat are occupied by leucine. Positions e and g are often charged residues (Cohen & Parry, 1986) whose side chains can also participate

in interhelical electrostatic interactions (Glover & Harrison, 1995; Lumb & Kim, 1995), while residues at positions b, c and f are generally exterior and exposed to solvent. Coiled-coils with as few as 14 residues (GAL4-DNA complex; Marmorstein et al., 1992) and as many as 1086 residues (myosin; McLachlan & Karn, 1982) occur in proteins. However, the majority of studies investigating the factors that contribute to the formation and stability of coiled-coils use synthetic peptides of *de novo* design containing 5-7 hydrophobic heptad repeats (Monera et al., 1993; Thompson et al., 1993). Recently Su et al. (1994) have reported that a minimum of three heptads corresponding to six helical turns is required for a peptide to adopt a two-stranded α-helical coiled-coil conformation in aqueous solution.

Amphipathic α-helices have a pronounced tendency to self-associate. The equilibrium constant for self-association of amphipathic peptides results from two antagonistic effects: a favorable hydrophobic effect that leads to burial of nonpolar residues within the oligomer, and an unfavorable electrostatic force due to proximity of highly charged surfaces. Self-association is promoted by screening or suppressing the charges and also by increasing the size of the nonpolar face in the peptides. Synthetic helices have been designed that aggregate to form dimers, trimers, tetramers, and hexamers with parallel and antiparallel orientations (Lau et al., 1984; Regan & DeGrado, 1988; Hill et al., 1990; Chin et al., 1992; Harbury et al., 1993). Also, in a number of proteins of diverse function (Cohen & Parry, 1986; Lupas et al., 1991), the leucine zipper domain has been identified as the noncovalent dimerization surface for homodimer (O'Shea et al., 1992) and heterodimer assembly (O'Shea et al., 1989).

Using a minimalist approach (Zhou et al., 1992; Åkerfeldt et al., 1993), we previously designed amphipathic α-helical peptides with heptad repeats that have high antimicrobial activity (Javadpour et al., 1996). In this chapter, we examine the

self-association of these and other related peptides. We investigate the effects of the size of the nonpolar domain and of substitution of phenylalanine at positions a and d on the stability and aggregation state of the coiled-coil in solution. Circular dichroism spectroscopy was used to monitor peptide secondary structure. Analytical ultracentrifugation was used to determine the oligomerization model and equilibrium constant. Thermodynamic properties of the self-association were derived from the temperature dependence of the equilibrium constant. We also determined the aggregation state of carboxyfluorescein-labeled peptides in DPC micelles. Finally, the effect of self-assembly on antimicrobial activity of the peptides was considered.

3.2 Materials and Methods

Peptide Synthesis and Labeling. The peptides were synthesized by a solid-phase method using a MilliGen 9050 PepSynthesizer. 9-Fluorenylmethyloxycarbonyl amino acids protected at the α-amino function were purchased from MilliGen, and activated as pentafluorophenyl esters at the carboxyl function. Coupling was performed using HOBt (Fisher Biotech, Fair Lawn, NJ) in an extended cycle. Polystyrene resin with a peptide amide linker was purchased from MilliGen. The peptide was cleaved from the resin and deprotected by treatment with a trifluoroacetic acid reagent (88% TFA, 5% water, 5% phenol, 2% triisopropylsilane) for 2-4 h, extracted in cold 20% acetic acid and diethyl ether, and lyophilized.

Peptides were purified by reverse phase chromatography on a Waters 15 μ Deltapak C_4 column, 200 x 25 mm, using a gradient of 10-50% acetonitrile (0.05% v/v TFA) and water (0.05% v/v TFA). Purity was checked on an analytical Vydac 5 μ C_{18} column monitored with a UV detector at 220 nm. The molecular mass of purified peptide was verified by plasma desorption mass spectrometry. Peptides were stored as lyophilized powders at -20 °C. Peptide concentrations were

determined by quantitative amino acid analysis. Peptide samples containing a norleucine standard were hydrolyzed in 6N HCl, 0.1% phenol, for 24 h at 110 °C, followed by sodium cation exchange chromatography on a Pickering 3 x 250 mm column and derivatization with ninhydrin at 130 °C.

Peptides were labeled at their N-termini with carboxyfluorescein (Eastman Kodak, Rochester NY). Derivitization was carried out on the resin using the following coupling cycle: 1 mol equivalent each of carboxyfluorescein, BOP (Castro's reagent; Millipore, Bedford, MA), and HOBt and 1.5 mol equivalent NMM in dry DMF for 3-5 hrs. Peptides were cleaved from the resin, deprotected, and purified by HPLC as described above. Fluorescent-labled peptides were monitored during HPLC using UV (220 nm) and fluorescence (excitation 470 nm, emission 520 nm) detectors. The correct product was confirmed by plasma desorption mass spectrometry and peptide concentration was determined by quantitative amino acid analysis.

Circular Dichroism. CD measurements were made using an Aviv 60DS spectropolarimeter. The wavelength was calibrated with (+)-10-camphorsulfonic acid (Chen & Yang, 1977). Measurements were made over the 250-190-nm range in quartz cells of 0.1-0.001 cm path length. All CD spectra were recorded at room temperature with 1-nm bandwidth, 10-nm/min scan speed, and 5-s time constant. Duplicate scans were acquired to improve the signal-to-noise ratio. A baseline was recorded and subtracted after each spectrum. The mean residue ellipticity $[\theta]$ in deg cm^2 $dmole^{-1}$ was calculated from

$$[\theta] = [\theta]_{obs} (MRW/10lc) \qquad (3.1)$$

where $[\theta]_{obs}$ is the ellipticity measured in millideg, MRW is the mean residue molecular mass of the peptide (molecular mass divided by the number of amino acid residues), l is the optical pathlength of the cell in cm, and c is the concentration of

the sample in mg/mL. Since all the peptides were synthesized as C-terminal amides, the C -terminus acts as an extra residue (W. C. Johnson, personal communication). % Helicity was calculated from (McLean et al., 1991)

$$\% \text{ Helicity} = [-100(\theta_{222} + 3000)]/33{,}000 \qquad (3.2)$$

Analytical Ultracentrifugation. Sedimentation equilibrium experiments were performed using a Beckman XL-A analytical ultracentrifuge. Experiments were run in a 4-hole rotor with a counterbalance and 3 carbon-filled epoxy double-sector cells at a rotor speed of 40,000 rpm at 10 temperatures from 2-38 °C. Peptides were dissolved in 2.5 mM sodium phosphate buffer, pH 7.4, 0.4 M NaCl. Peptide concentrations were 1 and 2 mM for 21-mer leucine peptides, 2 and 4 mM for 21-mer phenylalanine peptides, and 0.1 mM for 28-mer peptides. The concentration distribution of peptide within the cell was determined by absorbance at 254 or 236 nm for the 21- or 28-mer leucine peptides and 273 or 222 nm for the 21- or 28-mer phenylalanine peptides. Baseline scans were measured at 365 nm, where the peptides have no absorption. The time required to attain equilibrium at the first temperature was established by running until the scans were invarient for 12 h; this was achieved by 34 h. The temperature was increased in 4 °C increments and scans were taken after 24 h at each temperature. It was verified that this time was sufficient for reequilibration at the new temperature. Scans were collected with a radial step size of 0.001; 10 acquisitions were averaged at each radial position to give the final data for analysis.

Concentration distributions were fit to various mathematical models by least squares using MLAB (Civilized Software, Inc., Bethesda, MD; Knott, 1979) . Best fit criteria were minimum sum of squares of the residuals, random distribution of the residuals, and minimum standard errors of parameters.

A mathematical model for a reversible monomer-tetramer interaction was used.

$$c_r = c_{b,1} \exp[AM_1(r^2 - r_b^2)] + c_{b,1}^4 \exp[\ln k_{14} + 4AM_1(r^2 - r_b^2)] + \varepsilon \qquad (3.3)$$

where $c_{b,1}$ is the concentration of monomer at r_b, the radial position of the cell bottom; M_1 is the molecular mass of the monomer calculated from the amino acid sequence; and ε is a small base-line error correction term. $A = (1 - \bar{\nu}\rho)\omega^2/2RT$, where $\bar{\nu}$ is the partial specific volume of the monomer, ρ is solvent density, ω is angular velocity, R is the gas constant, and T is absolute temperature. The partial specific volume of the monomer was calculated from the amino acid sequence of the peptide (Zamyatnin, 1984). It was also measured using H_2O and D_2O containing buffers (Edelstein & Schachman, 1967) and fitting the two sedimentation equilibrium data sets simultaneously to eq 3.3 with $\bar{\nu}$ as an adjustable parameter. Eq 3.3 assumes that monomer and tetramer have the same partial specific volume. The value of $\bar{\nu}$ determined from both methods was the same within experimental error. The equilibrium constant for tetramer formation is defined as $k_{14} = c_{b,4}/c_{b,1}^4$, where $c_{b,1}$ and $c_{b,4}$ refer to the concentration of monomer and tetramer, respectively, at the radial position of the cell bottom and are given in terms of absorbance at the scanning wavelength. If the tetramer species is absent, the fitting procedure will return a large negative number for the value of $\ln k_{14}$.

The mathematical formulation in eq 3.3 ensures that the values of the equilibrium constants are physically meaningful by virtue of having positive values. The use of the logarithm of the equilibrium constant acts as an implicit constraint. Fitting for $\ln k_{14}$ also has the advantage that the logarithm of the molar equilibrium constant, taking 1 mol of the monomer as the standard state, is given by $\ln K_{14} = \ln k_{14} + \ln(E_1^3/4)$ where E_1 is the molar extinction coefficient of the monomer at the scanning wavelength. These values are used to calculate thermodynamic parameters

such as the standard free energy change, since $\Delta G^\circ = -RT\ln K$. Finally, the standard error estimates for fits to the logarithm of the equilibrium constant appear to be more nearly valid (Lewis, 1992).

The temperature dependence of ln K_{14} was analyzed by the method of Clarke and Glew (1966) using the Taylor's series expansion about the reference temperature θ

$$R \ln K = -\Delta G^\circ_\theta/\theta + \Delta H^\circ_\theta (1/\theta - 1/T) + \Delta C^\circ_{P,\theta} [\theta/T - 1 + \ln(T/\theta)]$$
$$+ \theta/2 \, (d\Delta C^\circ_P/dT)_\theta [(T/\theta) - (\theta/T) - 2\ln(T/\theta)]$$
$$+ \theta^2/12 \, (d^2\Delta C^\circ_P/dT^2)_\theta [(T/\theta)^2 - 6(T/\theta) + 3 + 2(\theta/T) + 6\ln(T/\theta)] \quad (3.4)$$

Eq 3.4 describes the temperature dependence of the equilibrium constant. The fitting parameters are ΔG°_θ, ΔH°_θ, $\Delta C^\circ_{P,\theta}$, $d\Delta C^\circ_P/dT$, and $d^2\Delta C^\circ_P/dT^2$. The reference temperature was taken to be 298.15 K. Data were weighted by the normalized reciprocals of the variances of the values of ln k calculated from the approximate standard errors obtained when fitting the sedimentation equilibrium concentration distribution. Best fit criteria were minimum sum of squares and minimum standard errors of parameters.

The molecular weight of peptides in micelles was measured at 22 °C by the method of Reynolds and Tanford (1976). Samples were made from stock solutions of DPC and carboxyfluorescein-labeled peptide diluted to the final concentration with different ratios of H_2O/D_2O to yield a range of densities. Stock solutions were 0.3 M DPC in 25 mM sodium phosphate buffer, 0.1 M NaCl, pH 7.4; 0.01 M CF-(KLAKKLA)$_3$ in water; and 0.01 M CF-(KLGKKLG)$_3$ in water. Final concentrations were 2.5 mM sodium phosphate buffer, pH 7.4, 0.01 M NaCl containing 2×10^{-4} M CF-peptide and 3×10^{-2} M DPC. This DPC concentration was > 20-fold above the critical micelle concentration of 1.1×10^{-3} M (Lauterwein et al., 1979). Reference solutions contained 3×10^{-2} M DPC and buffer at the same

density as the sample. Densities ranging from 0.99905-1.09236 g/cm^3 were measured on a Paar DMA 58 density meter. Concentration distributions of carboxyfluorescein-labled peptides within the cell were determined from absorbance at 537 nm. Baseline scans were measured at 634 nm, where the peptides and the detergent have no absorbance. Experiments were run at a rotor speed of 40,000 rpm. The time required to reach equilibrium was 34 h. Partial specific volumes of carboxyfluorescein-labeled peptides in solution were measured as described above. The partial specific volume of $(KLAKLAK)_3$ was measured in 15% hexafluoroisopropanol to model the micelle environment.

Fluorescence Quantum Yields. Quantum yields were determined relative to ANS in ethanol on an SLM 8000 spectrofluorometer interfaced to a Macintosh IIcx computer. Temperature was maintained at 25 °C with a Lauda circulating bath. Samples were excited at 360 nm and emission spectra were scanned from 410-600 nm in ratio mode with single excitation and emission monochromators set at 4- and 8-nm bandpass, respectively. Magic angle polarizers were set to 55° on the excitation side and 0° on the emission side to avoid the Wood's anomaly of the emission grating. A solvent blank was subtracted, and spectra were corrected for wavelength-dependent instrument response using correction factors determined with a standard lamp from Optronics.

Biological Activity. MICs of peptides were determined against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25723. Peptide 1:2 serial dilutions were prepared from 512 μg/mL stock solutions to give a range of 256 - 2 μg/mL in the culture media. Bacterial cultures were grown to mid-log phase in nutrient broth and were standardized to a 0.5 McFarland turbidity tube before dilution. To each sterile well containing 5×10^4 cells in 50 μL, an equal volume of peptide solution was added. The MIC is the lowest peptide concentration that

inhibits cell growth as evidenced by absence of turbidity after 4 h (Waitz et al., 1990). The median assay values are reported for 3-7 separate tests. Assay results vary by no more than one dilution from the median value.

MBCs of peptides were tested against *E. coli* and *S. aureus* at 100, 10, and 0.1 μM. Bacteria were grown to mid-log phase in nutrient broth, washed in 10 mM phosphate buffer, pH 7, and standardized to a McFarland tube so that the number of cells approximated 10^5 in each test tube. Tubes containing the buffer, cells, and peptide were incubated at 37 °C for 1 h. Serial 1:10 dilutions were made and aliquots were spread on duplicate tryptic soy agar plates. Surviving cells were counted after 18 h of incubation. A set of control plates containing the same number of cells but no peptide were also counted to determine the actual number of cells present and the survival rate. The MBC is the peptide concentration needed to kill ≥99% of the cells.

The efficacy of peptides in killing 3T3 mouse fibroblasts was determined by a 2-fold serial dilution assay. 50 μL of a stock peptide solution was diluted with an equal volume of MEM and 1:2 serial dilutions in MEM were prepared. Each dilution was applied to a 1-day old monolayer of 3T3 cells (approximately 1×10^4 cells/well) maintained in a 96-well plate with fresh MEM (50 μl/well). Peptide-treated and peptide-free control cells were incubated at 37 °C for 30 min. The supernatant was removed and the cells were gently treated with 0.2% trypan blue stain and viewed in an inverted light microscope. Inclusion of trypan blue dye within a cell is indicative of cell death. A sub-lethal dose is defined as the highest dilution in which only 1-10 adherent cells are not stained.

3.3 Results

Peptide Design. Peptides with the general sequences $[(KNpNp)(KNpNp)K]_n$ and $[(KNpNp)K(KNpNp)]_n$, (n = 3, 4), where Np = A/G and

L/F were designed to form amphipathic α-helices (Figure 3.1). These sequences are isomeric and correspond to 3-4 and 4-3 hydrophobic repeats. In general, most designed amphipathic helices use alanine and leucine residues to form the hydrophobic face of the helix. These residues have high helix propensity (Chou & Fasman, 1978; Scheraga, 1978; Sueki et al., 1984). Lysine is chosen as the polar residue not only because of high helix propensity (Cohen & Parry, 1986), but also because the presence of lysine and alanine on adjacent turns of the helix stabilizes the coiled-coil. The leucine peptides have the sequences $(KLAKLAK)_3$ and $(KLAKKLA)_n$, while the phenylalanine peptides substitute phenylalanine for leucine, $(KFAKFAK)_3$ and $(KFAKKFA)_n$. Since the hydrophobicities of leucine and phenylalanine appear to be the same (Guo et al., 1986; Parker et al., 1986), the question we are probing is the effect of the shape of the side chains at positions a and d on the stability and number of strands in the coiled-coil. A peptide substituting glycine for alanine, $(KLGKKLG)_3$ was also synthesized. Glycine is a helix destabilizing amino acid (Lyu et al., 1990; O'Neil & DeGrado, 1990; Lyu et al., 1991).

Secondary Structure. Circular dichroism spectroscopy was used to monitor secondary structure of the peptides. CD spectra were measured in two buffers: 2.5 mM sodium phosphate buffer, pH 7.4 or 10 mM Tris buffer, pH 7.4 at 25°C. The 21-mer peptides have a random structure with a characteristic minimum near 200 nm in both buffers. The 28-mer peptides form large insoluble aggregates in phosphate buffer. However, the 28-mers are soluble in Tris buffer with ~ 36% α-helix. The designed amphipathic α-helices have a polar face consisting entirely of lysines with a total charge of +9 and +12, respectively, for the 21 and 28-mer peptides. Repulsive forces between the charged lysines favor the extended random structure. According to polyelectrolyte theory, increasing the salt concentration should promote α-helix

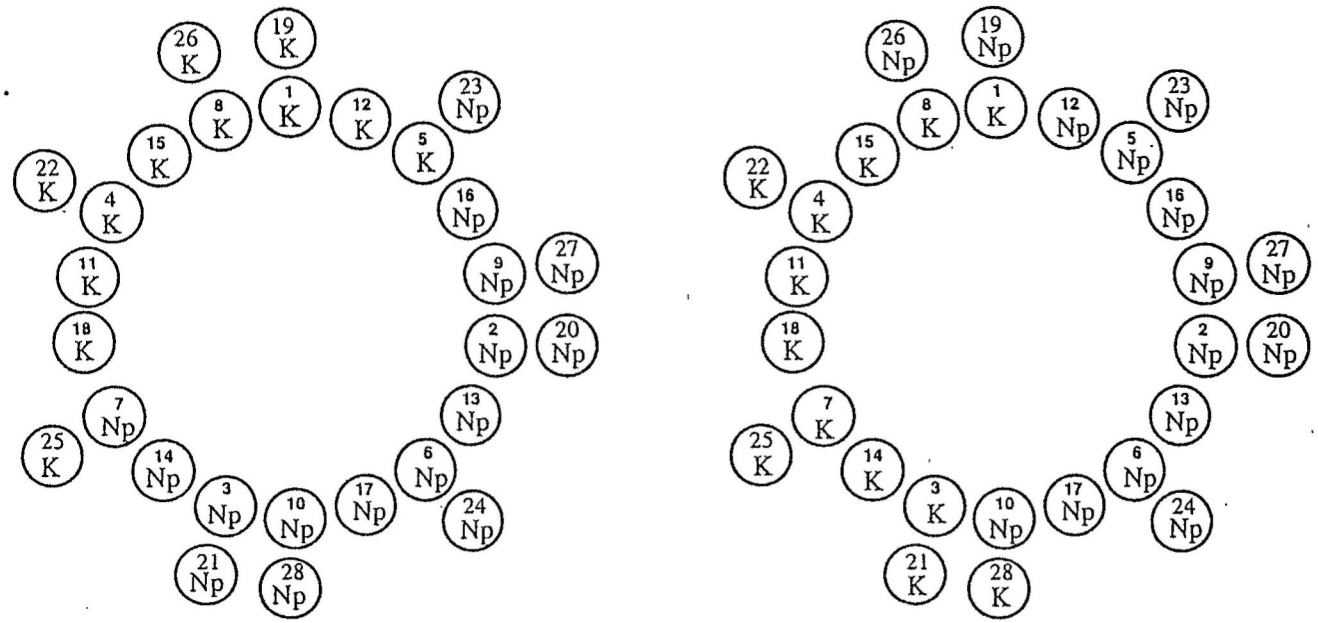


Figure 3.1: Wheel Diagram of the designed peptides with the general sequences $[(KNpNp)(KNpNp)K]_4$ and $[(KNpNp)K(KNpNp)]_4$

formation by screening the positive charges of the lysines (Record et al., 1978). The CD spectra of the peptides were recorded as a function of NaCl concentration from 0-1 M at constant peptide concentration. All of the peptides except $(KLGKKLG)_3$ become more α-helical with increasing NaCl concentration in both phosphate and Tris buffers. Their CD spectra are characteristic of an α-helix with a distinct minimum at 222 nm (n-π^* transition), a second minimum close to 208 nm (superposition of the random coil π–π^* transition at 200 nm and the α-helix π–π^* transition at 208 nm), and the α-helix maximum at 193 nm. An isodichroic point is observed at 203 nm indicating a local two-state equilibrium with each residue being in one of two states, helix or random coil (not shown). The salt dependence of the molar ellipticity at 222 nm is shown in Figure 3.2. The extent of helix formation of the peptides appears to be independent of buffer. The 28-mers are more helical at lower salt concentrations than the 21-mers, suggesting a larger contribution from the nonelectrostatic component of the free energy of helix formation.

Helix formation is coupled to aggregation in peptides with heptad repeats. An aggregation-induced increase in helicity with increasing peptide concentration is detected by circular dichroism (Osterhout Jr. et al., 1992; Lutgring & Chmielewski, 1994; Su et al., 1994). To determine if the salt-dependent helical form of the 21 and 28-mer peptides is monomeric or oligomeric, the concentration dependence of the CD spectra was determined in 2.5 mM phosphate buffer, pH 7.4, 0.4 M NaCl at 25 °C. The molar ellipticity of these peptides depends on concentration, showing that helix formation is accompanied by self-association (Figure 3.3). The driving force for self-assembly is the association of hydrophobic side chains (Bryson et al., 1995). In $(KLAKLAK)_n$ and $(KLAKKLA)_n$, (n=3, 4), the hydrophobic leucine residues are presumed to form the intermolecular interface, while in $(KFAKFAK)_n$ and $(KFAKKFA)_n$, (n=3, 4), the phenylalanine residues would be at the interface. The

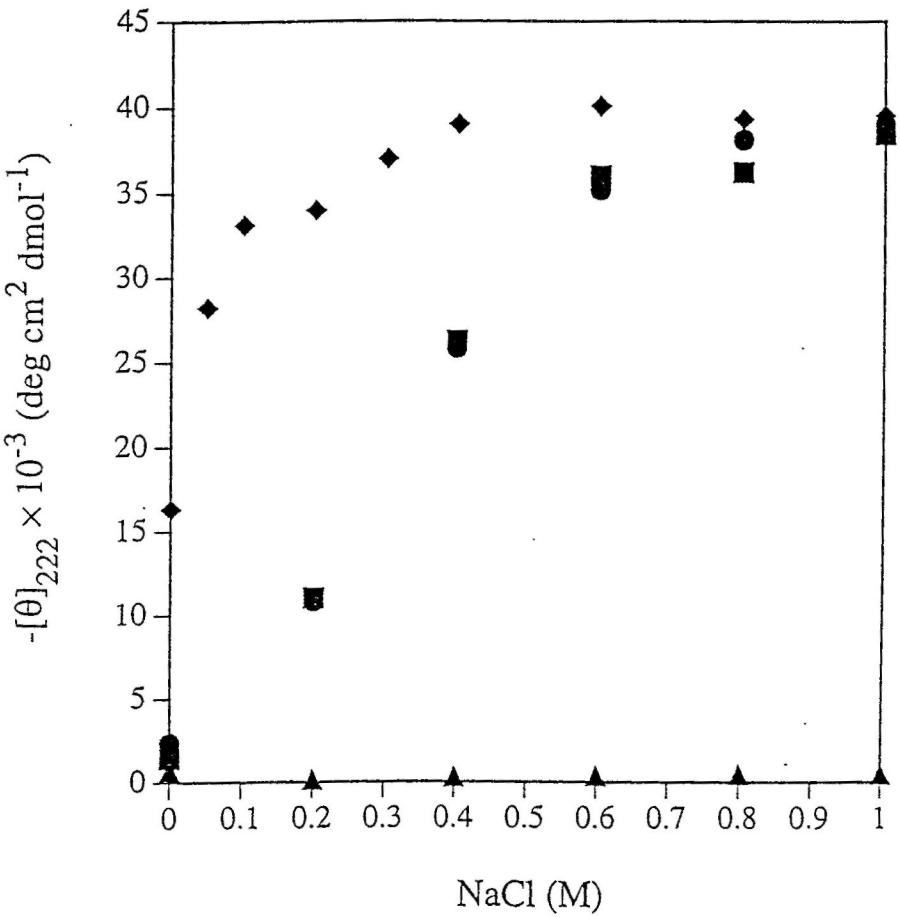


Figure 3.2: Molar ellipticity of peptides at 222 nm as a function of NaCl. Peptide concentration is 6.4×10^{-4} M. (▲)$(KLGKKLG)_3$ and (●) $(KLAKKLA)_3$ in 2.5 mM sodium phosphate buffer, pH 7.4; (◆) $(KLAKKLA)_4$ and (■) $(KLAKKLA)_3$ in 10 mM Tris buffer, pH 7.4.

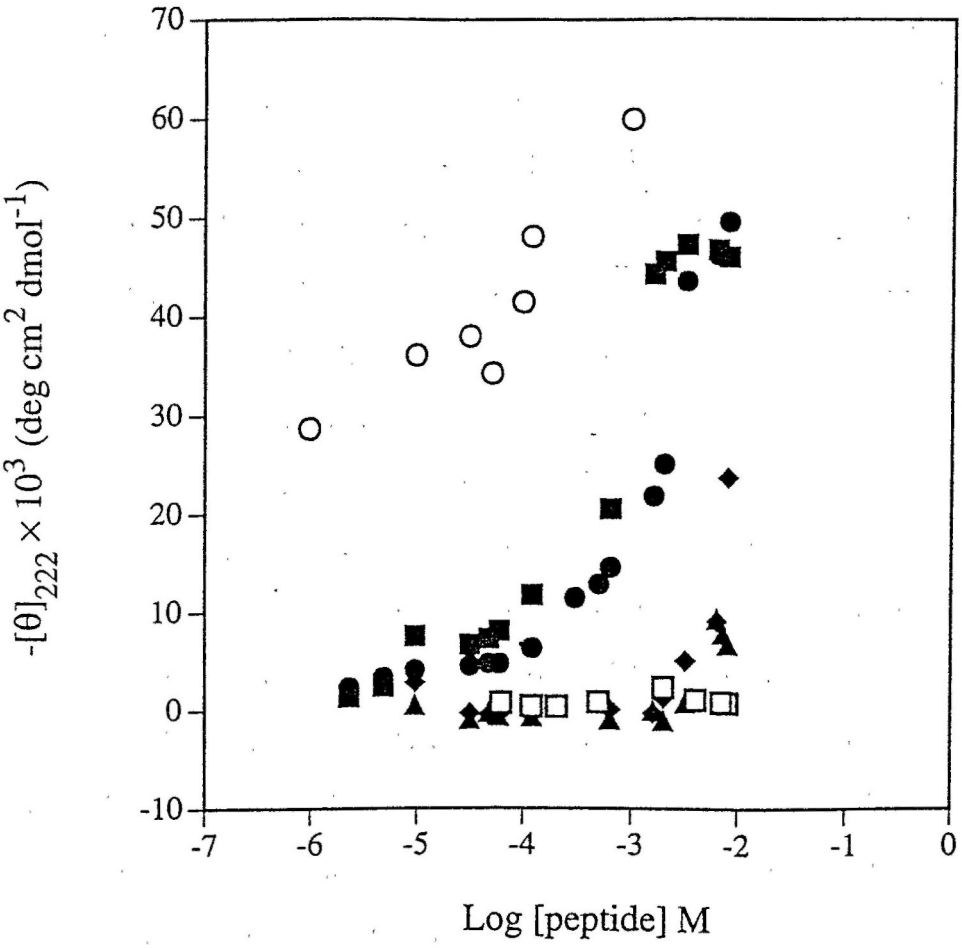
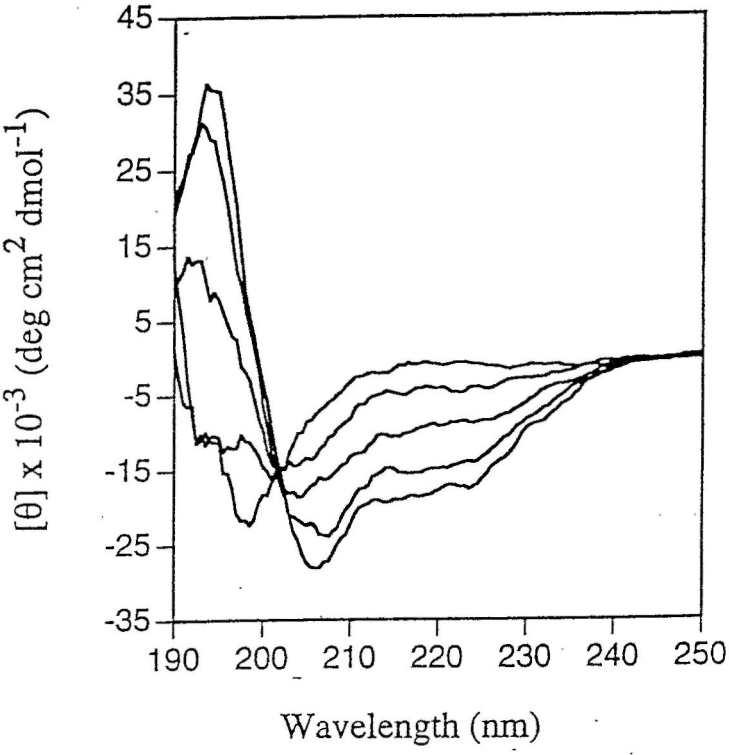


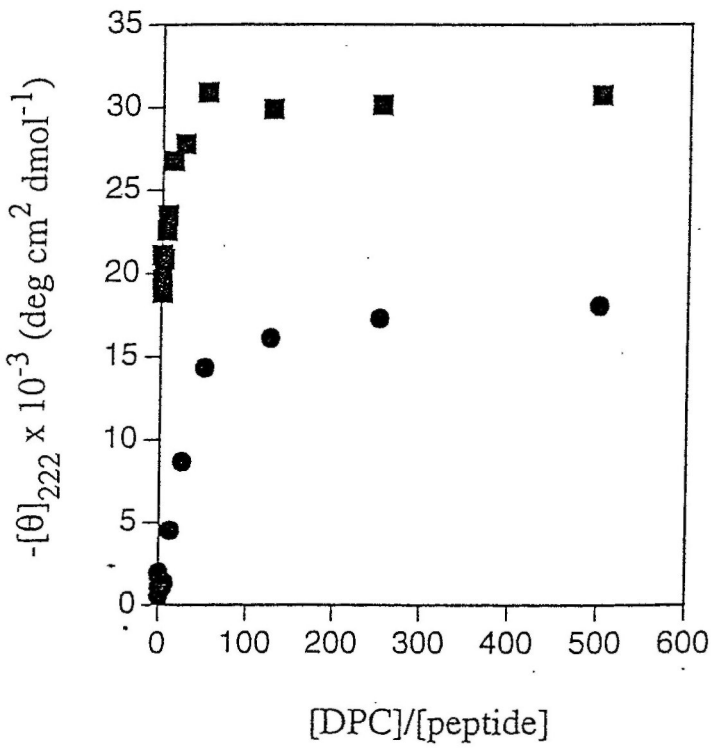
Figure 3.3: Molar ellipticity of peptides at 222 nm as a function of concentration. 2.5 mM phosphate buffer, pH 7.4, 0.4 M NaCl. (●) $(KLAKKLA)_3$, (■) $(KLAKLAK)_3$, (◆) $(KFAKFAK)_3$, (▲) $(KFAKKFA)_3$, (O) $(KLAKKLA)_4$ and (□) $(KLGKKLG)_3$.

28-mer peptides self-associate at concentrations as low as 1×10^{-6} M. The extra heptad is responsible for the higher helical content of the 28-mer peptides relative to the 21-mers. Even though helices 21-28 residues in length are sufficient to form coiled coils, an increase in the size of the hydrophobic segment results in greater stability of interchain hydrophobic interactions (Lau et al., 1984; Lumb et al., 1994; Su et al., 1994). The aromatic residues of phenylalanine induce positive CD signals causing significant errors in the estimation of the helical content from the ellipticity values at 222 nm (Manning & Woody, 1989; Chakrabartty et al., 1993). Therefore, the extent of helix formation in the leucine and phenylalanine zipper peptides cannot be directly compared. However, our main interest here is the concentration dependence of the CD. In contrast to the other designed peptides, $(KLGKKLG)_3$ shows no concentration- or salt-dependent changes in the CD spectrum. This indicates that the intermolecular hydrophobic interactions are not strong enough to compensate for the helix destabilization of the glycine residues.

The carboxyfluorescein-labeled peptides also have random coil conformation in 2.5 mM phosphate buffer, pH 7.4, 0.01 M NaCl. Association of the peptides with DPC micelles induces α-helical secondary structure. CD spectra of CF-$(KLAKKLA)_3$ and CF-$(KLGKKLG)_3$ were measured as a function of DPC concentration by increasing the detergent/peptide ratio from 0 to 500:1. An isodichroic point was observed at 203 nm indicating a local two-state equilibrium between random coil and α-helical conformations (Figure 3.4). Minimum molar ellipticity at 222 nm was reached for CF-$(KLAKKLA)_3$ and CF-$(KLGKKLG)_3$ at detergent/peptide ratios of 50:1 and 125:1, respectively (Figure 3.4). The helix contents of detergent-bound CF-$(KLAKKLA)_3$ and CF-$(KLGKKLG)_3$ are 82% and 40%. These values are close to the helix contents of 85% and 42% obtained for unlabeled $(KLAKKLA)_3$ and $(KLGKKLG)_3$ in DPC micelles (not shown).

Figure 3.4: Upper panel: circular dichroism spectra of 2×10^{-4} M CF-$(KLGKKLG)_3$ as a function of DPC concentration. DPC concentration increases top to bottom in the order: 0, 2.5, 5, 10, and 100 mM. Isodichroic point at 203 nm. Lower panel: molar ellipticity of (●) CF-$(KLGKKLG)_3$ and (■) CF-$(KLAKKLA)_3$ at 222 nm as a function of DPC concentration. Peptide concentration is 2×10^{-4} M.


45
35
25
15
5
-5
-15
-25
-35
190
200
210
220
230
240
250
[θ] x 10-3 (deg cm2 dmol-1)
Wavelength (nm)


35
30
25
20
15
10
5
0
0
100
200
300
400
500
600
-[θ]222 x 10-3 (deg cm2 dmol-1)
[DPC]/[peptide]

Self-assembly in Solution. Sedimentation equilibrium was used to examine the association state of the peptides in aqueous solution and to measure the related association constants. Experiments were performed as a function of temperature at 4° intervals from 2-38 °C. The data were fit to various models for the self-assembly, assuming the same partial specific volume for all species. As shown in Table 3.1, the partial specific volumes calculated from the amino acid sequence of the peptide (Zamyatnin, 1984) were the same within experimental error as the values measured in the ultracentrifuge (Edelstein & Schachman, 1967). This confirms that the partial specific volume of the peptides does not vary with association state. It also indicates that dV = 0 for the association reaction, so that the value of the association constant is not pertubed by sedimentation in the XL-A. The parameters ln k_{14}, $c_{b,1}$, and ε were treated as adjustable parameters in the analysis. A, M_1, and r_b were fixed at their known values. To ensure correct models for the association reaction and accurate values for the association constants, some of the peptides were run at two different concentrations and the data were evaluated in a global fit. $(KLGKKLG)_3$ is monomeric at all temperatures (Figure 3.5). The rest of the peptides are in a monomer-tetramer equilibrium (Figure 3.6). During the analysis $M_1 \leftrightarrow M_2$ and $M_1 \leftrightarrow M_2 \leftrightarrow M_4$ were also considered as possible models. However, the fits for $M_1 \leftrightarrow M_2$ were poor (Figure 3.6). The fits for $M_1 \leftrightarrow M_2 \leftrightarrow M_4$ were much better than for the $M_1 \leftrightarrow M_2$ model, but gave either very small values of ln k_{12} or worse fits than the $M_1 \leftrightarrow M_4$ model. Moreover, the temperature dependence of the equilibrium constants was poorly behaved for the $M_1 \leftrightarrow M_2 \leftrightarrow M_4$ model. Thus, while we cannot completely eliminate the possibility that the peptides may dimerize as well, the dimer concentration appears to be too low to resolve two equilibrium constants. Models for $M_1 \leftrightarrow M_3$, $M_1 \leftrightarrow M_6$, and $M_1 \leftrightarrow M_8$ were also considered, but negative

Table 3.1: Partial specific volume of designed peptides.		
Peptide	Calculated[a]	Measured[b]
$(KFAKKFA)_3$	0.76352	
$(KFAKFAK)_3$	0.76352	
$(KLAKKLA)_3$	0.79575	0.78909 ± 0.00327
$(KLAKLAK)_3$	0.79575	
$(KLGKKLG)_3$	0.78040	
$(KLAKKLA)_4$	0.79920	
$(KFAKKFA)_4$	0.76539	
CF-$(KLAKKLA)_3$		0.74055 ± 0.00521
CF-$(KLGKKLG)_3$		0.77750 ± 0.00234

[a]Zamyatnin, 1984. [b]Edelstein & Schachman, 1967.

Figure 3.5: Sedimentation equilibrium of $(KLGKKLG)_3$ at 26 °C. Upper panel: concentration distribution of 4 mM $(KLGKKLG)_3$ in 2.5 mM phosphate buffer, pH 7.4, 0.4 M NaCl. The line shows the best fit for the monomer model. Lower panel: residuals for the fit.

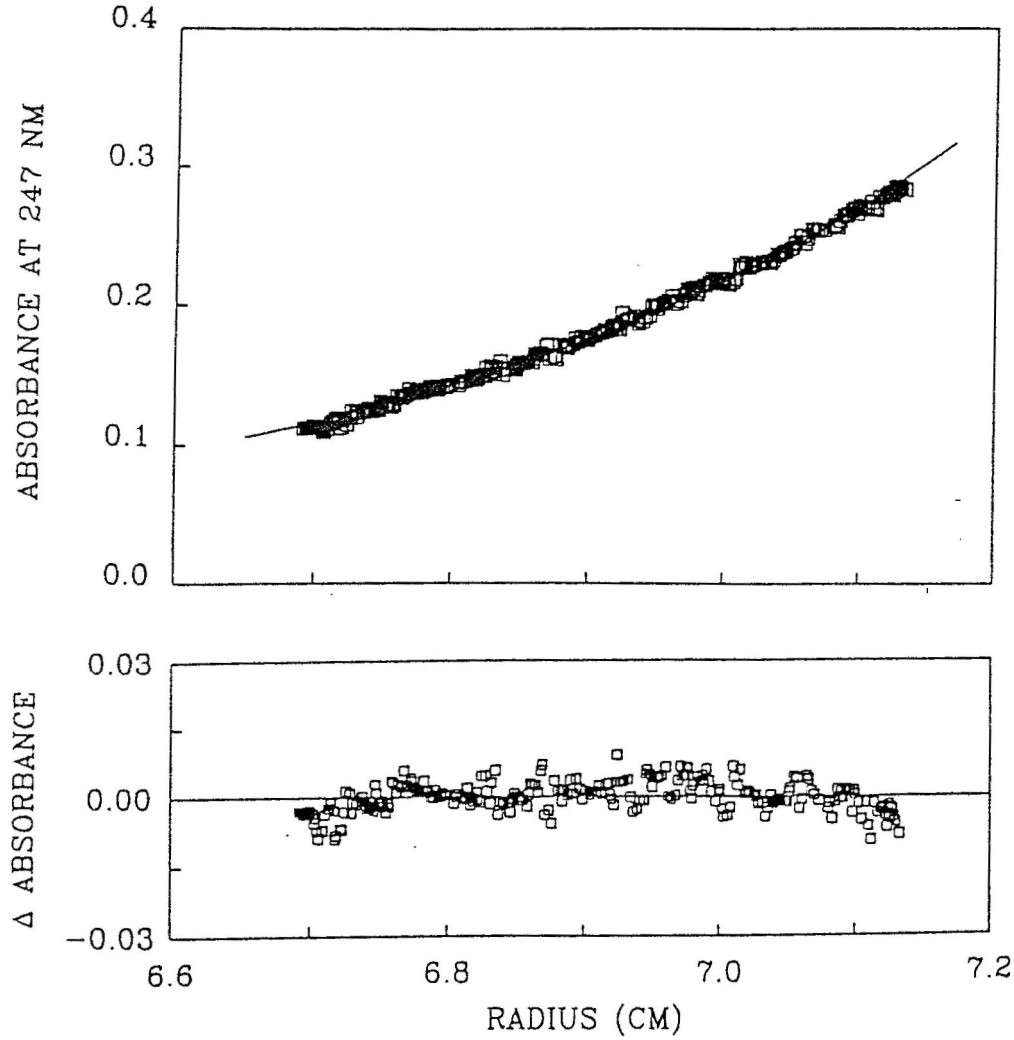
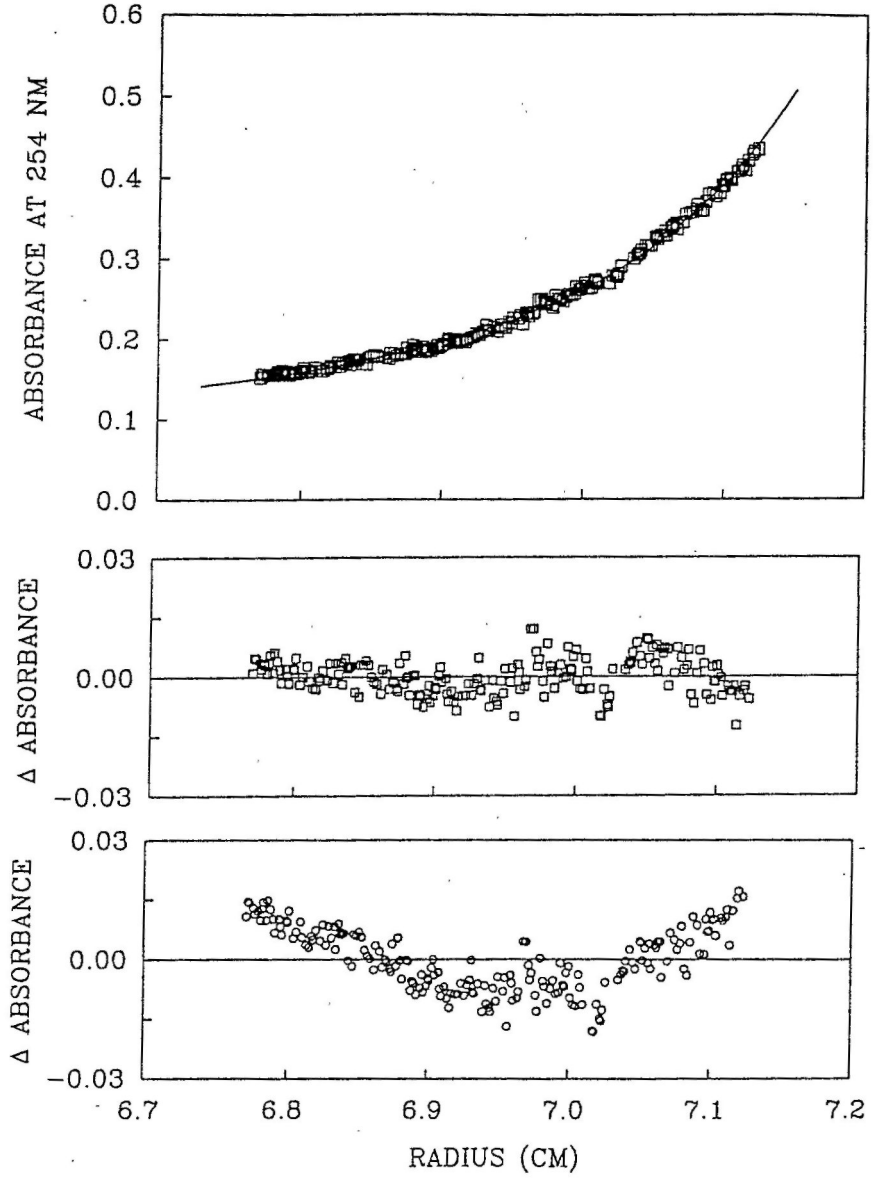

0.4
0.3
0.2
0.1
0.0
ABSORBANCE AT 247 NM
0.03
0.00
−0.03
Δ ABSORBANCE
6.6
6.8
7.0
7.2
RADIUS (CM)

Figure 3.6: Sedimentation equilibrium of $(KLAKKLA)_3$ at 26 °C. Upper panel: concentration distribution of 2 mM $(KLAKKLA)_3$ in 2.5 mM phosphate buffer, pH 7.4, 0.4 M NaCl. The line shows the best fit for the monomer-tetramer equilibrium model. Middle panel: residuals for fit to monomer-tetramer equilibrium. Lower panel: residuals for fit to monomer-dimer equilibrium.


ABSORBANCE AT 254 NM
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Δ ABSORBANCE
0.03
0.00
−0.03
Δ ABSORBANCE
0.03
0.00
−0.03
6.7
6.8
6.9
7.0
7.1
7.2
RADIUS (CM)

values or positive values with unacceptable errors were obtained for the respective values of ln k.

Eq 3.4 was used to determine the thermodynamic parameters from the temperature dependence of $R\ln k$. In the Clark and Glew method, the data are fit by including successively higher order terms in the Taylor expansion. For the leucine zipper peptides, $(KLAKLAK)_3$ and $(KLAKKLA)_n$ (n=3, 4), the best fit was obtained with terms for ΔG°_θ and ΔH°_θ. Adding the term for $\Delta C^\circ_{p,\theta}$ increased the sum of squares of the fit 2-fold, so $\Delta C^\circ_{P,\theta}$ was set to zero. This means that the value of ΔC°_P over the temperature range studied is very small. On the other hand, for the phenylalanine zipper peptides $(KFAKFAK)_3$ and $(KFAKKFA)_n$ (n=3, 4), the $\Delta C^\circ_{P,\theta}$, $(d\Delta C^\circ_P/dT)_\theta$, and $(d^2\Delta C^\circ_P/dT^2)_\theta$ terms were necessary for a good fit. We also included the next higher term $(d^3\Delta C^\circ_P/dT^3)_\theta$ in the fit, but this increased the errors indicating $(d^3\Delta C^\circ_P/dT^3)_\theta = 0$. The nonzero values for $(d\Delta C^\circ_P/dT)_\theta$ and $(d^2\Delta C^\circ_P/dT^2)_\theta$ mean that ΔC°_P is a parabolic function of temperature T. Figure 3.7 plots $\Delta G^\circ = -RT\ln k$ as a function of temperature. The curvature of the plot for the phenylalanine peptides clearly demonstrates that ΔC°_P is nonzero, whereas the straight line for the leucine peptides indicates that ΔH and ΔS are independent of temperature . Table 3.2 contains the derived thermodynamic parameters of the peptides at 25 °C.

Self-assembly in Micelles. According to the CD spectra, the designed peptides adopt an α-helical confomation in solution and in DPC micelles. We have just shown that α-helix formation in solution is coupled to self-association to the tetramer. The method of Reynolds and Tanford (Reynolds & Tanford, 1976) was used to determine the aggregation state of CF-$(KLAKKLA)_3$ and CF-$(KLGKKLG)_3$ bound to DPC micelles by sedimentation equilibrium. Briefly, at a solvent density matching the detergent density the apparent molecular weight of the peptide-DPC

Figure 3.7: Temperature dependence of free energy for monomer-tetramer equilibrium. Upper panel: (□) $(KFAKKFA)_3$, (○) $(KFAKFAK)_3$, (◇) $(KLAKKLA)_3$, and (★) $(KLAKLAK)_3$. Lower panel: (□) $(KFAKKFA)_4$ and (◇) $(KLAKKLA)_4$. Error bars are standard errors obtained from fits of equilibrium concentration distributions.

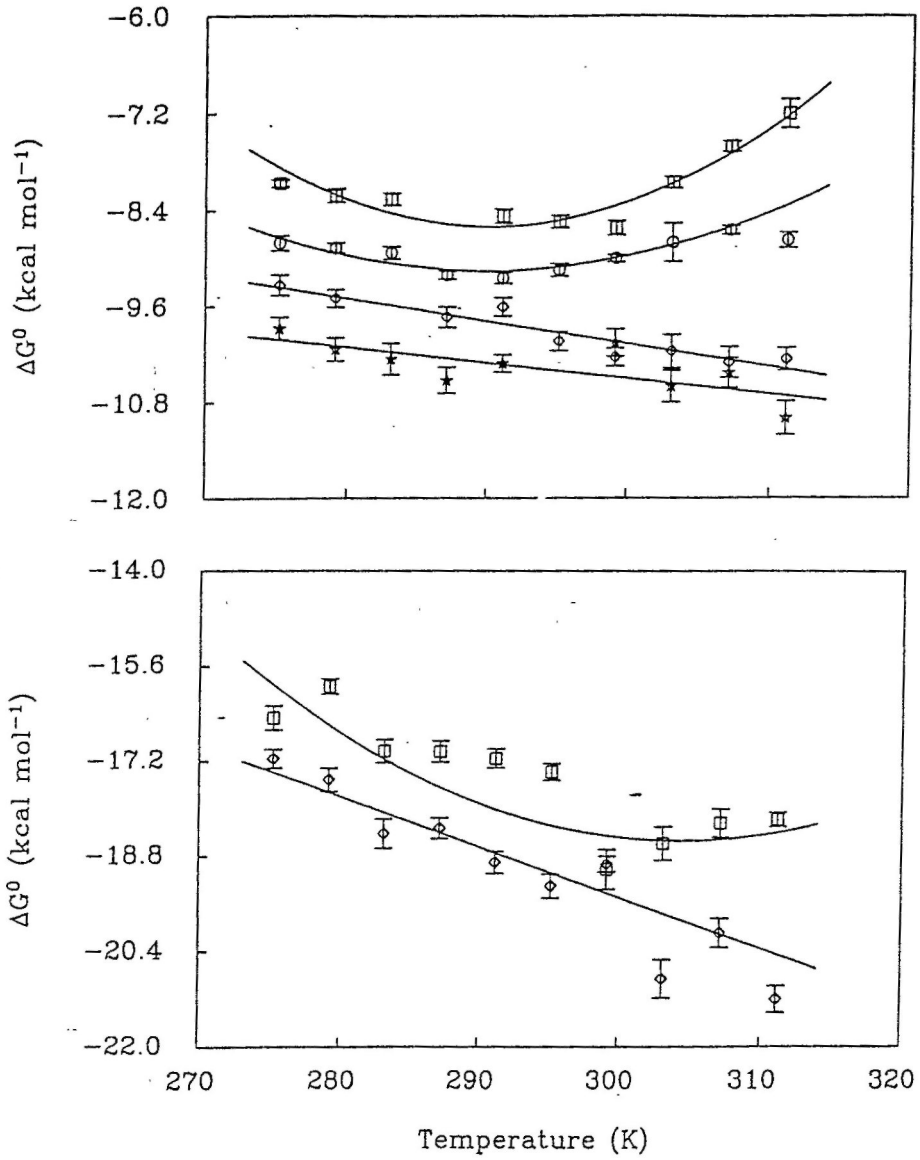

−6.0
−7.2
−8.4
−9.6
−10.8
−12.0
ΔG⁰ (kcal mol⁻¹)
−14.0
−15.6
−17.2
−18.8
−20.4
−22.0
ΔG⁰ (kcal mol⁻¹)
270
280
290
300
310
320
Temperature (K)

Table 3.2: Thermodynamic parameters for the monomer-tetramer equilibrium of the designed peptides at $\theta = 298.15$ K[a]

Peptide	$\Delta G^{\circ}_{\theta}$ (kcal/mol)	$\Delta H^{\circ}_{\theta}$ (kcal/mol)	$\Delta S^{\circ}_{\theta}$ (cal/mol deg)	$\Delta C^{\circ}_{P,\theta}$ (kcal/mol deg)	$(\Delta C^{\circ}_P/dT)_{\theta}$ (kcal/mol deg^2)	$(d^2\Delta C^{\circ}_P/dT^2)_{\theta}$ (kcal/mol deg^3)
$(KFAKKFA)_3$	-8.5 ± 0.1	-21.0 ± 4.8	-41.9 ± 10.4	-2.8 ± 0.6	-0.05 ± 0.1	-0.02 ± 0.03
$(KFAKFAK)_3$	-9.1 ± 0.02	-22.9 ± 1.0	-46.3 ± 2.0	-1.3 ± 0.2	0.37 ± 0.04	0.06 ± 0.001
$(KLAKKLA)_3$	-10.0 ± 0.1	-1.5 ± 0.1	28.5 ± 1.9	0	0	0
$(KLAKLAK)_3$	-10.5 ± 0.1	-4.7 ± 2.0	19.5 ± 8.3	0	0	0
$(KFAKKFA)_4$	-18.4 ± 0.3	10.1 ± 16.7	95.6 ± 261	-3.5 ± 2.4	-0.56 ± 0.7	-0.01 ± 0.01
$(KLAKKLA)_4$	-19.4 ± 0.1	6.2 ± 3.0	85.9 ± 41.5	0	0	0
CF-$(KLAKKLA)_3$	-6.8 ± 0.2					

[a]:2.5 mM sodium phosphate buffer, 0.4 M NaCl, pH 7.4

complex becomes equal to the molecular weight of the protein component. At this density the detergent makes no contribution to the sedimentation potential of the complex. If the chemical potential of all solvent components were kept constant the experimentally observed concentration gradient is a measure of $M_p(1 - \phi'\rho)$, where M_p is the molecular weight of the peptide moiety in the sedimenting particle, ρ is the solvent density, and ϕ' is the volume increment per gram of peptide. Since the measurement of ϕ' is difficult $M_p(1 - \phi'\rho)$ is expressed in terms of the individual contributions in the sedimenting particle.

$$M_p(1 - \phi'\rho) = M_p[(1 - \bar{v}_p\rho) + \delta_D(1 - \bar{v}_D\rho)] \qquad (3.5)$$

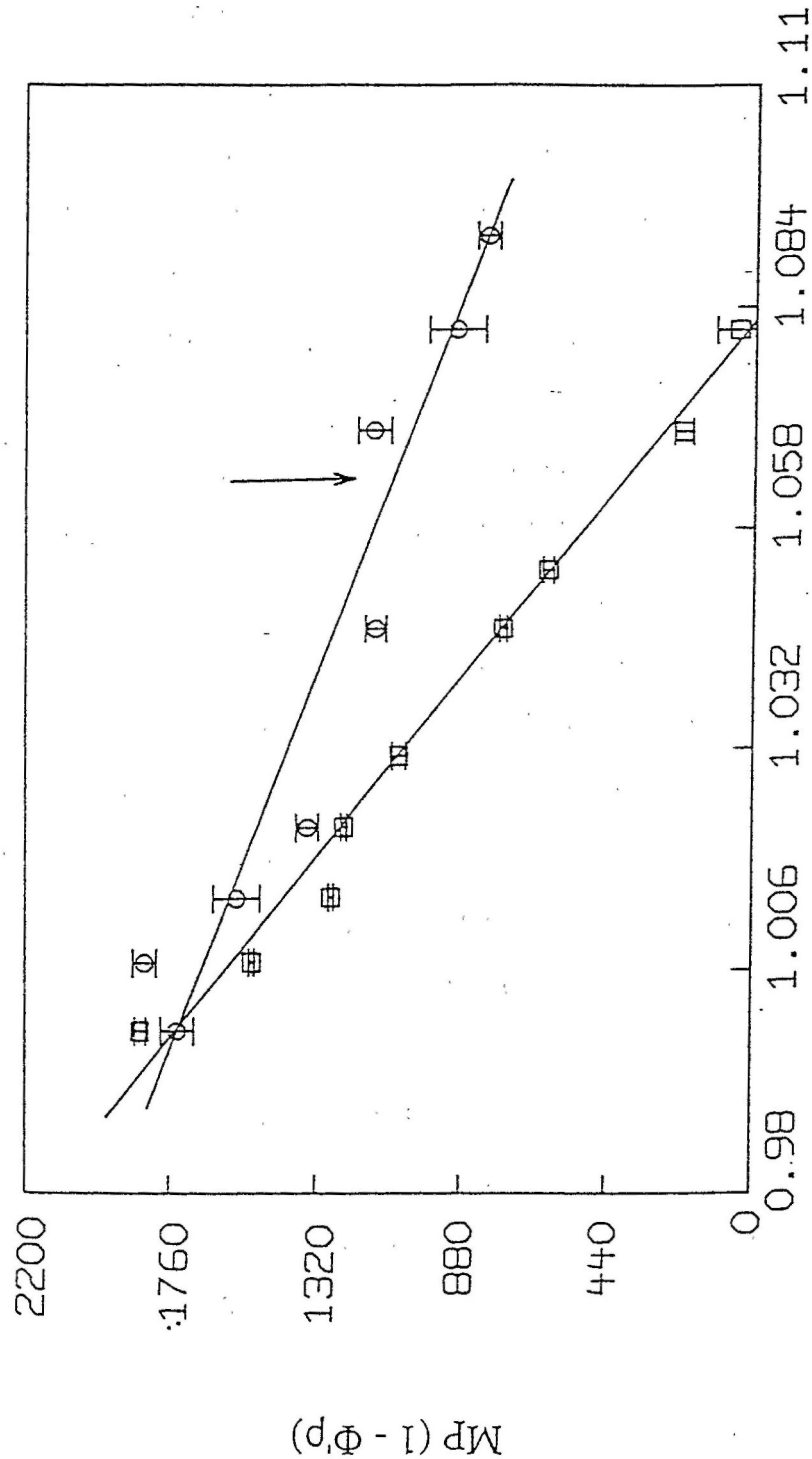
where $\bar{v}_p$ is the partial specific volume of the peptide, $\bar{v}_D$ is the partial specific volume of DPC, δ_D is gram of detergent in the sedimenting particle. At $\rho = 1/\bar{v}_D$ the contribution of the detergent vanishes $1 - \bar{v}_D\rho = 0$ and

$$M_p(1 - \phi'\rho) = M_p[(1 - \bar{v}_p\rho) \qquad (3.6)$$

This condition is attained by adjusting the density with D_2O.

Our experiments take advantage of an optical probe covalently bound to the peptides. By monitoring wavelengths where only carboxyfluorescein absorbs, the concentration distribution of the peptide can be determined without background absorption from the detergent. Figure 3.8 shows plots of the apparent value of $M_p(1 - \phi'\rho)$ for CF-(KLAKKLA)$_3$ and CF-(KLGKKLG)$_3$ as a function of solvent density. DPC has a partial specific volume of 0.937 cm^3/g (Lauterwein et al., 1979). The molecular weights are obtained from eq 3.6 at $\rho = 1/\bar{v}_D$. The monomer molecular weights of CF-(KLAKKLA)$_3$ and CF-(KLGKKLG)$_3$ are 2634 and 2562, respectively. Based on sedimentation equilibrium, the values of 5050 ± 80 and 1870 ± 70 for CF-(KLAKKLA)$_3$ and CF-(KLGKKLG)$_3$ measured in DPC micelles correspond to dimers and monomers, respectively.

Figure 3.8: Sedimentation equilibrium of (O) CF-(KLGKKLG)$_3$ and (□) CF-(KLAKKLA)$_3$ in DPC micelles by the method of Reynolds and Tanford (1976). $M_p(1 - \phi'\rho)$ is plotted versus solvent density ρ, which is varied by mixing different proportions of H_2O and D_2O buffers. The arrow indicates the solvent density for which $\rho = 1/\bar{v}$ of the DPC.


Mp (1 - Φ'p)
2200
1760
1320
880
440
0
0.98
1.006
1.032
1.058
1.084
1.11
Density

ANS Fluorescence. ANS is a hydrophobic fluorescent probe, whose fluorescence intensity increases and emission spectrum shifts to shorter wavelengths in nonpolar environments (Slavik, 1982; Semisotnov et al., 1991). It is practically nonfluorescent in water. As seen in Figure 3.9, the fluorescence of ANS increases in the presence of the self-associating peptides, indicative of binding to a hydrophobic environment. The quantum yields of the peptide-ANS complex was measured relative to ANS in ethanol. The quantum yield is higher for ANS bound to phenylalanine peptides than to leucine peptides: 0.189 and 0.276 for $(KFAKFAK)_3$ and $(KFAKKFA)_3$, compared to 0.066 and 0.126 for $(KLAKLAK)_3$ and $(KLAKKLA)_3$, respectively. These results suggest that the designed amphipathic peptides aggregate through the association of their hydrophobic side chains to form a hydrophobic core. The fluorescence of ANS also increases slightly in the presence of $(KLGKKLG)_3$ with a quantum yield of 0.024. This peptide does not self-associate. The increase in ANS fluorescence could be due to interaction of the probe with the hydrophobic leucine residues. In the presence of a control peptide $(KKAKKKA)_3$ lacking the leucine residues, the quantum yield of ANS is 0.01, which is the same as the value in buffer.

Biological Activity. The designed peptides were tested against Gram negative and Gram positive bacteria and also against mammalian cells (Table 3.3). Two antibacterial assays were done. MIC assay measures inhibition of cell growth and MBC assay measures cell death. The MIC data of the 21-mer peptides are all about the same for Gram positive and Gram negative bacteria with a slight improvement for the 21-mer phenylalanine peptides. The MBC data show that the 21-mer leucine peptides are more active towards Gram negative bacteria relative to Gram positive bacteria. The phenylalanine peptides show greater antibacterial activity compared to the leucine peptides, but no selectivity. The mammalian cell

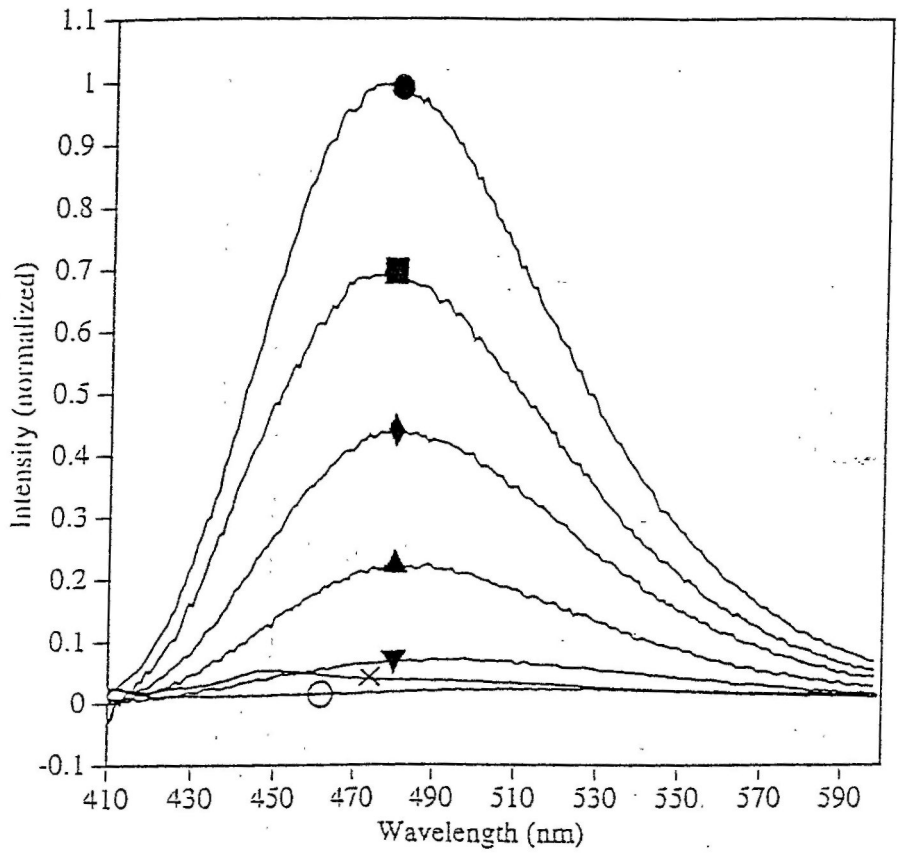


Figure 3.9: Emission spectra of 1×10^{-5} M ANS in the presence of 2×10^{-3} M peptide. Top to bottom: (●) $(KFAKKFA)_3$, (■) $(KFAKFAK)_3$, (◆) $(KLAKKLA)_3$, and (▲) $(KLAKLAK)_3$, (▼) $(KLGKKLG)_3$, (✖) $(KKAKKKA)_3$, and (O) 2.5 mM phosphate buffer, pH 7.4, 0.4 M NaCl.

Table 3.3: Biological activity of designed peptides[a].

Peptide	MIC		MBC		3T3
	E. coli	*S. aureus*	*E. coli*	*S. aureus*	
$(KLGKKLG)_3$	4	4	10	100	>393
$(KLAKKLA)_3$	4	4	1	100	11
$(KLAKLAK)_3$	4	4	1	10	9
$(KFAKKFA)_3$	2	3	1	1	5
$(KFAKFAK)_3$	3	3	1	1	11
$(KLAKKLA)_4$			10	10	10
$(KFAKKFA)_4$			1	1	4
CF-$(KLAKKLA)_3$	6	6			
CF-$(KLGKKLG)_3$	6	3			

[a]: Bacterial lysis is measured as both minimum inhibitore concentration assays (MICs) and minimum bacterial concentration assays (MBCs), and 3T3 mouse fibroblast lysis is measured as sublethal concentration (μM), as described in Materials and Methods section.

assay determines the efficacy of peptides to induce cell death in 3T3 mouse fibroblasts. The most dramatic difference in cytotoxicity of the designed peptides is exhibited by $(KLGKKLG)_3$. This peptide is about 40 times less potent compared to the other peptides in killing 3T3 cells. The CF-peptides had the same biological activity as their equivalent unlabled peptides.

3.4 Discussion

Hydrophobic interactions contribute to the stability of α-helices in aqueous solution (Kanehisa & Tsang, 1980; Hodges et al., 1981; Eisenberg et al., 1984; Lau et al., 1984; DeGrado & Lear, 1985; DeGrado et al., 1989; Hodges et al., 1990; Zhou et al., 1992). The driving force for the formation and association of amphipathic helices is the association of their hydrophobic side chains. The nature of the hydrophobic interactions depends on solvent environment. Most heptad repeats use leucines to form the hydrophobic sides of helices. The helix stabilizing capabilities of other hydrophobic residues, such as phenylalanine, have not been examined in detail. In this study we have demonstrated that phenylalanine zippers stabilize the self-association of peptides. These phenylalanine zippers are 1.0-1.5 kcal/mol less stable than the equivalent leucine zipper in the presence of 0.4 M NaCl. The aromatic residues may restrict the side-chain conformation and thereby destabilize the α-helix.

All of the designed peptides except for $(KLGKKLG)_3$ associate to form tetramers in 2.5 mM phosphate buffer, 0.4 M NaCl, pH 7.4. Unlike the amphipathic alanine-based peptides 16-17 residues in length that form monomeric helices (Marqusee & Baldwin, 1987; Marqusee et al., 1989), $(KLGKKLG)_3$ is a random coil in aqueous solution. Apparently, the intermolecular hydrophobic interactions are not strong enough to compensate for the helix destabilization of the six glycine residues in this peptide and consequently no conformational transition occurs at high peptide

or salt concentration. The designed peptides that self-associate have α-helical conformations in solution. The phenylalanine peptides $(KFAKFAK)_3$ and $(KFAKKFA)_3$ are capable of self-associating, however, their circular dichroism spectra have relatively small negative ellipticities at 222 nm; the % helicities calculated from eq 2 are 6% and 0%, respectively. Assuming that the tetramer is 100% helical, we can estimate helix content from the equilibrium constants for self-assembly: 20% and 7% for $(KFAKFAK)_3$ and $(KFAKKFA)_3$. The lower helix contents derived from circular dichroism may be due to the positive signals induced by the phenylalanine residues (Chakrabartty et al., 1993). On the other hand, circular dichroism overestimates the helix contents of the leucine peptides $(KLAKLAK)_3$ and $(KLAKKLA)_3$: 134% and 122%, respectively, compared to only 49% and 43% calculated from the association constants. Two possible reasons for a higher helix content estimated by circular dichroism are: (1) monomeric helices in equilibrium with tetramer or (2) anomalously large negative ellipticities of helical aggregates. Ellipticities at 222 nm as large as -60,000 are seen in the concentration studies of these peptides.

The designed amphipathic peptides aggregate through the association of their hydrophobic side chains to form a hydrophobic core. The fluorescence of ANS is enhanced upon binding to hydrophobic regions of proteins. The increase in quantum yield of the peptide-ANS complex is greater for the phenylalanine zippers than the leucine zippers. Computer modeling suggests that the bulky aromatic rings of the phenylalanine residues form a larger hydrophobic core that is more accessible to ANS. Morden et al. (1996) used sedimentation equilibrium and NMR spectroscopy to study the solution conformation of $(KLAKLAK)_3$ in 15% hexafluoroisopropanol. Under their experimental conditions this peptide exists in a monomer-octamer

equilibrium, and the hydrogen exchange results confirm the presence of a hydrophobic core.

The aggregation state of the peptides depends on solvent environment. In aqueous solution all the peptides except $(KLGKKLG)_3$ form tetrameric helical bundles; in hexafluoroisopropanol they form octameric bundles. In DPC micelles CF-$(KLAKKLA)_3$ exists as a dimer and CF-$(KLGKKLG)_3$ is a monomer. The carboxyfluorescein label does not affect the aggregation state. Helix content is also environmentally sensitive. In the presence of micelles CF-$(KLAKKLA)_3$ has a helix content of 82% compared to 85% for the unlabeled peptide. CF-$(KLGKKLG)_3$ is 40% α-helix and the unlabeled peptide is 42%. Bishop et al. (Bishop et al., 1996) have studied the binding and helical properties of $(KLAKKLA)_3$ and $(KLGKKLG)_3$ in neutral vesicles. The fraction of $(KLAKKLA)_3$ that binds to the vesicles has a larger helical content of 90%, compared to 63% for $(KLGKKLG)_3$.

The thermodynamic parameters quantify the self-assembly of the designed peptides. The 21-mer peptides with the general sequence of $[(KNpNp)(KNpNp)K]_n$ (n=3, 4), having a hydrophobic 3-4 repeat, are ~ 0.5 kcal/mol more stable compared to $[(KNpNp)K(KNpNp)]_n$, which has a hydrophobic 4-3 repeat. Attachment of carboxyfluorescein to the N-terminus of $(KLAKKLA)_3$ has no effect on aggregation state, but destabilizes the tetramer by ~ 3 kcal/mol. The 28-mer peptides are more stable compared to the 21-mer peptides by ~ 10.0 kcal/mol. This can be attributed to the additional heptad repeat present in the 28-mer peptides. An increase in the length of the nonpolar domain does not affect the aggregation state of the peptides, but it provides a greater region of hydrophobic interactions and thus additional stability. Besides the difference in stability between leucine and phenylalanine zippers, the $\Delta C^{\circ}_{P,\theta}$ values provide information about the exposure of nonpolar residues to water (Spolar & Record Jr., 1994). For the leucine peptides $\Delta C^{\circ}_{P,\theta} = 0$ and the

temperature melts of these peptides are not cooperative (data not shown). However, the curvature in the plot of ΔG° as a function of temperature and the negative value of $\Delta C^\circ_{P,\theta}$ for the phenylalanine peptides resembles the behavior of natural proteins. Negative $\Delta C^\circ_{P,\theta}$ values result from withdrawal of nonpolar groups from the aqueous environment (Spolar et al., 1989; Livingstone et al., 1991). The difference in the $\Delta C^\circ_{P,\theta}$ values of the leucine and phenyalanine peptides can be attributed to the size of the water-accessible nonpolar surface area of leucine and phenylalanine residues. Since leucine residues have a smaller water-accessible nonpolar surface area compared to phenylalanine (Livingstone et al., 1991), the $\Delta C^\circ_{P,\theta}$ value may too small to be measured accurately over the narrow temperature range studied.

The signs and magnitudes of the ΔH° and ΔS° values for the association reactions of the designed peptides can be attributed to the strength and extent of the hydrophobic interactions between the nonpolar residues. Ross and Subramanian (1981) envision peptide association as a two step process proceeding from isolated hydrated species to a state where the peptides partially interact with mutual penetration of their hydration layers, and from there to a fully interacting association complex. Positive enthalpy and entropy changes occur in the first step as a result of partial withdrawal of the nonpolar groups from water accompanied by an increase in the number of unstructured water molecules (Gill et al., 1967; Gill et al., 1976; Ross & Subramanian, 1981). Negative enthalpy and entropy changes arise in the second step from non-bonded short-range interactions such as strengthened van der Waals interactions and hydrogen-bond formation (Gill et al., 1967; Gill et al., 1976; Lauterwein et al., 1979; Ross & Subramanian, 1981). Since the hydrophobicities of leucine and phenylalanine are about the same (Guo et al., 1986; Parker et al., 1986), the leucine and phenylalanine zippers should give approximately the same positive values for ΔH° and ΔS° to form the hydrophobically associated tetramer. However,

the overall values of ΔH° and ΔS° for the 21-mer peptides are more negative in the phenylalanine zippers compared to the leucine zippers due to the larger nonpolar surface area (Livingstone et al., 1991) and stronger van der Waals interactions of phenylalanine relative to leucine. In the case of the 28-mer peptides the positive ΔH° and ΔS° values suggest that the strong hydrophobic interactions taking place between the nonpolar residues overwelm the negative contributions from van der Waals interactions.

The importance of helix content in the biological activity of the designed peptides is well established (Javadpour et al., 1996), but the role of self-association of the amphipathic α-helices remains unclear. The only significant difference in biological activity is seen with $(KLGKKLG)_3$. The antibacterial activity of the designed peptides has been measured by both MIC and MBC assays. All the peptides inhibit bacterial growth to the same extent based on the MICs. However, $(KLGKKLG)_3$ appears to be slightly less effective in killing bacteria in the MBCs. Unlike the other designed peptides, this peptide is very selective in discriminating between bacterial and mammalian cells. It kills both Gram positive and Gram negative bacteria, but is 40 times less potent than the other peptides against mammalian cells. Bishop et al. (Bishop et al., 1996) have extensively studied the interaction of $(KLGKKLG)_3$ and $(KLAKKLA)_3$ with model membranes. Their findings suggest that the helix propensity of the peptides in a membrane environment is an important factor in the binding affinity, which correlates with biological activity. Our studies suggest that aggregation may contribute to the selectivity of the peptides. The peptide that does not aggregate in solution or neutral micelles has a high selectivity and does not kill mammalian cells at the highest concentration tested.

3.5 References to Chapter 3

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Appendix A

Abbreviations

ANS	1-anilino-naphthalene-8-sulfonate
ATCC	American type culture collection
BOP	benzotriazol-1-yloxytris(dimethylamino)-phosphonium-hexafluorophosphate
CD	circular dichroism
CF	carboxyfluorescein
cmc	critical micelle concentration
DLPC	dilauryl phosphatidyl choline
DLPG	dilauryl phosphatidyl glycerol
DMF	N,N'-dimethylformamide
DPC	dodecylphosphocholine
HOBt	1-hydroxybenzotriazole
MBC	minimum bactericidal concentration
MEM	minimal essential medium
MIC	minimum inhibitory concentration
NMM	N-Methyl morpholine
SDS	sodium dodecylsulfate
TFA	trifluoroacetic acid
Tris	tris-(hydroxymethyl)-aminomethane

Appendix B

Self-assembly of (KLAKLAK)$_3$ in Hexafluoroisopropanol

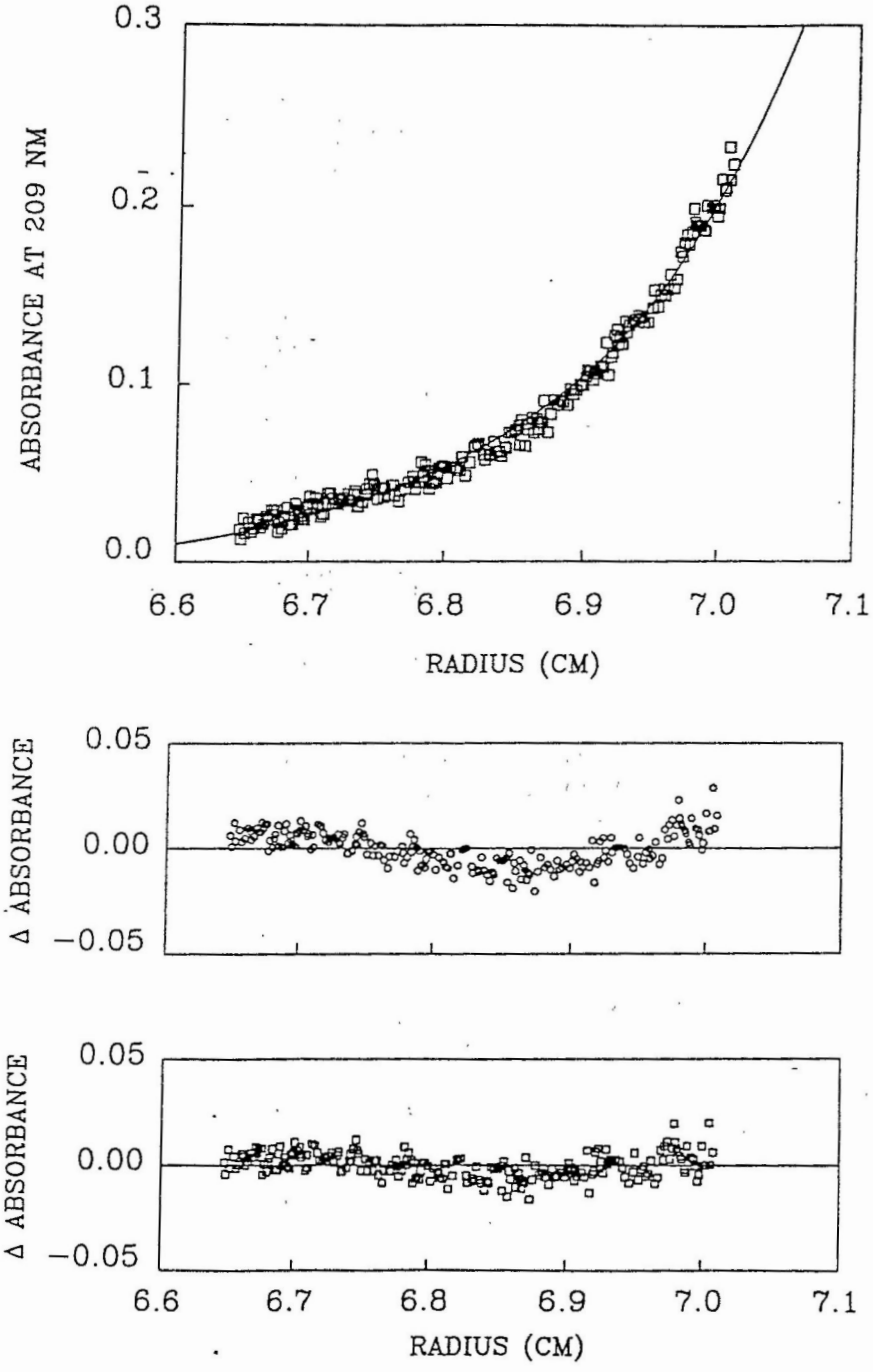
The aggregation state of (KLAKLAK)$_3$ was determined in 0.85 mM Na_2HPO_4, 0.085 mM NaCl, 8.5 μM EDTA, 15% HFIP (v/v) and pH 3.3 at 25° C using a Beckman XL-A. The peptide is soluble in aqueous solution with no specific secondary structure as determined by circular dichroism spectroscopy. The addition of hexafluoroisopropanol (HFIP) promotes the formation of secondary structure and the peptide adops an α-helical conformation. Minimum ellipticity at 208 nm and 222 nmis obtainedin aqueous solutions with greater than or equal to 15% HFIP (v/v). Analytical ultracentrifugation was performed at 25° C using a Beckman XL-A, a four-hole rotor with a counterbalance and three double-sector cells at a rotor spped of 30,000 RPM. Equilibrium was established after 17 hours. The distribution of the peptide within the cell was determined by measuring the absorbance at 209 nm with baseline scans measured at 281 nm where the peptide has no absorption. Ten scans were collected and averaged to give the final data for analysis. Based upon these results the best fit to the data is a monomer-octamer equilibrium (Figure B1). The mathematical model which was used to obtain the equilibrium constant has the form:

$$C_r = C_{b,1} \exp[AM_1(r^2 - r_b^2)] + C_{b,1}^8 \exp[\ln k_{1,8} + 8AM_1(^2 - r_b^2)] + \varepsilon$$

where $C_{b,1}$ is the concentration of the monomer at r_b, the radial position of the cell bottom, M_1 is the molecular mass of the monomer calculated from the amino acid sequence, ε is a small baseline error correction term and $A = (1-\upsilon\rho)\omega^2/2RT$, where υ is the compositional partial specific volume of the monomer, ρ is the solvent density, ω is the rotor angular velocity, R is the gas constant, and T is the absolute tem,perature. For aqueous solutions the partial specific volume is typically calculated based upon the sequence of the peptide, but because of the solvent system used in this study data was collected in two different densities of solvents (H_2O and

D_2O in 15% HFIP (v/v)) and globally fit the data to experimentally determine the partial specific volume of the peptide. The experimentally determined partial specific volume of the peptide (0.79353 ± 0.00536) and the calculated value (0.79575) are within experimental error. Thus the 15% HFIP does not seem to have a large effect on the hydration of the peptide. The association constant determined from the ultracentrifugation is 5×10^{41} M^7.

Figure B1: Distribution of concentration and residuals of $(KLAKLAK)_3$. Upper panel: concentration distribution of 7.9×10^{-6} M $(KLAKLAK)_3$.in 0.85 mM Na_2HPO_4, 0.085 mM NaCl, 8.5 μM EDTA, 15% HFIP (v/v) and pH 3.3. The line shows the best fit fot the monomer-octamer equlibrium model. Middle panel: residuals for fit to monomer-hexamer equilibrium. Lower panel: residuals for fit to monomer-octamer equlibrium.


ABSORBANCE AT 209 NM
0.3
0.2
0.1
0.0
6.6
6.7
6.8
6.9
7.0
7.1
RADIUS (CM)
Δ ABSORBANCE
0.05
0.00
−0.05
Δ ABSORBANCE
0.05
0.00
−0.05
6.6
6.7
6.8
6.9
7.0
7.1
RADIUS (CM)

Appendix C

Letter of Permission

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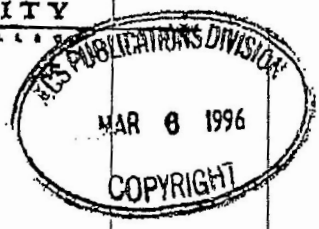

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Department of Chemistry
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Louisiana State University
Baton Rouge, LA 70803
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VITA

Maryam Javadpour, known as "Maria" was born in Tehran, Iran, in 1966, the daughter of Mahin Farahi and Seyyed Hossein Javadpour. She received her Bachelor of Science degree in Chemistry from University of Shiraz, Iran, in May 1990. She spent a year working as a research assistant in The Royal Melbourne Institute of Technology, Australia. She began pursuing her doctorate degree in Biophysics at Louisiana State University in Spring of 1992 under the supervision of Professor Mary D. Barkley. Currently, she is a candidate for the degree of Doctor of Philosophy in the Department of Chemistry.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Maryam Javadpour

Major Field: Chemistry

Title of Dissertation: Solution Conformation of de novo Antimicrobial Peptides

Approved:

Mary Dicky Barkley
Major Professor and Chairman

John M. Larkin
Dean of the Graduate School

EXAMINING COMMITTEE:

Bruce J. Hales

Kathleen M. Merz

Mark L. McLaughlin

Ronald K. Gough

Date of Examination:

03/29/96