
SCHOOL *of* GRADUATE STUDIES
EAST TENNESSEE STATE UNIVERSITY

East Tennessee State University
Digital Commons @ East Tennessee State University

Electronic Theses and Dissertations | Student Works

5-2011

Requirement of ßDELSEED-Motif of *Escherichia coli* F_1F_O ATP Synthase in Antimicrobial Peptide Binding.

Junior Kom Tayou
East Tennessee State University

Follow this and additional works at: https://dc.etsu.edu/etd

 Part of the Bacteriology Commons, and the Microbial Physiology Commons

Recommended Citation
Tayou, Junior Kom, "Requirement of ßDELSEED-Motif of *Escherichia coli* F_1F_O ATP Synthase in Antimicrobial Peptide Binding." (2011). *Electronic Theses and Dissertations.* Paper 1260. https://dc.etsu.edu/etd/1260

This Thesis - Open Access is brought to you for free and open access by the Student Works at Digital Commons @ East Tennessee State University. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of Digital Commons @ East Tennessee State University. For more information, please contact digilib@etsu.edu.

Requirement of βDELSEED-Motif of *Escherichia coli* F_1F_O ATP Synthase in Antimicrobial Peptide Binding

---

A thesis

presented to

the faculty of the Department of Biological Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Biology

---

by

Junior K. Tayou

May 2011

Michael Zavada, Ph.D., Chair

Dhirendra Kumar, Ph.D.

Thomas F. Laughlin, Ph.D.

Key words: F_1F_O ATP Synthase, Antimicrobial Peptides; Melittin-NH_2 and MRP-NH_2, ATPase Activity

ABSTRACT

Requirement of βDELSEED-Motif of *Escherichia coli* F_1F_O ATP Synthase in Antimicrobial Peptide Binding

by

Junior K. Tayou

F_1F_O ATP synthase is a membrane bound enzyme capable of synthesizing and hydrolyzing ATP. Lately, α-helical cationic peptides such as melittin and melittin related peptide (MRP) were shown to inhibit *E. coli* ATP synthase. The proposed but unconfirmed site of inhibition is βDELSEED-motif formed by the residues 380-386, located at the interface of α/β subunit of ATP synthase. This project was a mutagenic analysis of βDELSEED –motif residues to understand the binding mechanism and mode of action of peptide inhibitors. The study addressed 2 main questions: Are the antibacterial/anticancer effects of these peptides related to their inhibitory action on ATP synthase through interaction with the βDELSEED –motif? If so, which amino acid residues play critical role in peptide binding?

The findings demonstrated that the βDELSEED –motif is the binding site of the above peptides on ATP synthase and Glutamate residues are more important in peptide binding than the Aspartate residues.

ACKNOWLEDGEMENTS

I will like to acknowledge my committee members; Dr. Thomas Laughlin, for his intellectual input during the course of this project and for critical review of this manuscript; Dr. Dhirendra Kumar, for providing me with bench space in his lab to complete part of this project, for his intellectual input, and also for reviewing this manuscript; Dr. Michael Zavada, for reviewing this manuscript and all his advice through the course of this project. I would also like to acknowledge Dr. Zulfiqar Ahmad for being the originator of the idea behind this project and for his guidance and advice. I will like to express my appreciation to the faculty, staff, and student body in the Department of Biological Sciences for making ETSU a wonderful learning environment.

This work was supported in part by National Institutes of Health grant GM 085771 to Dr. Zulfiqar Ahmad. I will also like to thank the Graduate School for providing me with graduate assistantship throughout my stay at ETSU.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Background Information on ATP Synthase

F_1F_O ATP synthase is a multisubunit membrane-associated enzyme complex that is responsible for cellular energy production in animals, plants, and almost all microorganisms by oxidative phosphorylation and photophosphorylation. In energy-transducing membranes, this enzyme complex uses energy from a proton motive force generated by the electron transport chain to catalyze the formation of ATP from ADP and inorganic phosphate. In most prokaryotic organisms, depending on the nutritional need, this complex can work in the reverse direction generating an electrochemical proton gradient at the expense of ATP hydrolysis. Thus, this enzyme is capable of coupling reversibly the hydrolysis or synthesis of ATP, respectively, with an endergonic and exergonic efflux and influx of protons across the membrane (Boyer 1997; Fillingame et al. 2000; Capaldi and Aggeler 2002). ATP synthase is present in all living organisms and is located in the inner membrane of the mitochondria, the thylakoid membrane of the chloroplast, plasma membrane of bacteria, and on the surfaces of various mammalian cell types, including endothelial cells, adipocytes, and keratinocytes. The overall topology of ATP synthases is well conserved in evolution. In *E. coli*, the molecular weight of this enzyme is ~ 530 kDa, consisting of 2 rotary sectors, the F_1 (hydrophilic or extrinsic) portion formed by 5 polypeptides in the stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$ and an F_O (hydrophobic or intrinsic) portion formed by three polypeptides in the stoichiometry of ab_2c_{10} (figure 1). On the F_1 sector are located 3 active sites of this enzyme at the 3 interfaces of the α and β

subunits that have alternating affinities for ATP, ADP and P_i. There is a high similarity in the ATP synthase subunits from different bacteria and chloroplasts. The mitochondrial counterpart is much more complex; 17 different types of subunits have been identified so far. Subunits $\alpha_3\beta_3\gamma$ in the F_1 portion and subunits *a* and *c* in the F_O portion have high similarity to the bacterial and chloroplast counterparts. These are the subunits that are directly involved in proton transport, subunit rotation and ATP synthesis/hydrolysis (Frasch 2000; Ren 2000; Noji and Yoshida 2001; Weber and Senior 2003; Ahmad and Senior 2005; Feniouk et al. 2006; Pedersen 2007; Senior 2007).

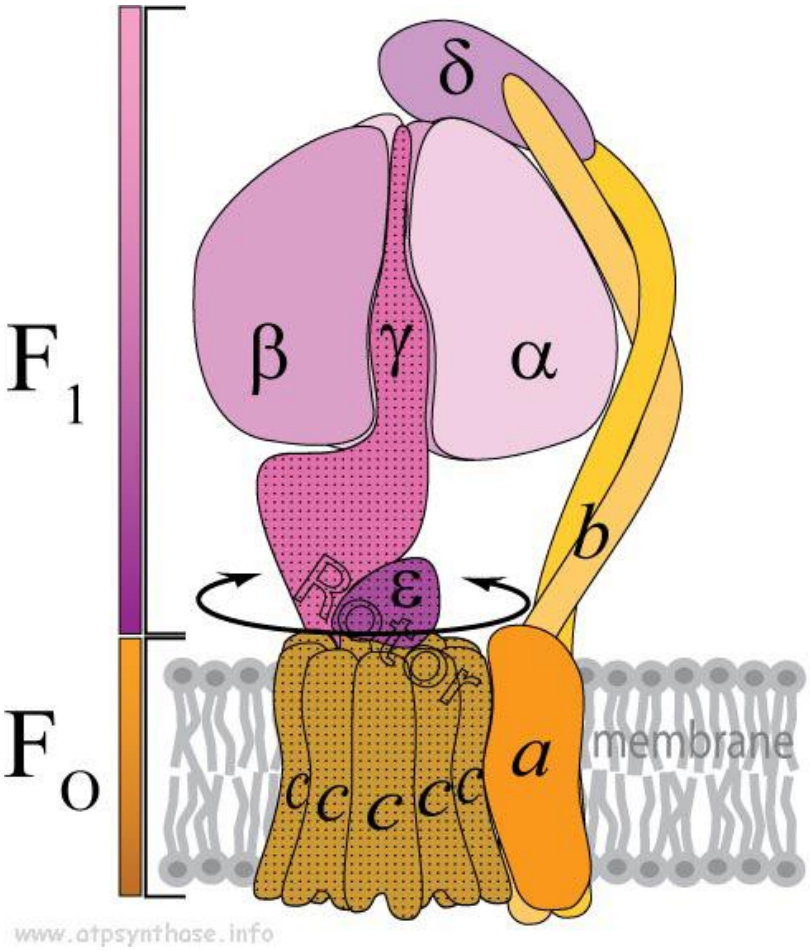


Figure 1: Structure of bacterial F_1F_O ATP synthase by Feniouk, B.A. F_1 subunits α_3 and β_3 hexamer form the catalytic portion. The γ-subunit forms the shaft of the rotor. Proton translocation take place at the interface of subunits a and c.
Figure used with permission from Feniouk 2006.

Proton flow down the gradient through F_O is coupled to ATP synthesis on the F_1 portion by a unique rotary mechanism. The protons flow through channels at the interface of the a and c subunits, which drives rotation of the ring of c subunits. The c_{10} ring, together with γ and ε subunits, forms the rotor while the "stator" composed of $b_2\delta$, prevents the simultaneous rotation of catalytic sites and the *a* subunit with the rotor. Rotation of γ leads to conformational changes in the catalytic nucleotide binding sites on

the β subunits where ADP and P_i are bound. Proton driven clockwise rotation of the γ subunit leads to a conformational change that results in the formation and release of ATP. Thus, ATP synthase converts electrochemical energy, the proton gradient, into mechanical energy in the form of subunit rotation and back into chemical energy in the form of phosphoanhydride bonds in ATP, making this enzyme the smallest known biological nanomotor. In *E. coli* this enzyme can function reversibly depending on the physiological needs of the cell. A plasma membrane proton motive force required for mechanical processes such as nutrient transport and locomotion can be generated by hydrolysis of glycolytically derived ATP by the F_1F_O-ATPase function, with the result that protons are pumped from the cytoplasm to the intermembrane space of the plasma membrane (Hara et al. 2001; Noji and Yoshida 2001; Weber and Senior 2003; Wilkens 2005; Dimroth et al. 2006; Weber 2006).

Importance of This Study

ATP synthase is the fundamental means of cellular energy production in animals, plants, and almost all microorganisms. In order to synthesize ATP, the cell's energy currency, through oxidative phosphorylation and photophosphorylation, a mechanical rotation mechanism is used in which subunits rotate at approximately 100 times per second in order to convert foodstuffs into energy by oxidation.

Many studies have portrayed ATP synthase as a critical enzyme in human health and in the growth of disease causing organisms in the human body. Malfunction of this enzyme complex has been directly or indirectly associated with various disease conditions (Hong and Pedersen 2008). A low expression of the β subunit on the F_1

portion of this complex and the accumulation of the α subunit in the cytosol of neuronal cells is characteristic of Alzheimer's disease, a progressive and neurodegenetive disorder (Sone et al. 1979; Chandrasekaran et al. 1997; Kim et al. 2000). Leigh syndrome is also another example of a neurodegenerative disorder that is associated with a mutation in subunit a of the F_O sector of this complex leading to impaired ATP synthesis (de Vries et al. 1993). Dysfunction of ATP synthase as a result of a mutation in the a subunit is also implicated in ataxia, retinitis pigmentosa syndrome, and neuropathy (De Meirleir et al. 1995; Thyagarajan et al. 1995). A dysfunctional c subunit is implicated in Kufs' disease, which is a lysosomal storage disease (Palmer et al. 1986). Additionally, the α subunit has being characterized as a molecular target for innate antitumor cytotoxicity mediated by natural interleukin 2-activated killer cells (Das et al. 1994). Intravascular circulation of ATP synthase F_6 subunit is associated with increased blood pressure (Pal and Coleman 1990).

Nonmitochondrial ATP synthases that are located on the surface of endothelial cells, adipocytes, and keratinocytes have been shown to be involved in cell mediated processes such as angiogenesis, pH homeostasis, lipid metabolism, and apoptosis (Arakaki et al. 2003; Hong and Pedersen 2008; Laughlin and Ahmad 2010). These associations make ATP synthase a good molecular target for drugs in the treatment of different diseases and the regulation of energy metabolism. Examples of such drugs include Benzodiazepine (Bz-423) used as a therapy for systemic lupus erythematosus, an autoimmune disorder. Bz-423 selectively kills pathogenic lymphocytes by inducing apoptosis in lymphoid cells. Bz-423 has been shown to bind to the oligomycin sensitivity-conferring subunit of ATP synthase and significantly reduce its activity (Blatt,

Bednarski et al. 2002). Diarylquinoline drugs used in treating tuberculosis are another example, as they function through blocking the synthesis of ATP by binding to subunit c of ATP synthase and thereby preventing rotation of the γ subunit (Andries, Verhasselt et al. 2005). Recent studies have also shown that inhibition of nonmitochondrial ATP synthase results in inhibition of cytosolic lipid droplet accumulation in adipocytes and inhibition of tumor angiogenesis in endothelial cancer cells, thereby preventing their proliferation and migration (metastasis) (Moser, Stack et al. 1999; Moser, Kenan et al. 2001; Moser, Stack et al. 2002; Arakaki et al. 2007).

Peptides and Other Inhibitors of ATP Synthase

Many organic and inorganic compounds, both natural and synthetic, have been shown to bind and inhibit the synthetic and hydrolytic activity of ATP synthase. These inhibitors include sodium azide, fluoroaluminate, bioflavinolic compounds, polyketide, peptides, and a host of other organic molecules (Hong and Pedersen 2008; Dadi et al. 2009; Laughlin and Ahmad 2010). Antimicrobial α helical basic peptides such as Melittin, (figure 3) the principal component of honeybee venom, and Melittin Related Peptide, a frog skin peptide were shown to inhibit the growth of *E. coli* cells and hydrolytic activity of both membrane-associated *E. coli* F_1F_O ATP synthase and purified F_1-ATPase (Laughlin and Ahmad 2010). The binding site of these peptides, based on indirect evidence, is the βDELSEED-motif (figure 2) located in a loop at the carboxyl terminal of the β subunit. This motif has been well conserved in all forms of F_1 with minor variations; DELSDED in TF_1-β, DELSEED in MF_1-β, and DELSEED in the β

subunit of F_1 from *Escherichia coli* (EF_1). This loop is also highly conserved in many species (Table 1). In the closed conformation of the β subunit, this region has contact with the γ subunit. The complement contact region of the γ subunit is mainly in the short helix that forms a small protrusion from the straight coiled-coil structure. Based on these observations, the βDELSEED-motif has been assumed to play an essential role in the rotation of the γ subunit and hence the coupling between catalysis and transport (Hara et al 2001; Mnatsakanyan et al. 2009). The ε subunit is an intrinsic inhibitor of F_1-ATPase and it rotates along with the γ subunit. The ε subunit was unable to inhibit the F_1-ATPase activity of a mutant thermophilic ATP synthase (TF_1) in which all the acidic amino acid residues of the βDELSEED-motif were replaced by alanine residues and a similar loss of inhibition by the ε subunit was observed when the basic residues in the C-terminal of ε subunit were replaced by alanine residues (Hara et al. 2001).

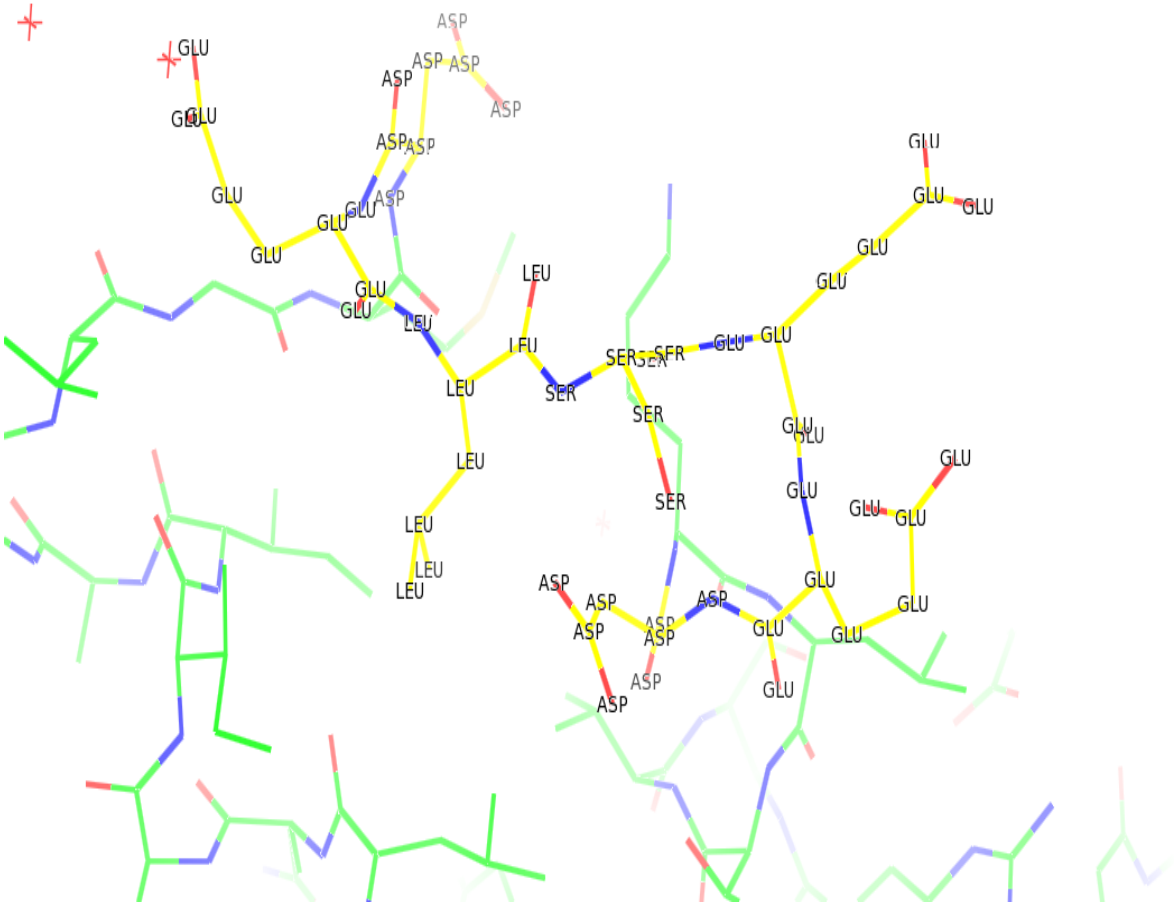


Figure 2a: X-ray Crystallographic structure of the βDELSEED loop showing the negatively charged amino acids residues. This figure was generated using the PyMOL software. The *E. coli* numbers for the DELSEED residues are 380 – 386.

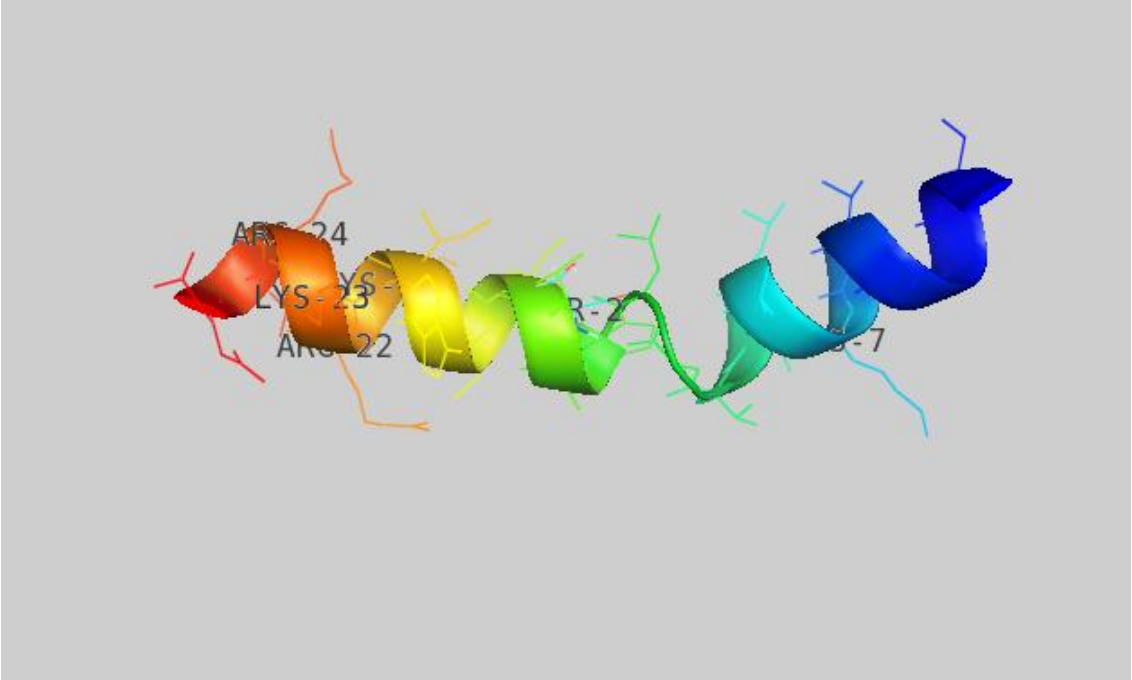


Figure 2b: The x-ray structure of Melittin showing the five positively charged amino acid residues. Most AMPs are known to have α-helical-amphipathic structures similar to that of melittin. This figure was also generated using the PyMOL software.

Table 1: Amino acid sequence of the evolutionary conserved βDELSEED loop. The starting residue tyrosine shown here for E. coli is βY355

Escherichia coli	YDTARG VQSILQRYQE LKDIIAILGM DELSEEDKLV VARARKIQRF LSQPFF
Shigella dysenteriae	YDTARG VQSILQRYQE LKDIIAILGM DELSEEDKLV VARARKIQRF LSQPFF
Salmonella typhimurium	YDTARG VQSILQRYQE LKDVIAILGM DELSEEDKLV VARARKIQRF LSQPFF
Haemophilus influenzae	YDVARG VQGILQRYKE LKDIIAILGM DELSEEDKLV VARARKIERF LSQPFF
Klebsiella pneumonia	YDTARG VQSILQRYQE LKDIIAILGM DELSEEDKLV VARARKIQRF LSQPFF
Photorhabdus asymbiotica	YNVARG VQSILQRYQE LKDIIAILGM DELSEDDKLV VARARKIQRF LSQPFF
Erwinia tracheiphila	YDVARG VQSLLQRYQE LKDIIAILGM DELSEEDKLV VARSRKMQRF LSQPFF
Mycobacterium tuberculosis	YRVAQE VIRILQRYKD LQDIIAILGI DELSEEDKQL VNRARRIERF LSQNMM
Thyrsopteris elegans (chl)	YETAQG VKQTLQRYKE PQDIIAIPGL DELSEEDRLT VARARKIERF LSQPFL
Beta vulgaris	YEIAQR VKQTLQRYKE LQDIIAILGL DELSEEDRLT VARARKIERF LSQPFF
Trypanosoma brucei gambiense	YNVAQD VVQMLTKYRE LQDIIAVLGI DELSEEDKLI VDRARKLVKF LSQPFQ
Rattus norvegicus	YDVARG VQKILQDYKS LQDIIAILGM DELSEEDKLT VSRARKIQRF LSQPFQ
Mus musculus	YDVARG VQKILQDYKS LQDIIAILGM DELSEEDKLT VSRARKIQRF LSQPFQ
Homo sapiens	YDVARG VQKILQDYKS LQDIIAILGM DELSEEDKLT VSRARKIQRF LSQPFQ

Antimicrobial Peptides

Antimicrobial peptides are innate components of the immune response that are present in all eukaryotic classes. These peptides have been shown to kill gram positive and negative bacteria, enveloped viruses, fungi, and cancerous cells. Some of these peptides are thought to function as immunomodulators, enhancing or repressing the immune response. Most AMPs have a broad range of activity targeting 2 or more cellular structures or processes (Sitaram and Nagaraj 2002).

There are over 1500 identified AMPs in the antimicrobial database, most of which exist as α-helical amphipathic molecule of between 12 and 50 amino acid residues. They usually contain 2 or more positively charged residues provided by arginine and/or lysine.

This cationicity allows for ionic interaction with the phosphate head of the lipid bilayer, while the hydrophobic amino acid residues interact with the hydrophobic tails enabling their transport into microbial cells (Sitaram and Nagaraj 2002; Laughlin and Ahmad 2010). These membrane targeting peptides have a potent bacteriocidal activity against multidrug resistant pathogens in a controlled environment, but little is known about their effects within a living organism (Hancock and Sahl 2006). This selectivity might be because the cell membranes of bacteria are more negatively charged compare to mammalian cells; bacteria membranes are richer in acidic phospholipids, such as phosphatidylglycerol and cardiolipin, causing AMPs to have an increased affinity to bacterial membrane. Secondly, cholesterol is a stabilizing agent found in mammalian plasma membranes but which is absent in bacteria membranes. Cholesterol may reduce the activities of AMPs by stabilization of the lipid bilayer and/or its interaction with the peptide. Additionally, the transmembrane potential of bacterial cells is more negative than that of normal mammalian cells, making bacteria more prone to membrane permeabilization by the insertion of positively charged AMPs (Sitaram and Nagaraj 2002; Tennessen 2005; Hancock and Sahl 2006; Matsuzaki 2008). AMPs are thought have varied mechanisms by which they carry out their antimicrobial activities, ranging from membrane permeabilization to effects on a range of cytoplasmic targets including interference with metabolism (Matsuzaki et al. 1995).

Hypotheses

Recently it was shown that some AMPs including melittin, melittin related peptide (MRP), magainin, and their derivatives differentially inhibit the hydrolytic

activity of *E. coli* F_1F_O ATP synthase (Hong and Pedersen 2008; Laughlin and Ahmad 2010). These same peptides were also shown to differentially inhibit the growth of wild type *E. coli* strain pBWU13.4/DK8, with melittin and MRP and their C-terminal amide derivatives being more potent inhibitors compared to magainin (Laughlin and Ahmad 2010). The proposed binding site of these peptides on F_1F_O ATP synthase is the βDELSEED-motif. This study was a mutagenic analysis of the βDELSEED-motif to understand the mechanism by which these AMPs inhibit F_1-ATPase. Mechanistic knowledge of peptide binding will be helpful in understanding the antibacterial/anticancer nature of the above peptides. The main questions were;

1. Does the inhibition of F_1-ATPase by AMPs involve the binding of these AMPs to the βDELSEED-motif? And if so,
2. Which of the amino acid residue(s) of this motif are most essential for peptide binding?

These questions were answered through substitution mutagenesis of the amino acids in the βDELSEED-motif and comparative inhibitory biochemical assays of AMPs to wild-type and mutant F_1F_O ATP synthase. The acidic amino acid residues (Aspartate or Glutamate) were changed to Alanine residues, Leucine and Serine were also changed to Alanine, Arginine, Glutamate, or Glutamine. Comparative inhibitory biochemical assays of AMPs to wild type and mutant F_1F_O ATP synthase were performed to assess the effect of these mutations on AMP binding.

CHAPTER 2

MATERIALS AND METHODS

Construction of Wild Type and Mutant Strains of *E. coli*

The wild-type strain was pBWU13.4/DK8 (Ketchum et al. 1998; Li et al. 2009). The template for oligonucleotide-directed mutagenesis was pSN6. pSN6 is a plasmid with the βY331W mutation from plasmid pSWM4 introduced on a SacI-EagI fragment into pBWU13.4 (Weber et al 1993; Li et al. 2009). This plasmid was used as the template to generate the following substitution mutations; βL382A, βL382R, βL382Q, βL382E, βS383A, βS383R, βS383Q, βS383E, βD380A, βE381A, βE384A, βE385A, and βE386A. Substitutions were introduced by polymerase chain reaction using the Stratagene quikchange lightning mutagenesis kit from Agilent Technologies (catalog #210519) (see appendix A for essential steps). The mutagenic oligonucleotides were designed using an online (http://www.stratagene.com/qcprimerdesign) software package provided by Stratagene (appendix D shows the forward primers for the different mutants that were designed) and purchased from Fischer Scientific. Wild-type and mutated plasmids were transformed into *E. coli* DK8 competent cells (see appendix E), which does not express *E. coli* ATP synthase (Weber et al. 1993). DNA sequencing was performed on plasmids from the transformed DK8 strains to confirm the presence of mutations and the absence of undesired changes in sequence. It should be noted that all the mutant strains contain the βY331W mutation, which does not affect the function of this enzyme significantly, and the presence of this mutation was included for future studies on flourimetric estimations of ADP and/or ATP binding and transition-state formation.

Measurement of Growth Yield in a Limiting Glucose Medium

Oxidative and/or photophosphorylation were measured on limiting glucose media. Limiting glucose is a phosphate buffer containing 3–5 mM glucose with other micro- and macronutrients (See details in appendix F). *E. coli* strain DK8 cells harboring wild-type or mutated plasmids were aerobically cultivated at 37^{o}C for 16 – 24 hours in a limiting glucose medium and growth was measured spectrophotometrically at OD_{595} as in (Senior et al. 1983; Ahmad and Senior 2005).

Preparation of *E. coli* Membrane Associate F_1F_O ATP Synthase

Wild-type or mutant *E. coli* membrane associate F_1F_O ATP synthase were isolated and purified as in [(Senior et al. 1983; Ahmad and Senior 2005) and appendix B]. In this procedure, loop full wild type *E. coli* or mutant was aerobically cultivated for 18 – 24 hours (until late log phase of growth) at 37^{o}C, 250 rpm.

The cells were harvested by centrifugation (X2) at 9500 rpm, 4^{o}C for 15 – 25 minutes. Prior to the second centrifugation, the cells were resuspended in STEM buffer containing 20 mM Sucrose, and the pellet obtained was resuspended in 2 ml STEM/g of wet cells.

20 mg of DNase was added to cells that were then resuspended in STEM. The cells were disrupted by 2 passages through a chilled French Press cell fractionator at 20,000 psi. Cell debris were removed by centrifugation at 22,000 rpm for 20 minutes at 2^{o}C and the membrane bound enzyme was obtained by ultracentrifugation at 60,000 rpm for 120 minutes at 2^{o}C.

The isolated membrane bound F_1F_O ATP synthase was washed 3 times through ultracentrifugation at 60,000 rpm. The first wash was performed in a buffer containing 50 mM TES pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM p-aminobenzamidine. The subsequent 2 washes were performed in a buffer containing 5 mM TES pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM p-aminobenzamidine, 0.5 mM DTT, 0.5 mM EDTA. The final membrane bound F_1F_O ATP synthase was resuspended and stored in 50 mM TrisSO_4 pH 8.0, 2.5 mM $MgSO_4$ (Noji and Yoshida 2001).

Measurement of Membrane Associated F_1F_O ATP Synthase Activity

Enzyme concentrations were found by plotting the absorbance at 595 nm using Bradford reagent against a standard bovine serum albumin curve.

F_1-ATPase activity was measured in 1 ml assay buffer containing 10 mM NaATP, 4 mM $MgCl_2$, 50 mM TrisSO_4, at pH 8.5 and 37^{o}C. Reactions were started by addition of 20 µg membrane associated F_1F_O ATP synthase and stopped by addition of SDS to 3.3% (V/V) final concentration. P_i release was assayed spectrophotometrically by the addition of T&S reagent containing 10 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 250 mM $Fe(NH_4)_2(SO_4)_2\cdot 6H_2O$, and 1.2N H_2SO_4 which reacts with P_i to form a blue color complex the intensity of which was measured at OD_{700} (Taussky and Shorr 1953). The hydrolytic activity of ATP synthase in µmol/min/mg was calculated as:

$$ATPase\ Activity = \frac{\text{Average Sample OD} - \text{Average Blank OD}}{\text{amount of protein (mg) x time (min)}}$$

Source of Peptides and Other Chemicals

Melittin related peptide-amide (MRP-amide, an amide modified derivative of the amphibian melittin related peptide) and Melittin-amide (an amide modified derivative of the honey bee venom peptide melittin) were custom ordered from Biomatik. Both peptides were received as lyophilized powder shipped on dry ice and their purity determined by HPLC was greater than 95%. Once received, they were immediately stored at -20^{o}C and resuspended in autoclaved deionized water as needed. Other chemicals used in this study were ordered from Fischer Scientific Company, Agilent Technologies, or Sigma-Aldrich Chemical Company and were of ultra-pure analytical grade.

Inhibition of ATPase Activity by Amphibian Melittin Related Peptide-Amide or Honey Bee Venom Peptide Melittin-Amide

Wild-type or mutant membrane bound F_1F_o ATP synthase were preincubated with varied concentrations of MRP-amide or melittin-amide for 60 minutes at room temperature in 50 mM TrisSO_4 pH 8.0. 500 µl ATPase assay buffer contained 10 mM NaATP, 4 mM $MgCl_2$, 50 mM TrisSO_4, and pH 8.5. The reaction was stopped by the addition of SDS to a final concentration of 3.3% (v/v). The addition of an equal volume of T&S reagent gave rise to a blue color that was assayed spectrophotometrically at OD_{700}. Inhibitory exponential decay and piecewise curves were generated with Sigma plot 10.0 (Taussky and Shorr 1953; Laughlin and Ahmad 2010).

CHAPTER 3

RESULTS

Growth Properties of βD380A, βE381A, βE384A, βE385A, and βD386A Mutants of *E. coli* ATP Synthase

βD380A, βE381A, βE384A, βE385A, and βD386A mutants were generated and the presence of the desired mutations and absence of unwanted mutations were confirmed through sequencing of the C-terminal of the β-subunit. The Glutamate and Aspartate residues shown here are highly conserved in the βDELSEED-loop **(**figure 2 and Table 1**)** of ATP synthase from all sources and organisms. βD380A, βE381A, βE384A, βE385A, and βD386A mutations were designed to understand the possible effect of the smaller neutral side chain of Alanine in the binding/inhibition by AMPs.

The introduction of βE384A or βD386A mutation resulted in approximately 26% or 32% loss of oxidative phosphorylation respectively, but the growth yield of βD380A, βE381A, and βE385A was within ±15% that of the wild-type (Table 2). This slight loss in oxidative phosphorylation might be caused by the fact that the βDELSEED motif is not located in catalytic site of this enzyme.

Table 2: Growth Properties of βD380A, βE381A, βE384A, βE385A, and βD386A Mutants of *E. coli* ATP Synthase on Limiting Glucose

Strains	Mutations	Growth yield on limiting glucose
pBWU13.4 (Wild type)	-	100
pSN6	βY331W	98
pUC118 (Null)	-	42
pJT01	βD380A/ βY331W	92
pJT04	βE381A/ βY331W	93
pJT17	βE384A/ βY331W	84
pJT20	βE385A/ βY331W	91
pJT23	βD386A/ βY331W	81

*Wild type (pBWU13.4/DK8) contains UNC^{+} gene encoding ATP synthase; Null (pUC118/DK8) with UNC^{-} gene and lacks ATP synthase.

Table 2 shows the effects of βD380A, βE381A, βE384A, βE385A, or βD386A mutation on cell growth in a limiting glucose media. Limiting glucose contain 3 – 5 mM Glucose (see materials and method and appendix F). βD380A, βE381A, βE384A, βE385A, or βD386A mutant was expressed with βY331W also present, which has being shown to significantly affect cell growth (Weber and Senior 2003; Ahmad and Senior 2005; Li et al. 2009). Each data point is the mean of 4 experimental values.

Growth Properties of βL382A, βL382R, βL382Q, βL382E, βS383A, βS383R, βS383Q, and βS383E Mutants of *E. coli* ATP Synthase

The introduction of βL382A, βL382R, βL382Q, βL382E, βS383A, βS383R, βS383Q, or βS383E mutation resulted in between 2 and 17% loss of growth on a limiting glucose media (Table 3). This slight loss in oxidative phosphorylation again might be

caused by the fact that the βDELSEED motif is not located in catalytic site of this enzyme.

Table 3: Effects of βL382A, βL382R, βL382Q, βL382E, βS383A, βS383R, βS383Q, or βS383E mutation on cell growth in a limiting glucose media.

Strains	Mutations	Growth yield on Limiting Glucose
pBWU13.4	-	100
pSN6	βY331W	98
pUC118	-	42
pJT07	βL382A/ βY331W	90
pJT08	βL382R/ βY331W	85
pJT09	βL382Q/ βY331W	83
pJT11	βL382E/ βY331W	89
pJT12	βS383A/ βY331W	95
pJT13	βS383R/ βY331W	85
pJT14	βS383Q/ βY331W	93
pJT16	βS383E/ βY331W	93

*Wild type (pBWU13.4/DK8) contains UNC^{+} gene encoding ATP synthase; Null (pUC118/DK8) with UNC^{-} gene and lacks ATP synthase.

Table 3 shows the effects of βL382A, βL382R, βL382Q, βL382E, βS383A, βS383R, βS383Q, or βS383E mutation on cell growth in a limiting glucose media. Limiting glucose contain 3 – 5 mM Glucose (see materials and method and appendix F). Each data point is the mean of 4 experimental values.

ATPase Activity of Wild Type and Mutant F_1F_O ATP Synthase

Specific F_1-ATPase activities of membrane preparations containing mutant enzymes were compared with wild type and null control at 37°C (Table 4). Introduction of βL382Q reduced the ATPase activity by 2 fold while the other mutations that were introduced into this βDELSEED motif resulted in approximately 4 – 5 fold loss of ATPase activity.

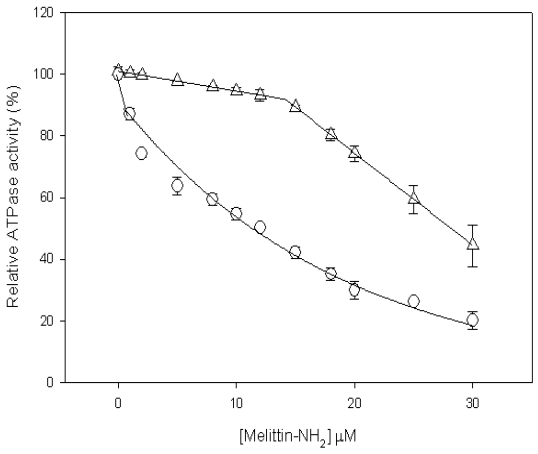
Table 4: ATPase Activity of Wild Type and Mutant F_1F_O ATP Synthase

Strains	***Mutations***		***ATPase Activity (μmol/mg/min)***
WT	-	DELSEED	20
pJT01	βD380A/ βY331W	AELSEED	4.7
pJT04	βE381A/ βY331W	DALSEED	2.9
pJT17	βE384E/ βY331W	DELSAED	5.3
pJT20	βE385A/ βY331W	DELSEAD	6.6
pJT23	βE386R/ βY331W	DELSEEA	3.5
pJT07	βL382A/ βY331W	DEASEED	5.7
pJT08	βL382R/ βY331W	DERSEED	7.4
pJT09	βL382Q/ βY331W	DEQSEED	12.5
pJT11	βL382E/ βY331W	DEESEED	4.8
pJT12	βS383A/ βY331W	DELAEED	3.4
pJT13	βS383R/ βY331W	DELREED	4.6
pJT14	βS383Q/ βY331W	DELQEED	4.1
pJT16	βS383E/ βY331W	DELEEED	6.9
Null	-	-	0

Table 4 shows ATPase Activity of wild type and mutant enzymes. The ATPase activity was measured at 37°C and expressed as μmol of ATP hydrolyzed/min/mg of protein present. Each individual experimental point is the mean of duplicate assay tubes.

Inhibition of ATPase Activity of Membrane bound βD380A and βD386A Mutant F_1F_O ATP Synthase by Melittin-Amide and MRP-Amide

Figure 3a shows the inhibition of ATPase activity of pJT01/DK8 (βD380A, or AELSEED) and figure 3b pJT23/DK8 (βD386A, or DELSEEA) membrane bound F_1F_O ATP synthase in the presence of varied concentrations of melittin-amide. The results with the wild type enzyme was within ± 10% that reported in previous studies involving inhibition of wild type (pBWU13.4/DK8) F_1F_O ATP synthase with Melittin-amide (Li et al. 2009). The result also shows that in the presence of βD380A or βD386A mutation, the inhibitory profile with melittin-amide was significantly changed. 20 µM melittin-amide inhibited 75% of wild type (DELSEED) activity, 30% of pJT01/DK8 (AELSEED), and 28% of pJT23/DK8 (DELSEEA) activity.



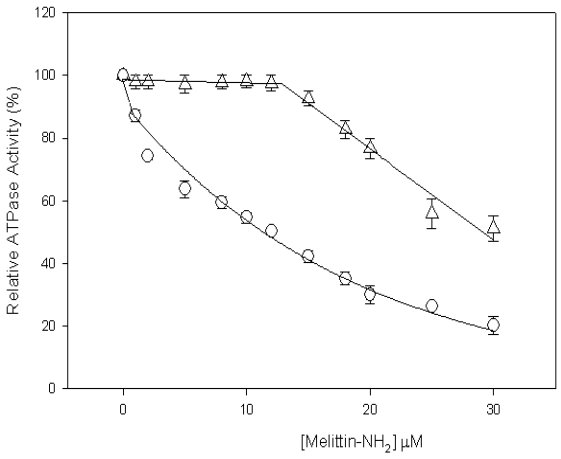


Figure 3a: βD380A (AELSEED) Figure. 3b**:** βD380A (DELSEEA)

Figure 3: Inhibition of ATPase activity in F_1F_O ATP synthase by melittin-amide on; the membrane bound enzymes were preincubated with varied concentration of melittin-amide for 60 min at 26 °C and then 1 ml of assay buffer was added and ATPase activity determined. See details in material and methods. Symbols used are: (○, Wild-type (DELSEED) and Δ, βD380A (AELSEED) or βD386A (DELSEEA)). Each data point represents average of at least 2 experiments done in duplicate and results agreed within ±10%.

Figure 4a shows the inhibition of ATPase activity of pJT01/DK8 (AELSEED) and Figure 4b pJT23/DK8 (DELSEEA) membrane bound F_1F_O ATP synthase in the presence of varied concentrations of MRP-amide. The results with the wild-type enzyme was within ± 10% of that reported in previous studies involving inhibition of the wild-type strain (pBWU13.4/DK8) with MRP-amide (Laughlin and Ahmad 2010). The result also shows that in the presence of βD380A or βD386A mutation, the inhibitory profile with MRP-amide was significantly changed. 20 µM MRP-amide was inhibited to 70% of wild type (DELSEED) ATPase activity, 58% of pJT01/DK8 (AELSEED), and 50% of pJT23/DK8 (DELSEEA) ATPase activity.

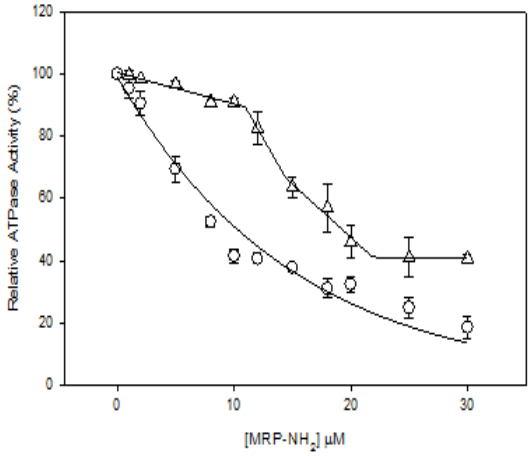


Figure 4a: βD380A (AELSEED)

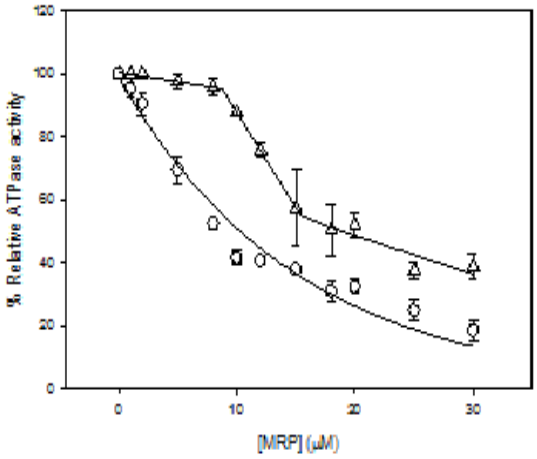


Figure 4b: βD380A (DELSEEA)

Figure 4: Inhibition of ATPase activity in F_1F_O ATP synthase by MRP-amide. Symbols used are: (○, Wild type (DELSEED) and Δ, βD380A (AELSEED) or βD386A (DELSEEA)). Each data point represents average of at least 2 experiments done in duplicate and results agreed within ±10%.

Inhibition of ATPase Activity of Membrane bound βE381A, βE384A and βE385A Mutant F_1F_O ATP Synthase by Melittin-Amide and MRP-Amide

Figure 5a shows the inhibition of ATPase activity pJT04/DK8 (DALSEED), Figure 5b: pJT17/DK8 (DELSAED) and Figure 5c: pJT20/DK8 (DELSEAD) of membrane bound F_1F_O ATP synthase in the presence of varied concentrations of melittin-amide. The result also shows that in the presence of βE381A, βE384A, or βE385A mutation, the inhibitory profile with MRP-amide was significantly changed. 20 µM melittin-amide inhibited 5% of pJT04/DK8 (DALSEED), 20% of pJT17/DK8 (DELSEAD), and 22% of pJT20/DK8 (DELSEAD) ATPase activity.

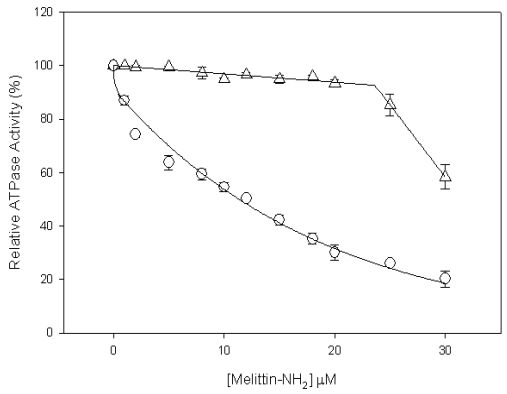


Figure 5a: βE381A (DALSEED)

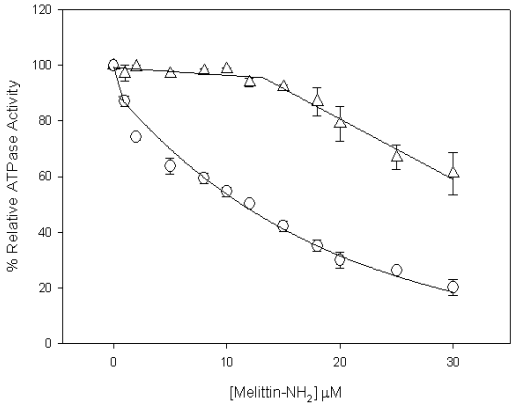


Figure 5b: βE384A (DELSAED)

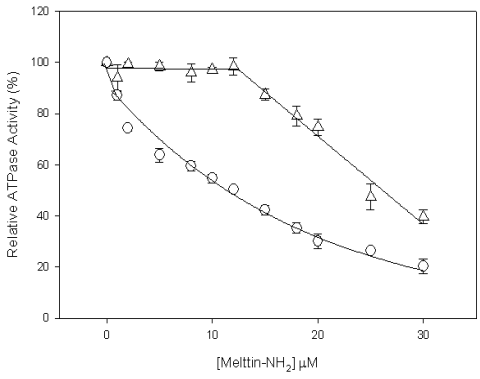


Figure 5c**:** βE385A (DELSEAD)

Figure 5: Inhibition of ATPase activity in F_1F_O ATP synthase by melittin-amide. Symbols used are: (○, Wild type (DELSEED) and Δ, βE381A (DALSEED), βE384A (DELSAED), or βE385A (DELSEAD)). Each data point represents average of at least 2 experiments done in duplicate and results agreed within ±10%.

Figure 6c shows the inhibition of ATPase activity of pJT04/DK8 (DALSEED), Figure 6b: pJT17/DK8 (DELSAED) and Figure 6c: pJT20/DK8 (DELSEAD) membrane bound F_1F_O ATP synthase in the presence of varied concentrations of MRP-amide. The result also shows that in the presence of βE381A, βE384A, or βE385A mutation the inhibitory profile with MRP-amide was significantly changed. 20 μM MRP-amide was inhibited to 40% of pJT04/DK8 (DALSEED), 25% of pJT17/DK8 (DELSEAD), and 55% of pJT20/DK8 (DELSEAD) ATPase activity.

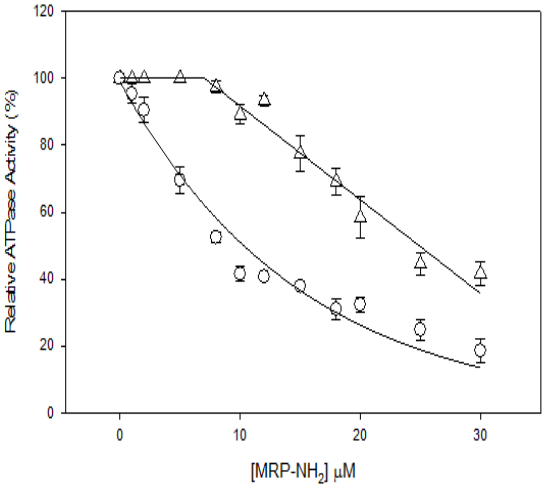


Figure 6a: βE381A (DALSEED)

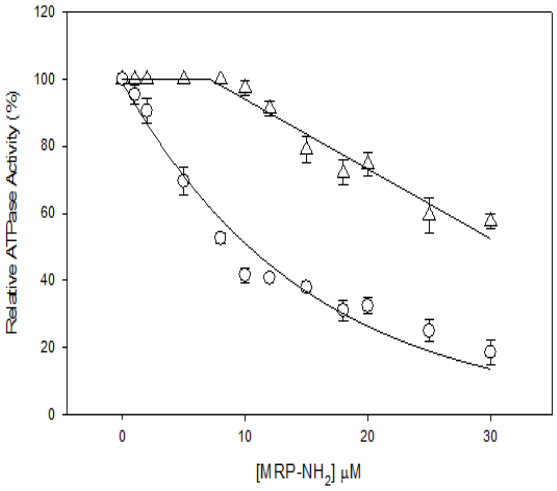


Figure 6b: βE384A (DELSAED)

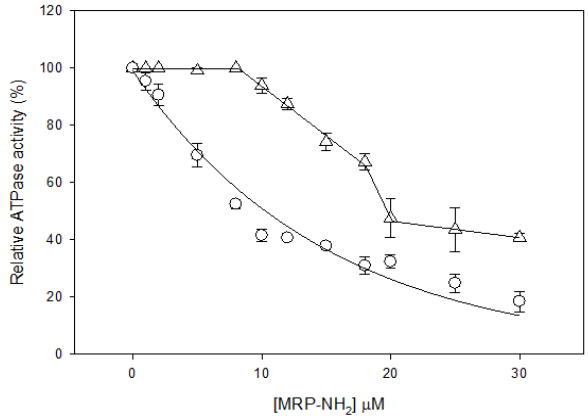


Figure 6c**:** βE385A (DELSEAD)

Figure 6: Inhibition of ATPase activity in F_1F_O ATP synthase by MRP-amide. Symbols used are: (○, Wild type (DELSEED) and Δ, βE381A (DALSEED), βE384A (DELSAED), or βE385A (DELSEAD)). Each data point represents average of at least 2 experiments done in duplicate and results agreed within ±10%.

Inhibition of ATPase Activity of Membrane bound βL382A, βL382R, βL382Q or βL382E, Mutant F_1F_O ATP Synthase by MRP-Amide

The introduction of βL382A or βL382Q mutation did not significantly change the inhibitory profile compared to wild-type. The introduction of βL382R mutation resulted in a significant loss of inhibition by MRP-NH_2. Interestingly 20 µM MRP-NH2 inhibited 82% of βL382E mutant ATPase activity compared to 76% activity in wild-type (figure 7).

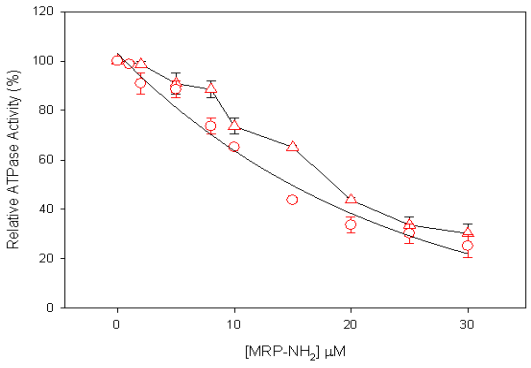


Figure 7a: βL382A (DEASEED)

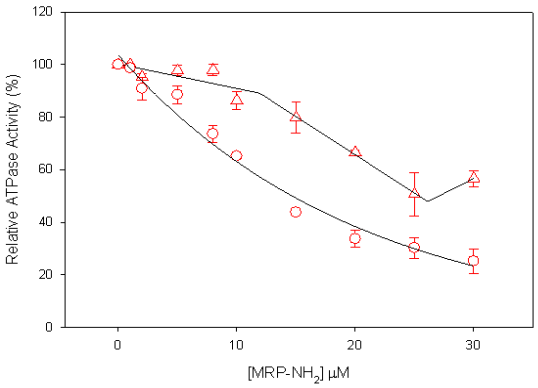


Figure 7b: βL382R (DERSEED)

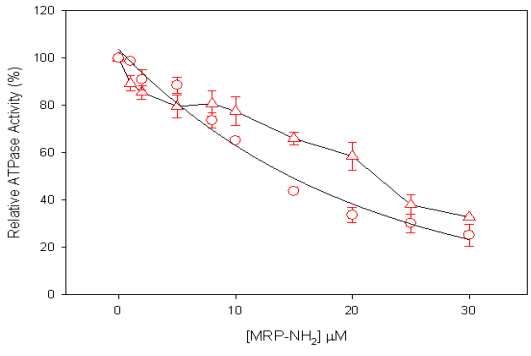


Figure 7c: βL382Q (DEQSEED)

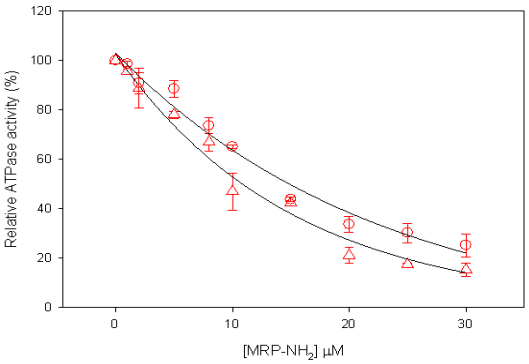


Figure 7d: βL382E (DEESEED)

Figure 7: Inhibition of ATPase activity in F_1F_O ATP synthase by MRP-amide. Symbols used are: (○, Wild type (DELSEED) and Δ, βL382A (DEASEED), βL382R (DERSEED), βL382Q (DEQSEED), or βL382A (DEESEED)). Each data point represents average of at least 2 experiments done in duplicate and results agreed within ±10%. Spectroscopic readings were obtained using Synergy HT spectrophotometer from Biomatik.

Inhibition of ATPase Activity of Membrane bound βS383A, βS383R, βS383Q or βS383E Mutant F_1F_O ATP Synthase by MRP-Amide

The introduction of βS383A or βS383R mutation did not significantly change the inhibitory profile compared to wild-type. But contrary to what was expected, 20 µM MRP-NH_2 could not significantly inhibit the ATPase activity of βS383E mutant enzyme.

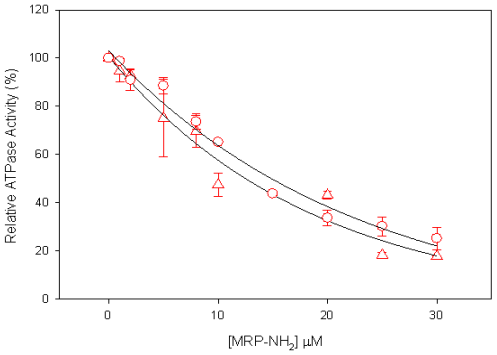


Figure 8a: βS383A (DELAEED)

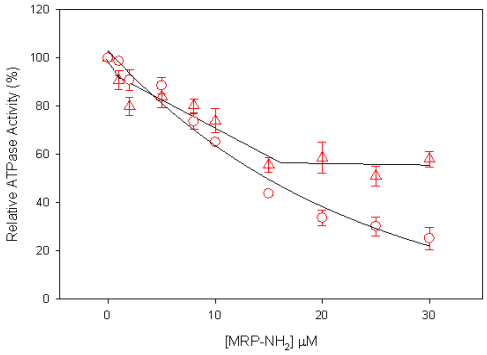


Figure 8b: βS383R (DELREED)

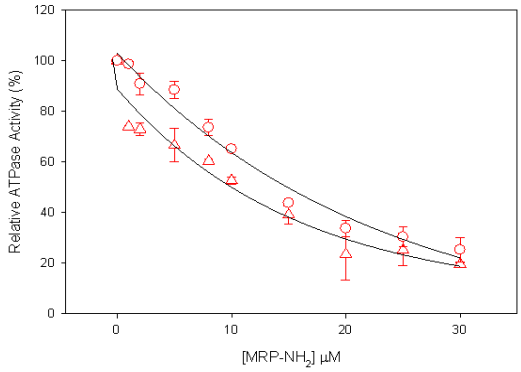


Figure 8c: βS383Q (DELQEED)

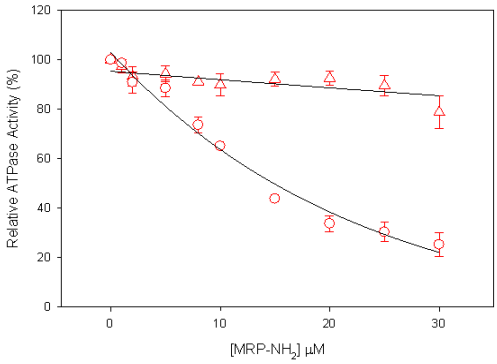


Figure 8d: βS383E (DELEEED)

Figure 8: Inhibition of ATPase activity in F_1F_O ATP synthase by MRP-amide. Symbols used are: (○, Wild type (DELSEED) and Δ, βS383A (DELAEED), βS383R (DELREED), βS383Q (DELQEED), or βS383E (DELEEED)). Each data point represents average of at least 2 experiments done in duplicate and results agreed within ±10%. Spectroscopic readings were obtained using Synergy HT spectrophotometer from Biomatik.

CHAPTER 4

DISCUSSION

The purpose of this study was to examine the mechanism by which α-helical-amphipathic antimicrobial peptides bind and inhibit F_1F_O ATP synthase. Previously, it was shown that some insect and amphibian antimicrobial peptides differentially bind and inhibit F_1-ATPase activity (Laughlin and Ahmad 2010). It was proposed that the binding site of these peptides on F_1F_O ATP synthase was the conserved βDELSEED-motif found at the carboxyl terminal domain of the beta subunit (Table 1 and figure 2). This motif was previously identified as the binding site for many peptide inhibitors (Hong and Pedersen 2008). Melittin or cationic phenothiazine was shown to bind to calmodulin, a calcium binding protein expressed in many eukaryotic cells, by interacting with the negatively charged sequence EEEIRE, which resembles the negatively charge βDELSEED-motif
(Malencik and Anderson 1984; Gresh and Pullman 1986; Strynadka and James 1988). Also ε-subunit, an intrinsic inhibitor of F_1-ATPase that is characterized by the presence of positively charge amino acid residues at its carboxyl terminus, was shown to exert its inhibitory effect by ionic interactions with the βDELSEED-motif (Noji and Yoshida 2001).

We introduced the following mutations βL382A, βL382R, βL382Q, βL382E, βS383A, βS383R, βS383Q, βS383E, βD380A, βE381A, βE384A, βE385A, and βE386A to examine the requirement of the βDELSEED-motif in peptide binding and the role played by individual amino acid residues in this binding mechanism. Introduction of any of the said mutations resulted in a loss of between 5 - 18% of growth in a limiting glucose

medium (Tables 2 and 3). The probable reason for this relatively small decrease in growth compared to previous mutational studies of ATP synthase (Li et al. 2009) is that the βDELSEED residues are not present in the active site of the enzyme.

The introduction of βD380A (AELSEED), βE381A (DALSEED), βE384A (DELSAED), βE385A (DELSEAD), or βE386A (DELSEEA) resulted in a change in the inhibitory profile by melittin-NH_2 and MRP-NH_2 compared to the wild-type (DELSEED). The inhibition of *E. coli* F_1F_O ATP synthase after the introduction of either of these mutations was characterized by a 'biphasic' inhibitory profile; at concentrations < 10 µM the peptides did not inhibit F_1-ATPase activity of these mutants, but as the inhibitor concentrations were increased there were changes in the inhibitory profiles. This can be explained by the fact that either of these mutations resulted in a decrease in the number of negative charge amino acid residues in the βDELSEED-motif, likely making it less favorable for melittin-NH_2 and MRP-NH_2 at concentrations of < 10 µM to effectively bind and inhibit ATP synthase. But as the concentrations were increased the positively charged peptides did bind at the other negatively charged amino acid residues and thus inhibit F_1-ATPase activity.

The introduction of the βL382E mutation resulted in a slight increase in the inhibition of F_1-ATPase activity by MRP-NH_2 (figure 7) suggesting that Leucine at position 382 on the β subunit might be providing a steric hindrance and thereby preventing tight binding of AMPs at the βDELSEED-motif. Introduction of βS383E resulted in a complete loss of inhibition of F_1-ATPase activity by MRP-NH_2 (figure 8). This was contrary to what was expected. Because this mutation resulted in an increase in number of negatively charged amino acids, it was expected that there will be tighter

binding of the inhibitor unto this motif. The basis of this observation is unknown because this mutant enzyme had an activity of 6.9 μmolmin^{-1}mg^{-1} (Table 4) and the growth of the bacteria harboring this mutated enzyme on limiting glucose media was close to that of the wild type (Table 3), which suggests an intact functional enzyme.

The results also show that for each of the mutated enzymes, there was less F_1-ATPase activity in the presence of 20 μM MRP-NH_2 than in the presence of 20 μM Melttin-NH_2. This implies that MRP-NH_2, having 3 basic amino acid residues, requires the presence of fewer acidic amino acid residues to exert its effect compare to Melittin-NH_2 with 5 basic amino acid residues.

REFERENCES

Ahmad Z, Senior AE. 2005. Identification of phosphate binding residues of *Escherichia coli* ATP synthase. J Bioenerg Biomembr 37(6):437-40.

Ahmad Z, Senior AE. 2005. Modulation of charge in the phosphate binding site of *Escherichia coli* ATP synthase. J Biol Chem 280(30):27981-9.

Andries K, Verhasselt P, Guillemont J, Gohlmann HW, Neefs JM, Winkler H, Van Gestel J, Timmerman P, Zhu M, Lee E and others. 2005. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. Science 307(5707):223-7.

Arakaki N, Kita T, Shibata H, Higuti T. 2007. Cell-surface H^+-ATP synthase as a potential molecular target for anti-obesity drugs. FEBS Lett 581(18):3405-9.

Arakaki N, Nagao T, Niki R, Toyofuku A, Tanaka H, Kuramoto Y, Emoto Y, Shibata H, Magota K, Higuti T. 2003. Possible role of cell surface H^+ -ATP synthase in the extracellular ATP synthesis and proliferation of human umbilical vein endothelial cells. Mol Cancer Res 1(13):931-9.

Blatt NB, Bednarski JJ, Warner RE, Leonetti F, Johnson KM, Boitano A, Yung R, Richardson BC, Johnson KJ, Ellman JA and others. 2002. Benzodiazepine-induced superoxide signals B cell apoptosis: mechanistic insight and potential therapeutic utility. J Clin Invest 110(8):1123-32.

Boyer PD. 1997. The ATP synthase – A splendid molecular machine. Annu Rev Biochem 66:717-49.

Chandrasekaran K, Hatanpaa K, Rapoport SI, Brady DR. 1997. Decreased expression of nuclear and mitochondrial DNA-encoded genes of oxidative phosphorylation in association neocortex in Alzheimer disease. Brain Res Mol Brain Res 44(1):99-104.

Dadi PK, Ahmad M, Ahmad Z. 2009. Inhibition of ATPase activity of *Escherichia coli* ATP synthase by polyphenols. Int J Biol Macromol; 45(1):72-9.

Das B, Mondragon MO, Sadeghian M, Hatcher VB, Norin AJ. 1994. A novel ligand in lymphocyte-mediated cytotoxicity: expression of the beta subunit of H^+ transporting ATP synthase on the surface of tumor cell lines. J Exp Med 180(1):273-81.

De Meirleir L, Seneca S, Lissens W, Schoentjes E, Desprechins B. 1995. Bilateral striatal necrosis with a novel point mutation in the mitochondrial ATPase 6 gene. Pediatr Neurol 13(3):242-6.

de Vries DD, van Engelen BG, Gabreels FJ, Ruitenbeek W, van Oost BA. 1993. A second missense mutation in the mitochondrial ATPase 6 gene in Leigh's syndrome. Ann Neurol 34(3):410-2.

Dimroth P, von Ballmoos C, Meier T. 2006. Catalytic and mechanical cycles in F-ATP synthases. Fourth in the Cycles Review Series. EMBO Rep 7(3):276-82.

Feniouk BA, Kato-Yamada Y, Yoshida M, Suzuki T. 2006. Conformational transitions of subunit epsilon in ATP synthase from thermophilic Bacillus PS3. Biophys J; 98(3):434-42.

Fillingame RH, Jiang W, Dmitriev OY. 2000. Coupling H^+ transport to rotary catalysis in F-type ATP synthases: structure and organization of the transmembrane rotary motor. J Exp Biol 203(Pt 1):9-17.

Frasch WD. 2000. The participation of metals in the mechanism of the F_1-ATPase. Biochim Biophys Acta 1458(2-3):310-25.

Gresh N, Pullman B. 1986. A theoretical study of the binding of phenothiazine derivatives to residues 82-93 of calmodulin. Mol Pharmacol 29(4):355-62.

Hancock RE, Sahl HG. 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol 24(12):1551-7.

Hara KY, Kato-Yamada Y, Kikuchi Y, Hisabori T, Yoshida M. 2001. The role of the βDELSEED motif of F_1-ATPase: propagation of the inhibitory effect of the epsilon subunit. J Biol Chem 276(26):23969-73.

Hong S, Pedersen PL. 2008. ATP synthase and the actions of inhibitors utilized to study its roles in human health, disease, and other scientific areas. Microbiol Mol Biol Rev 72(4):590-641.

Ketchum CJ, Al-Shawi MK, Nakamoto RK. 1998. Intergenic suppression of the gammaM23K uncoupling mutation in F_OF_1 ATP synthase by βGlu-381

substitutions: the role of the β380DELSEED386 segment in energy coupling. Biochem J 330 (Pt 2):707-12.

Kim SH, Vlkolinsky R, Cairns N, Lubec G. 2000. Decreased levels of complex III core protein 1 and complex V beta chain in brains from patients with Alzheimer's disease and Down syndrome. Cell Mol Life Sci 57(12):1810-6.

Laughlin TF, Ahmad Z. 2010. Inhibition of *Escherichia coli* ATP synthase by amphibian antimicrobial peptides. Int J Biol Macromol; 46(3):367-74.

Li W, Brudecki LE, Senior AE, Ahmad Z. 2009. Role of α-subunit VISIT-DG sequence residues Ser-347 and Gly-351 in the catalytic sites of *Escherichia coli* ATP synthase. J Biol Chem 284(16):10747-54.

Malencik DA, Anderson SR. 1984. Peptide binding by calmodulin and its proteolytic fragments and by troponin C. Biochemistry 23(11):2420-8.

Matsuzaki K. 2009. Control of cell selectivity of antimicrobial peptides. Biochim Biophys Acta; 1788(8):1687-92.

Matsuzaki K, Sugishita K, Fujii N, Miyajima K. 1995. Molecular basis for membrane selectivity of an antimicrobial peptide, magainin 2. Biochemistry 34(10):3423-9.

Mnatsakanyan N, Krishnakumar AM, Suzuki T, Weber J. 2009. The role of the βDELSEED-loop of ATP synthase. J Biol Chem 284(17):11336-45.

Moser TL, Kenan DJ, Ashley TA, Roy JA, Goodman MD, Misra UK, Cheek DJ, Pizzo SV. 2001. Endothelial cell surface F_1F_O ATP synthase is active in ATP synthesis and is inhibited by angiostatin. Proc Natl Acad Sci USA 98(12):6656-61.

Moser TL, Stack MS, Asplin I, Enghild JJ, Hojrup P, Everitt L, Hubchak S, Schnaper HW, Pizzo SV. 1999. Angiostatin binds ATP synthase on the surface of human endothelial cells. Proc Natl Acad Sci USA 96(6):2811-6.

Moser TL, Stack MS, Wahl ML, Pizzo SV. 2002. The mechanism of action of angiostatin: can you teach an old dog new tricks? Thromb Haemost; 87(3):394-401.

Nakamoto RK, Baylis Scanlon JA, Al-Shawi MK. 2008. The rotary mechanism of the ATP synthase. Arch Biochem Biophys 476(1):43-50.

Noji H, Yoshida M. 2001. The rotary machine in the cell, ATP synthase. J Biol Chem 276(3):1665-8.

Pal PK, Coleman PS. 1990. Detecting precatalytic conformational changes in F1-ATPase with 4-benzoyl (benzoyl)-1-amidofluorescein, a novel fluorescent nucleotide site-specific photoaffinity label. J Biol Chem 265(25):14996-5002.

Palmer DN, Barns G, Husbands DR, Jolly RD. 1986. Ceroid lipofuscinosis in sheep. II. The major component of the lipopigment in liver, kidney, pancreas, and brain is low molecular weight protein. J Biol Chem 261(4):1773-7.

Pedersen PL. 2007. Transport ATPases into the year 2008: a brief overview related to types, structures, functions and roles in health and disease. J Bioenerg Biomembr 39(5-6):349-55.

Ren H, Allison WS. 2000. On what makes the gamma subunit spin during ATP hydrolysis by F_1. Biochim Biophys Acta 1458(2-3):221-33.

Senior AE. 2007. ATP synthase: motoring to the finish line. Cell 130(2):220-1.

Senior AE, Langman L, Cox GB, Gibson F. 1983. Oxidative phosphorylation in *Escherichia coli*. Characterization of mutant strains in which F_1-ATPase contains abnormal beta-subunits. Biochem J 210(2):395-403.

Sitaram N, Nagaraj R. 2002. Host-defense antimicrobial peptides: importance of structure for activity. Curr Pharm Des 8(9):727-42.

Sone N, Ikeba K, Kagawa Y. 1979. Inhibition of proton conduction by chemical modification of the membrane moiety of proton translocating ATPase. FEBS Lett 97(1):61-4.

Strynadka NC, James MN. 1988. Two trifluoperazine-binding sites on calmodulin predicted from comparative molecular modeling with troponin-C. Proteins 3(1):1-17.

Taussky HH, Shorr E. 1953. A microcolorimetric method for the determination of inorganic phosphorus. J Biol Chem 202(2):675-85.

Tennessen JA. 2005. Molecular evolution of animal antimicrobial peptides: widespread moderate positive selection. J Evol Biol 18(6):1387-94.

Thyagarajan D, Shanske S, Vazquez-Memije M, De Vivo D, DiMauro S. 1995. A novel mitochondrial ATPase 6 point mutation in familial bilateral striatal necrosis. Ann Neurol 38(3):468-72.

Weber J. 2006. ATP synthase: subunit-subunit interactions in the stator stalk. Biochim Biophys Acta 1757(9-10):1162-70.

Weber J, Senior AE. 2003. ATP synthesis driven by proton transport in F_1F_O-ATP synthase. FEBS Lett 545(1):61-70.

Weber J, Wilke-Mounts S, Lee RS, Grell E, Senior AE. 1993. Specific placement of tryptophan in the catalytic sites of *Escherichia coli* F_1-ATPase provides a direct probe of nucleotide binding: maximal ATP hydrolysis occurs with three sites occupied. J Biol Chem 268(27):20126-33.

Wilkens S. 2005. Rotary molecular motors. Adv Protein Chem 71:345-82.

APPENDICES

Appendix A

Quikchange Lightning Mutagenesis Procedure

The steps outlines here were used to generate the mutated plasmid used in this study. These steps are part of a protocol supplied with the mutagenesis kit from Stratagene purchased from Agilent Technologies catalog #210519.

Into a thin-walled reaction tube, add

- 5 µl of 10× reaction buffer
- 75 ng of dsDNA template
- 125 ng of oligonucleotide forward primer
- 125 ng of oligonucleotide reverse primer
- 1 µl of dNTP mix
- 1.5 µl of QuikSolution reagent
- ddH2O to a final volume of 50 µl

Then add:

- 1 µl of QuikChange Lightning Enzyme

PCR of reaction mix

Segment	Cycles	Temperature	Time
1	1	95°C	2 minutes
2	18	95°C	20 seconds
		60°C	10 seconds
		68°C	30 seconds/kb of plasmid
3	1	68°C	5 minutes

- Add 2 µl of *Dpn* I restriction enzyme directly to each amplification reaction. *Dpn* I digest the parental plasmid which is dam methylated.
- Pipette up and down the reaction mixture several times and immediately incubate at 37oC for 15 minutes to digest parental supercoiled dsDNA.

Transformation of XL10-Gold Ultracompetent Cells

- Place XL10-Gold ultracompetent cells on ice and allow to thaw.
- For each sample reaction to be transformed,
    - Aliquot 45 µl of the ultracompetent cells to a *prechilled* 14-ml BD Falcon polypropylene round-bottom tube.
    - Add 2 µl of the β-mercaptoethanol mix to the 45 µl of cells.
    - Swirl the contents of the tube gently and incubate on ice for 2 minutes.
    - Transfer 2 µl of the *Dpn* I-treated DNA from each control and sample reaction to separate aliquots of the ultracompetent cells.
    - Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 minutes.
    - Heat-pulse the tubes in a 42°C water bath for 30 seconds. Do not exceed 42°C.
    - Incubate the tubes on ice for 2 minutes.
    - Add 0.5 ml of preheated (42°C) NZY^+ broth to each tube and then incubate the tubes at 37°C for 1 hour with shaking at 250 rpm.
- Plate the appropriate volume of each transformation reaction on agar plates containing the appropriate antibiotic for the plasmid vector, in my case ampicillin.

Appendix B

A Schematic Representation of Preparation of Membrane Bound F_1F_O ATP Synthase

- Overnight *E. coli* cultures
- Centrifuge pellet at

  9500 rpm x 15 min
- Resuspend pellet in STEM

  Spin at 9500 rpm x 25 min
- Resuspend pellet in 2 ml STEM/g wet cells

  Add DNase to the pellet and French Press twice at

  20 K x 3-6 minutes
- Centrifuge at22K rpm x 20 minutes
- Centrifuge supernatant at

  60K rpm x 3 hrs at 2^oC
- Resuspend pellet in TES 50

  Spin at 60K rpm x 3 hrs at 2^oC
- Resuspend pellet in TES 5 + PBA

  Spin at 60K rpm x 2 hrs at 2^oC

  (Repeat this step)
- Resuspend in 50 mM TrisSO_4 pH 8.0, 2.5 mM $MgSO_4$

  Store at -70^oC or -20^oC for short-term usage

Appendix C

List of Antimicrobial Peptides

Table A3: A list of peptides with antimicrobial peptides that were shown to differential inhibits F_1-ATPase activity. Melittin-amide and MRP-amide were shown to inhibit more 90% of ATPase activity, Magainin II- amide 60%, and Caerin 1.8 0%. Melittin-amide and MRP-amide were the 2 inhibitors that were used in this study.

AMPs	*Sequence*	*length*	*Net positive charge*
Melittin-amide	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	26	5
MRP-amide	AIGSILGALAKGLPTLISWIKNR-NH_2	23	3
Magainin II-amide	GIGKFLHSAKKFGKAFVGEIMNS-NH_2	23	4
Caerin 1.8	GLFKVLGSVAKHLLPHVVPVIAEK	24	3

Appendix D

List of Oligonucleotide Used for Site Directed Mutagenesis

The forward and reverse primer for site directed mutagenesis were designed using online software provide by Agilent Technologies (www.genomics.agilent.com). The primers were between 25 and 45 bases in length, with a melting temperature (*T*m) of ≥78°C. The underline bases show the introduction of the desired mutation.

Strain	Mutation		Forward Primer
WT (pBWU13.4)	-	βDELSEED	NA
pSN6	βY331W	βDELSEED	NA
pJT01	βD380A	βAELSEED	CGCCATCCTGGGTATGGCTGAACTGTCTGAAGAAG
pJT04	βE381A	βDALSEED	CATCCTGGGTATGGATGCACTGTCTGAAGAAGACA
pJT07	βL382A	βDEASEED	GCCATCCTGGGTATGGATCGACTGTCTGAAGAAGAC
pJT08	βL382R	βDERSEED	CCTGGGTATGGATGAACGGTCTGAAGAAGACAAAC
pJT09	βL382Q	βDEQSEED	CTGGGTATGGATGAACAGTCTGAAGAAGACAAC
pJT11	βL382E	βDEESEED	CGCCATCCTGGGTATGGATGAAGAGTCTGAAGAAGAC
pJT12	βS383A	βDELAEED	CTGGGTATGGATGAACTGGCTGAAGAAGACAAACTGG
pJT13	βS383R	βDELREED	CCTGGGTATGGATGAACTGCGTGAAGAAGACAAACTGGTG
pJT14	βS383Q	βDELQEED	CATCCTGGGTATGGATGAACTGCAGGAAGAAGACAAACTGGTGGTAG
pJT16	βS383E	βDELEEED	CATCCTGGGTATGGATGAACTGGAGGAAGAAGACAAACTGGTGGTAG
pJT17	βE384A	βDELSAED	GTATGGATGAACTGTCTGCAGAAGACACAAACTGGTGGT
pJT20	βE385A	βDELSEAD	GGATGAACTGTCTGAAGCAGACAACTGGTGGTAG
pJT23	βE386A	βDELSEEA	GAACTGTCTGAAGAAGCCAAACTGGTGGTAGCGC

Appendix E

Electrotransformation of *E. coli* DK8 Strains

E. coli DK8 was transformed by electroporation using the MicroPulser electroporation apparatus from Bio-Rad cat # 165-2100.

Preparation of Electrocompetent Cells

- Inoculate 500 ml of LB-broth with 5 ml of a fresh overnight culture of *E. coli*.
- Grow the cells at 37^{o}C shaking at 300 rpm to an OD_{600} of ~0.6 (NB: the best results are obtained when the cells are harvested during mid-log phase in growth.)
- Keep cell on ice for 20 minutes. All subsequent steps are performed as close to 0^{o}C as possible.
- Harvest the cells by centrifuging at 500 rpm for 15 minutes at 4^{o}C.
- Pour off the supernatant and resuspend the cells in 500 ml of ice-cold 10% glycerol.
- Centrifuge at 5000 rpm for 15 minutes at 4^{o}C and discard the supernatant.
- Resuspend the cells in 250 ml ice-cold 10% glycerol, centrifuge at 5000 rpm for 15 minutes at 4^{o}C and pour off the supernatant.
- Resuspend the cells in 250 ml of ice-cold 10% glycerol, transfer to a 30 ml sterile Oakridge tube, centrifuge at 5000 rpm for 15 minutes at 4^{o}C and discard the supernatant.
- Resuspend the cell in final volume of 5% glycerol. The cell concentration should be about 1-3 x 10^{10} cells/ml.

- The cells can be transformed immediately or stored in aliquots on dry ice and stored at -70^{o}C for at least 6 months stability.

Electroporation

- In cold, 1.5 ml microfuge tube, mix 40 µl of the cell suspension and 2 µl of DNA. Mix and incubate on ice for ~1 minute.
- Set the MicroPulser to Ec1, Ecl2, or 3 when using a 0.1 or 0.2 cm cuvette respectively and pulse once with the cuvette sitting in the chamber slide.
- Remove the cuvette from the chamber and immediately add 1 ml of SOC medium to the cuvette.
- Transfer the cell suspension to a 17x100 mm polypylene tube and incubate at 37oC for ~1 hour, shaking at 225 rpm.
- Plate on selective medium; in my case ampicillin.

Appendix F

Buffers, Reagents, and Culture Media

The following quantities represent what is required for 1000 ml preparation

Argenine Ent Thimine

Use in preparing limiting and minimal glucose media

- To 800 ml ddH_2O add
- 6.17 g 2,3 Dihydroxy Benzoic acid
- 168.6 g L-Arginine
- 10 ml 20 mM Thiamine
- Add NaOH to completely dissolve the solutes
- Make final volume to 1000 ml with ddH_2O
- Filter sterile and store at room temperature

ATPase assay buffer

Use as an ATP generating solution to measure ATPase activity

- To 850 ml ddH_2O add
- 50 ml 1 M Tris
- 4 ml 1M $MgCl_2$
- 25 ml 0.4 Na ATP (Adenosine 5'-triphosphate disodium salt)
- Adjust pH to 8.5 with H_2SO4

- Bring to a final volume of 1000 ml with ddH_2O
- Store in plastic bottles at -20^oC

Isoleucine-Valine

Essential for bacteria growth, use in preparing limiting and miminal glucose media

- To 900 ml ddH_2O add
- 3.94 g Isoleucine
- 3.52 g Valine
- Make final volume to 1000 ml with H_2O
- Filter sterile and store at room temperature

LB ampicillin liquid media

Use to ensure selectivity since the plasmids contain ampicillin resistance gene

- 20 g of LB broth powder
- Add H_2O to bring to 1000 ml
- Autoclave for 30 minutes
- Cool the media to ~55^oC
- Add 1000 µl of 100 mg/ml Ampicillin and store at 4^oC

LB-agar (Solid) medium

To grow bacteria

- 20 g of LB broth powder
- Add 15g of agar

- Add ddH_2O to bring to 1000 ml
- Autoclave for 30 minutes
- Cool the media to ~55^oC
- Add 1000 µl of 100 mg/ml Ampicillin
- Pour into approximately 30 petridishes and store at 4^oC

Minimal Glucose Growth Media

Growth media use in cultivating bacteria prior to membrane bound enzyme purification

- To 850 ml ddH_2O add
- 10.45 g Potassium Phosphate Dibasic Trihydrate (K_2HPO_4)
- 4.8 g Sodium Phosphate Monobasic (NaH_2PO_4)
- 1.98 g Ammonium Sulfate ($(NH_4)_2SO_4$)
- Autoclave for 30 min, cool it to room temperature and add the following additions
    - 20 ml Uracil
    - 20 ml 27 % Glucose (Final glucose concentration of 10 mM)
    - 10 ml ILV (isoleucin-valine)
    - 1 ml TE (trace elements)

Store at 4^oC

NB: Preparation of limiting glucose media requires the addition of 6 ml of 27% Glucose solution to 1 liter of minimal media to have a glucose concentration of 3mM.

NZY$^+$ Broth

Use in enhancing transformation of XL10-Gold Ultracompetent Cells

- 10 g of NZ amine (casein hydrolysate)
- 5 g of yeast extract
- 5 g of NaCl
- Add ddH2O to a final volume of 1 liter
- Adjust to pH 7.5 using NaOH
- Autoclave

Add the following filer-sterilized supplements prior to use:

- 12.5 ml of 1 M MgCl2
- 12.5 ml of 1 M MgSO4
- 20 ml of 20% (w/v) glucose (or 10 ml of 2 M glucose)

10 % SDS

Use in stopping in vitro hydrolysis of ATP by ATP synthase

- 1000 mg Sodium dodecyl sulfate
- Bring to a final volume of 1000 ml with ddH_2O and store at room temperature

SOC

Use in enhancing electrotransformation of *E. coli* DK8

- To 865 ml of ddH_2O add
- 20 g tryptone
- 5 g yeast extract
- 0.5 g NaCl

- 20 ml of 1 M glucose
- Autoclave and add
    - 10 ml 0.25M KCl
    - 5 ml 2M MgSO4
    - 100 µl 5M NaOH

STEM

Use as a buffer to facilitate cell lyses during French press

- To 700 ml ddH_2O add
- 100 ml 1 M TES
- 4.29 g $Mg(CH3COO)_2 \cdot 4H_2O$
- 85.5 g sucrose
- 0.0951 g EGTA (Ethylene glycol-bis-(2-aminoethylether)-N,N,N,N-tetraacetic acid)
- 5 g EACA (6-Ainocaproic acid6-Ainocaproic acid)
- Adjust pH to 6.5 with NaOH
- Bring to a final volume of 1000 ml with ddH_2O
- Store in plastic bottles at -20^oC

TES 50

Use as a washing buffer during enzyme purification

- To 700 ml ddH_2O add
- 50 ml 1 M TES

- 150 ml glycerol
- 5 g 6-Ainocaproic acid 6-Ainocaproic acid (EACA)
- 1 g 4-Aminobenzamidine dihydrochloride (PAB)
- Adjust pH to 6.5 with NaOH
- Bring to a final volume of 1000 ml with ddH_2O
- Store in plastic bottles at -20^oC

TES 5 + PAB

Use as a washing buffer during enzyme purification

- To 700 ml ddH_2O add
- 5 ml 1 M TES
- 150 ml glycerol
- 1 ml 0.5 M Dithiothreitol (DTT)
- 5 g 6-Ainocaproic acid6-Ainocaproic acid (EACA)
- 1 g 4-Aminobenzamidine dihydrochloride (PAB)
- 2.5 ml 0.2 M Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA)
- Adjust pH to 6.5 with NaOH
- Store in plastic bottles at -20^oC

Trace Elements

Use in preparing limiting and minimal glucose media

- To 850 ml ddH_2O
- 2.51 g Zinc Sulfate ($ZnSO_4.7H_2O$)
- 0.17 g Manganese Sulfate ($MnSO_4.H_2O$)

- 0.29 g Boric acid (H_3BO_3)
- 0.12 g Calcium Sulfate ($CaSO_4.2H_2O$)
- 0.37 g Calcium Chloride ($CaCl_2.2H_2O$)
- 0.49 g Ferric Chloride ($FeCl_3.6H_2O$)
- Make final volume to 1000 ml with ddH_2O.
- Filter sterile and store at room temperature

50 mM Tris-SO4 buffer

Reaction buffer for ATPase activity assay

- To 850 ml ddH_2O add
- 6.1 g Tris
- Adjust pH to 8.0 with H_2SO_4
- Bring to a final volume of 1000 ml with ddH_2O
- Store at room temperature

(Taussky and Shorr) T&S reagent

- Solution A: 12 g Ammonium molybdate ($(NH4)_6Mo_7O_{24}\cdot 4H2O$ in 98 ml 12 N H_2SO_4)
- Solution B: 100 g Ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2\cdot 6H_2O$ in 700 ml H_2O)
- Add solution A to solution B while stirring
- Bring to a final volume of 1000 ml with ddH_2O
- Store at 4^oC

VITA

JUNIOR K. TAYOU

Personal Information:	Date of Birth: June 20, 1986 Place of Birth: Buea, Cameroon Marital Status: Single
Education:	B.Sc. Biochemistry, University of Buea, 2007 M.S. Biology, East Tennessee State University, 2011
Professional Experience:	Graduate Teaching Assistant, 2009 – 2011 East Tennessee State University
Presentations and Posters:	Tayou, J. K. (2010). "Requirement of βDELSEED-motif of *Escherichia coli* F_1F_O ATP Synthase in Antimicrobial Peptide Binding" Oral Presentation at Appalachian student research forum, East Tennessee State University, Johnson City, TN. Sherman, S. D., Bowers, M. I., Tayou, J. K., Laughlin, T. F. and Zulfiqar Ahmad (2010). "Antimicrobial/Anticancer Peptides Inhibit *Escherichia coli* ATP Synthase" Poster Presentation at Appalachian student research forum, East Tennessee State University, Johnson City, TN.
Honours and Awards:	B.S. 2nd Class upper division honours in Biochemistry, University of Buea, 2007 Tuition waiver scholarship/ Graduate teaching assistantship at ETSU from 2009 –2011 Second Place award in the 2010 Appalachian Research Forum, graduate Student Category (**Presentation Topic:** Requirement of βDELSEED-motif of *Escherichia coli* F_1F_O ATP synthase in antimicrobial peptide binding).