PROJECT SUMMARY

Title	Synthesis and Structural Analysis of Acylated Antimicrobial Lactoferricin Peptides by NMR Spectroscopy
Student Name	
Mentor Name	
School	University of Arkansas, Fayetteville
Classification	Junior
Area of Study	Biochemistry
Grade point average	

The goal of this research project is to study the structure of various lactoferricin analogues to determine which ones demonstrate the greatest amount of antimicrobial activity while remaining stable at the cell membrane interface. Lactoferricin is a peptide that inserts into the cell membrane of microbes, disrupting the membrane structure and ultimately leading to the rupture of the cell. The mechanism for this process is unknown, however, and it is difficult to consistently insert the peptide in the same orientation relative to the membrane. Not all orientations of the peptide exhibit an equal amount of antimicrobial activity, so it is important to devise a method for inserting the peptide in the orientation that produces the greatest antimicrobial effect. Fatty acid chains can be used to accomplish this because they readily embed in membrane bilayers, and when added to the end of the peptide these chains have a stabilizing effect. The focus of this project will be on finding the fatty acid chain with the greatest stabilizing effect on the peptide. The results can then be practically applied in the synthesis of new antimicrobial agents which can take the place of ineffective antibiotics in the treatment of certain diseases.

Synthesis and Structural Analysis of Acylated Antimicrobial Lactoferricin Peptides by Nuclear Magnetic Resonance Spectroscopy

Introduction

The resistance of microbes to antibiotic treatment has become one of the most significant problems facing scientists in recent years, and has led to an increased need to develop alternative methods of combating microbes. Scientists have found that certain proteins possess antimicrobial properties that would be useful in the development of such alternative treatments. One such protein is lactoferrin, an iron-binding protein found in the milk of cows and humans (Cavestro 2002). Analysis of lactoferrin has revealed that its antimicrobial properties come from a 25-amino acid sequence within the protein. The antimicrobial activity of the 25-residue peptide, called lactoferricin, has been further determined to stem from a six residue (or hexapeptide) sequence containing two amphipathic tryptophan (Trp) and three positively-charged arginine (Arg) residues (Schibli 1999).

The protein is thought to act on the microbe by embedding in and disrupting its cell membrane. Microbial cell membranes are composed of a negatively-charged phospholipid bilayer. This negative charge attracts the positively-charged arginine residues in the hexapeptide, and once the membrane and the peptide are in close proximity, the structure of the tryptophan allows it to embed within the bilayer at the surface of the membrane. These added tryptophans disrupt the bilayer and cause the membrane to rupture, killing the cell. The exact mechanism for this process is unknown. The problem with this method is that the tryptophan does not always embed in the membrane properly. If the tryptophan does not embed with the correct orientation, its ability to disrupt the membrane bilayer is affected. To solve this problem, a fatty acid

chain can be added to the end of the hexapeptide. This chain readily inserts into the membrane, stabilizing the rest of the hexapeptide and causing the tryptophan to embed in the proper orientation.

This project will explore the effectiveness of various fatty acid chains at stabilizing the orientation of the tryptophan within the membrane. The hexapeptide will be synthesized using four fatty acid chains of varying lengths: six (hexanoic acid), eight (octanoic acid), ten (decanoic acid), and twelve (dodecanoic acid) carbons. If the experiment is successful in identifying a fatty acid chain that stabilizes the tryptophan within the membrane, then the results can be used to better understand the mechanism by which the tryptophan causes the membrane to rupture. A proper understanding of this mechanism could lead to the development of new antimicrobial peptides that operate using a similar mechanism. The creation of such peptides would provide a practical alternative for fighting microbes that have developed resistance to traditional antibiotics.

Experimental Methods

Fmoc addition to 1-MeTrp: In order to allow for unhindered synthesis of the hexapeptide, an Fmoc group will be added to one of the Trp residues to prevent unwanted side reactions for occurring during peptide synthesis. The Fmoc group will be added by dissolving Fmoc-protected succinamide and 1-methyl-L-Trp in dimethoxyethane solvent. The product will be extracted by filtration, then dried on a rotovap machine, washed with methyl-*t*-butyl ether, and dried on a vacuum line. The product will then be dissolved in ethyl acetate and allowed to crystallize. The crystalline product will be pure Fmoc-1-methyl-Trp, which will be protected well enough to be used in the hexapeptide synthesis.

Peptide synthesis: The peptides proposed for this research project will be prepared by Fmoc solid phase methods on a peptide synthesizer equipped with a UV monitor. The first amino acid, Fmoc-Arg (Pmc) will be loaded to a Rink Amide AM resin, and the loading efficiency will be tested by measuring the amount of Fmoc protecting group present on a small sample of the resin. After capping unreacted sites on the resin, the remaining Fmoc-amino acids will be coupled. Side chain protecting groups will be N-pentamethylchroman-sulfonate (Pmc) for arginine, trityl (Trt) for glutamine, and *t*-butoxycarbonyl (Boc) for tryptophan. At each step, the amino Fmoc group will be removed from the growing chain with 20% piperidine in N-methylpyrollidinone.

Addition of Fatty acids to Peptides (Acylation): N-terminal acylation (addition of the fatty acid chain) will be accomplished by coupling fatty acids containing either 6 (hexanoic), 8 (octanoic), 10 (decanoic), or 12 (dodecanoic) carbons to the final Fmoc-deprotected amino acid.

Cleavage of Peptides from Resin: Upon completion the peptides will be removed from the resin using a trifluoroacetic acid (TFA) thiol cleavage cocktail under a blanket of N_2 for 4 hours at room temperature. After cleavage, the peptides will be separated from the resins by filtering into a polypropylene centrifuge tube. The peptides will be rinsed once with TFA, and the volume will be reduced under a stream of N_2. The peptide will then be precipitated with ice-cold methyl-*t*-butyl ether:hexane mixture, incubated on ice for 30 minutes, and centrifuged at 4 °C for 10 minutes at 1,700 rpm in a bench-top centrifuge. The supernatant will be discarded, and the precipitation-centrifugation steps will be repeated 3 times. The final peptide pellet will be lyophilized to a white powder from acetonitrile/H_2O mixture.

Deuteration of Trp and 1-MeTrp Residues in Peptides: To selectively and partially deuterate only the Trp and 1-MeTrp side chains, dried peptide powders will be dissolved in deuterated TFA and mixed for 4 hours at 4 °C. The volume of the TFA will be reduced under a stream of N_2 gas, and the peptides will be precipitated with ice-cold methyl-*t*-butyl ether:hexane solution, incubated for 30 minutes, and centrifuged. To ensure that back-exchange for all the peptide amide backbone protons occurs, the peptide will be lyophilized twice from acetonitrile/H_2O.

Characterization of Peptides: To confirm the quality of the peptides and the deuteration, the peptides will next be characterized by Reverse-Phase High Performance Liquid Chromatograph (RP-HPLC) and Electrospray or Matrix Assisted Laser Desorption Ionization Mass Spectrometry (ESI-MS or MALDI-MS) in dH_2O. The peptide will be quantitated using a Diode Array Spectrometer based on the absorbance of the Trp and 1-MeTrp residues at 280 nm.

Oriented Solid State ^{31}P and ^{2}H NMR: The acylated, partially deuterated peptides will next be used to prepare NMR samples. The peptide and lipid mixture will be dried under N_2 gas to remove the liquid, redissolved in 1mL chloroform, and evenly distributed over 50 glass slides arranged in a Petri dish. The Petri dishes will be dried under high vacuum for 48 hours, then hydrated and placed in a cuvette. The cuvette will be sealed and placed in a heating block at 45°C for about one week to allow the sample to become transparent. The samples will be measured by solid state ^{31}P NMR to confirm that the lipids have oriented, and by the solid state ^{2}H to investigate the orientation of the Trp or 1-MeTrp side chains.

2-Dimensional solution NMR in SDS-d25: Samples will also be prepared for 2 dimensional (2D) ^{1}H NMR in 90% dH_2O/10% D_2O and deuterated SDS (SDS-d25) micelles. To prepare the aqueous samples, each peptide will be dissolved in 90% dH_2O/10% D_2O, the pH will be adjusted to 4.5 using a pH meter, and the sample will be transferred to an NMR tube. For each sample, COESY (Correlated Spectroscopy), NOESY (Nuclear Overhauser Spectroscopy) and TOCSY (Total Correlated Spectroscopy) spectra will be obtained for structural analysis of the peptides.

Research Plan: This project will tentatively follow the schedule listed below.

2004
October—Prepare 2 batches of Fmoc-1MeTrp and analyze by 1H-NMR
Nov—Synthesize LfB 20-25 add the 6 carbon Fatty acid tail
Dec—Cleave LfB 20-25-FA6 from resin and treat with TFA-d1

2005
January— Analyze LfB 20-25(1MeTrp)-FA6 by HPLC and mass spectrometry
February—
a. Prepare oriented sample of LfB 20-25-FA6 in DOPC:DOPG (3:1) for solid state ^{2}H NMR
b. Prepare sample of LfB 20-25-FA6 in deuterated SDS micelles for 2 dimensional solution ^{1}H NMR and circular dichroism spectroscopy

March— Synthesize LfB 20-25(1MeTrp) and add the 12 carbon fatty acid tail
April—
a. Cleave LfB 20-25-FA12 from resin and treat with TFA-d1
b. Analyze LfB 20-25(1MeTrp)-FA12 by HPLC and mass spectrometry
September—
a. Prepare oriented sample of LfB 20-25-FA6 in DOPC:DOPG (3:1) for solid state ^{2}H NMR
b. Prepare sample of LfB 20-25-FA12 in deuterated SDS micelles for 2 dimensional solution ^{1}H NMR and circular dichroism spectroscopy
October—Work on data analysis

40 weeks, 10 hours a week = 400 hours

Bibliography

Cavestro, Giulia Martina, et al. "Lactoferrin: mechanism of action, clinical significance and therapeutic relevance." Acta Bio-Medica 73, number 5-6 (2002): 71-73.

Schibli, David J., et al. "The structure of the antimicrobial active center of lactoferricin B bound to sodium dodecyl sulfate micelles." FEBS Letters 446 (1999): 213-217.