5-2015

Venom Peptide Induced Inhibition of Escherichia coli ATP synthase

Sofiya Azim
East Tennessee State University

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By

Sofiya Azim
April 2015

An Undergraduate Thesis Submitted in Partial Fulfillment
of the Requirements for the
University Honors Scholars Program
East Tennessee State University

Sofiya Azim Date

Thomas F. Laughlin, Ph.D., Thesis Mentor Date
Department of Biological Sciences,
East Tennessee State University,
Johnson City, TN 37614, USA

Vineet K. Singh, Ph.D., Reader Date
Department of Microbiology & Immunology,
A.T. Still University of Health Sciences,
Kirksville, MO 63501, USA

Ismail O. Kady, Ph.D., Reader Date
Department of Chemistry,
East Tennessee State University,
Johnson City, TN 37614, USA

ABSTRACT

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ATP is the main cellular energy generated by the enzyme ATP synthase in almost all organisms from bacteria to vertebrates. While malfunction of the ATP synthase complex is responsible for several disease conditions, the enzyme itself can be used as a potent molecular drug target to combat many diseases including microbial infections, cancer, tuberculosis, and obesity. Recent widespread escalation of antibiotic resistant microbes in general and *E. coli* in particular demands novel alternative approaches to combat microbial infections. Inhibition of ATP synthase by inhibitors such as peptides is known to deprive microbes of required energy, resulting in microbial cell death. Therefore, we have examined the venom peptide induced inhibition of *E. coli* ATP synthase. It was found that venom peptides completely inhibited *E. coli* ATP synthase and the process of inhibition was found to be fully reversible. This study also links the antimicrobial properties of peptides in part to the inhibition of ATP synthase. Thus, selective use of ATP synthase as a molecular drug may have an important impact on biology and medicine.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Thomas Laughlin who was my thesis mentor during this process. I would also like to acknowledge the help and guidance provided to me by Dr. Vineet K. Singh and Dr. Ismail Kady. A special thanks needs to be given to the Honors College at East Tennessee State University, especially the University Honors Scholars program and Dr. Joy Wachs, the director. Finally, this thesis would not have been possible without the love and support from my parents, Zulfiqar and Mubeen Ahmad, or my sisters Sabiya, Samiya, and Anam who have provided me with love and support throughout my four years at East Tennessee State University.

TABLE OF CONTENTS

Page

ABSTRACT…… 2

ACKNOWLEDGMENTS…… 3

LIST OF FIGURES…… 6

Chapter

1. INTRODUCTION…….. 7

ATP synthase and its Role in Disease Conditions……... 8

Inhibition of ATP synthase…….. 9

Examples of ATP synthase Inhibition……. 9

Peptides…… 10

2. MATERIALS AND METHODS…… 12

Source of Peptides……. 12

Chemicals……. 12

Reagents…… 12

Experimental Methods…….. 12

Preparation of Membrane Bound *E. coli* ATP synthase…….. 13

Membrane Bound ATP Synthase Concentration and ATPase Assay…….. 14

Inhibition of *E. coli* ATP synthase by venom peptides, VP1 and VP2…… 15

Reversal of peptide inhibited ATPase activity……. 15

3. RESULTS…… 16

4. DISCUSSION……. 18

5. REFERENCES……... 21

LIST OF FIGURES

Figure	Page
1. Overall Structure of ATP synthase…….	7
2. Effect of Inhibition on Cell Survival……..	9
3. Peptide binding βDELSEED-motif…….	11
4. Venom peptides (VP1 and VP2) induced inhibition of ATP synthase……	16
5. Reversal of VP1 and VP2 induced inhibition of membrane bound *E. coli* ATP synthase enzyme…….	17

CHAPTER 1

INTRODUCTION

ATP synthase is the major source of energy production for all organisms from bacteria to vertebrates. Both the synthesis and hydrolysis of ATP occur on the catalytic sites of ATP synthase. This enzyme synthesizes ATP from ADP and Pi as well as hydrolyzes ATP depending on the physiological needs of the cell. ATP synthase works similarly to a motor and is actually the smallest known biological nanomotor found in living organisms. The clockwise rotation of ATP synthase generates ATP while hydrolysis of ATP occurs during counterclockwise rotation (1,2).

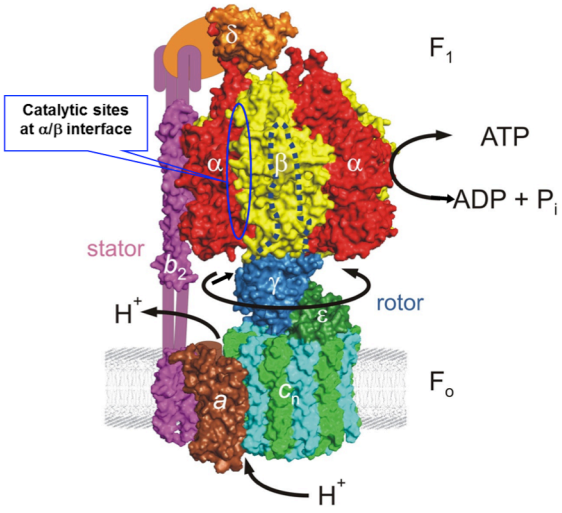

Figure 1. Overall structure of ATP synthase modified from (3)

ATP synthase (Figure 1) is a highly conserved enzyme among different species (3). In *E. coli*, ATP synthase has two sectors, F_1 and F_o. The F_1 sector has five different subunits, α_3, β_3, γ, δ, and ε. The F_o sector has three subunits, a, b_2, and $c_{10\text{-}14}$ (4).

In total, ATP synthase has six nucleotide binding sites on its F_1 sector. Three of them are noncatalytic while the other three are catalytic sites which are contributed by the α/β interface residues. ATP synthesis and hydrolysis takes place on the three catalytic sites. A proton gradient across the cell membrane moves protons through the F_o sector, which in turn rotates the γ-subunit. Rotation of the γ-subunit causes the conformational changes in the α and β-subunits allowing them to bind or release nucleotide reactants (ATP, ADP, or Pi). Functional significance of ATP synthase can be determined by the energy requirement of living organisms. For example, the total amount of ATP produced by a sedentary 70 kg human in about a 75 year life span is nearly 2 million kg. Such energy needs are fulfilled by the uninterrupted motor and catalytic function of ATP synthase (5).

ATP synthase plays an exceptionally important role in human health and diseases. Malfunction of ATP synthase is known to result in life threating and debilitating diseases such as cancer, tuberculosis, obesity, neuropathies, Alzheimer's, microbial infections, mitochondrial diseases, immune deficiency, cystic fibrosis, diabetes, ulcers, and Parkinson's. The functional importance of ATP synthase also makes it a potential molecular target for anti-bacterial and anti-cancer drugs (6,7).

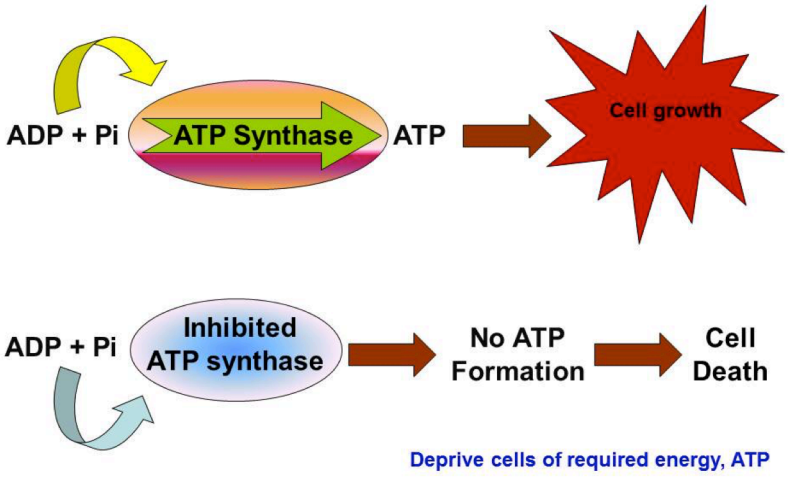

Figure 2. Effect of ATP synthase inhibition on cell survival

As shown in Figure 2, the inhibition of ATP synthase can lead to cell death. When ATP synthase is functional, it can synthesize ATP from ADP and Pi. This will then allow the cell to grow because it has its required energy. In the event that ATP synthase is inhibited, the reaction between ADP and Pi does not occur and ATP is not generated. This will cause cell death because the cell will be deprived of its required energy (8). There are a variety of natural and synthetic compounds that are known to inhibit ATP synthase, including peptides, such that it has potential as a therapeutic drug target (8-10).

Previously, ATP synthase inhibition has been used in a variety of beneficial ways. For example, *Streptococcus mutans* is a microbial agent that plays a major role in the pathogenesis of dental cavities by biofilm formation and acid production. Inhibition of *S. mutans* ATP synthase also inhibits biofilm formation and acid production, thus preventing the formation of cavities (11). It is the second largest killer after cancer. There are two mutations in the c-subunit

of ATP synthase of the *Mycobacterium* which make the bacteria resistant to most tuberculosis drugs. These mutations, D32V and A63P can occur together or individually (12,13). In 2012, the FDA approved an anti-tuberculosis drug, Bedaquiline. It is extremely selective in its inhibition of the F_o sector of the ATP synthase in *Mycobacterium tuberculosis*. This drug eradicates the infection quickly and effectively (14). ATP synthase inhibition also plays a role in angiogenesis. Angiogenesis is the process that involves the growth of new blood vessels from pre-existing vessels. This process is essential for tumor growth, and ATP synthase on the surface of endothelial cells is essential to angiogenesis in such cells. When non-mitochondrial endothelial cell surface ATP synthase is inhibited, formation of new vessels is prevented, thereby inhibiting tumor growth (15,16).

Inhibition by peptides was first described in insects as an inducible system of protection against bacterial infections (17). Peptides are known to have powerful activity against bacteria, fungi, parasites, and viruses as well as selective anticancer activity (18,19). Currently, antimicrobial peptides (AMPs) are being used in several clinical trials with mixed success (8,20-22). Widespread antibiotic-resistant microbial pathogens require new, alternative antimicrobial treatment choices (8,22). Some positively charged AMPs have been used against multidrug-resistant microbes such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *enterococci* (VRE), and multidrug resistant *Pseudomonas aeruginosa* (23-25). Cancer cells also exhibit numerous membrane protein targets including ectopic ATP synthases which peptide inhibitors could bind to as possible therapeutic molecules (6,26).

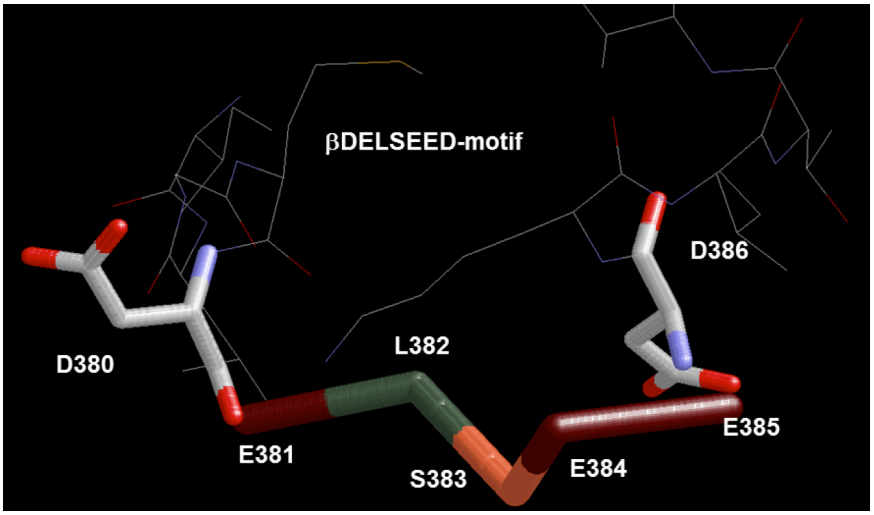

Figure 3 peptide binding βDELSEED-motif taken from reference (10)

Previously, it was found that melittin, melittin related peptide (MRP), and several structurally similar peptides inhibit the growth of *E. coli* cells by binding and inhibiting its ATP synthase. These cationic peptides were shown to bind at the βDELSEED-motif of ATP synthase (Fig. 3) (9,10).

In this study we examined the two cationic venom peptide (VP1 and VP2) induced inhibitions of *E. coli* ATP synthase. Based on VP1 and VP2 induced inhibitory profiles of ATP synthase, we present direct evidence that ATP synthase is a potent molecular drug target for antimicrobial peptides.

Hypothesis

We hypothesized that venom peptides will inhibit the *E. coli* ATP synthase due to the fact that cationic peptides should bind at the peptide bind βDELSEED-motif of ATP synthase.

CHAPTER 2

MATERIALS AND METHODS

Source of peptides:

Both venom peptides (VP1 and VP2) were purchased from Biomatik (http://www.biomatik.com). The peptides were determined to have greater than 95% purity by HPLC. Lyophilized powder was stored at -20 ^{o}C upon receipt and resuspended in deionized water for use as needed.

Chemicals:

ATP disodium salt, glucose, uracil, TES, TRIZMA, 4-aminobenzamidine dihydrochloride (PAB), and Sodium dodecyl sulfate (SDS) were all purchased from Fisher Scientific or Sigma–Aldrich Chemical Company.

Reagents:

50 mM Tris-H_2SO_4 pH 8 (T8), Taussky and Shorr Reagent (T&S), 10% SDS, STEM, TES 50, and TES5 +PAB were prepared as described (10).

Experimental Methods

Preparation of membrane bound *E. coli* ATP synthase

Grow wild-type E. coli in minimal media at 37^{o}C and 250 rpm

▼

Pellet at 9500 rpm for 15 min

▼

Resuspend pellet in STEM and spin at 9500 rpm for 25 min

▼

Resuspend pellet in 2 ml Stem/g wet cells

Add DNase and conduct Cell lysis by French press at 2000 psi

▼

Spin at 22K rpm for 20 min

▼

Spin supernatant at 60K rpm for 2 hours at 2^{o}C

▼

Resuspend pellet in TES 50 and spin at 60K rpm for 2hrs at 2^{o}C

▼

Resuspend pellet in TES 5 + PBA and spin at 60K rpm for 2 hours at 2^{o}C

(Repeat this step and store at - 70 ^{o}C

The wild type *E. coli* strain used in this study was pBWU13.4/DK8 (27).

Preparation of the membrane bound *E. coli* ATP synthase

50 mL of minimal media was inoculated with a loop full of the wild type *E. coli* strain and was grown over night at 37 °C and 250 rpm. This 50 mL of overnight starter culture was inoculated into 1L of minimal media and was grown at 37 ^{o}C at 250 rpm. Growth was measured at OD_{595} on an hourly basis until the late log phase was obtained.

Once no further growth was seen, the cells were harvested by spinning them at 9500 rpm at 4 ^{o}C in a super speed centrifuge for 15 minutes. The harvested cells were then resuspended in STEM and once again centrifuged at 9500 rpm for 25 minutes. The supernatant was discarded and the pellet was resuspended in 2 mL of STEM/g of cells and was stored at -80 ^{o}C overnight until the next step.

The stored cells were thawed and mixed with DNase to digest any nucleic acids. These cells were then disrupted by passing them through the French press at 2000 psi twice. Next, the lysed cells were centrifuged at 18K rpm for 20 minutes. Subsequently, the membrane bound *E. coli* ATP synthase was obtained by spinning the supernatant at 60K rpm for 120 minutes. The membrane bound *E. coli* ATP synthase was further resuspended in TES 50 and centrifuged at 60K rpm for 120 minutes. This pellet was then washed with TES 5 + PAB by spinning it twice at 60K rpm for 90 minutes. Finally, the purified membrane bound *E. coli* ATP synthase was resuspended in T8 buffer and stored at -80 ^{o}C for use in biochemical assays.

Membrane Bound ATP Synthase Concentration and ATPase Assay

Membrane bound *E. coli* ATP synthase concentrations were determined by plotting the absorbance at 595 nm using Bradford reagent against the standard BSA curve. ATPase activity was measured by adding 1 mL assay buffer containing 10 mM NaATP, 4 mM $MgCl_2$, and 50 mM TrisSO_4 (pH 8) to 20 µg of membrane bound *E. coli* ATP synthase at 37 °C. The reaction was stopped with SDS. The released Pi (inorganic phosphate) was measured by adding 1 mL of T&S reagent and was read at OD_{700}.

The enzymatic reaction of ATP synthase is as follows: ATP + ATP synthase ⇌ ADP + Pi and Pi + T & S → blue color. The intensity of the blue color observed at 700 nm is directly proportional to the activity of the enzyme.

Activity of ATP synthase was calculated by using the following formula

$$\frac{\text{Average of Sample OD - Average of Blank OD}}{\text{Amount of protein (mg) x Time (min)}} = \ldots\ldots\ldots\ldots\ldots \text{ µmol/min/g protein}$$

Inhibition of *E. coli* ATP synthase by venom peptides, VP1 and VP2

Wild-type membrane bound F_1F_o ATP synthase was preincubated with different concentrations of VP1 and VP2 for 60 minutes at room temperature (RT) in 50 mM TrisSO_4 at pH 8.0. 1 mL of ATPase cocktail was used to measure the enzyme activity. The reaction was stopped by the addition of 1 mL of SDS to a final concentration of 3.3%. 1 mL of T&S reagent was added to develop the blue color and was assayed at OD_{700} (28). Inhibitory exponential decay curves were generated with Sigma plot 10.0. The range of absolute specific activity for membrane bound F_1F_o ATP synthase was 15–20 µmol/min/mg at 37 ^oC for different preparations. Relative ATPase activity was calculated from the absolute values of wild type in the absence of peptides taken as 100%.

Reversal of peptide inhibited ATPase activity

Reversibility of inhibition was checked by dilution of the membrane bound enzyme. Membrane bound ATP synthase was first reacted with the maximal inhibitory concentration of peptides for 1 hour. Next, T8 buffer was used to bring the venom peptide concentrations to non-inhibitory levels and they were then incubated for one additional hour before measuring the ATPase activity.

CHAPTER 3

RESULTS

Venom peptide induced inhibition of *E. coli* ATP synthase

Figure 4 shows the inhibition of membrane bound *E. coli* ATP synthase in the presence of varied concentrations of venom peptides (VP1 and VP2). While VP1 caused approximately 70% inhibition with 30% residual activity, VP2 completely inhibited ATP synthase with essentially no residual activity (Figure 4).

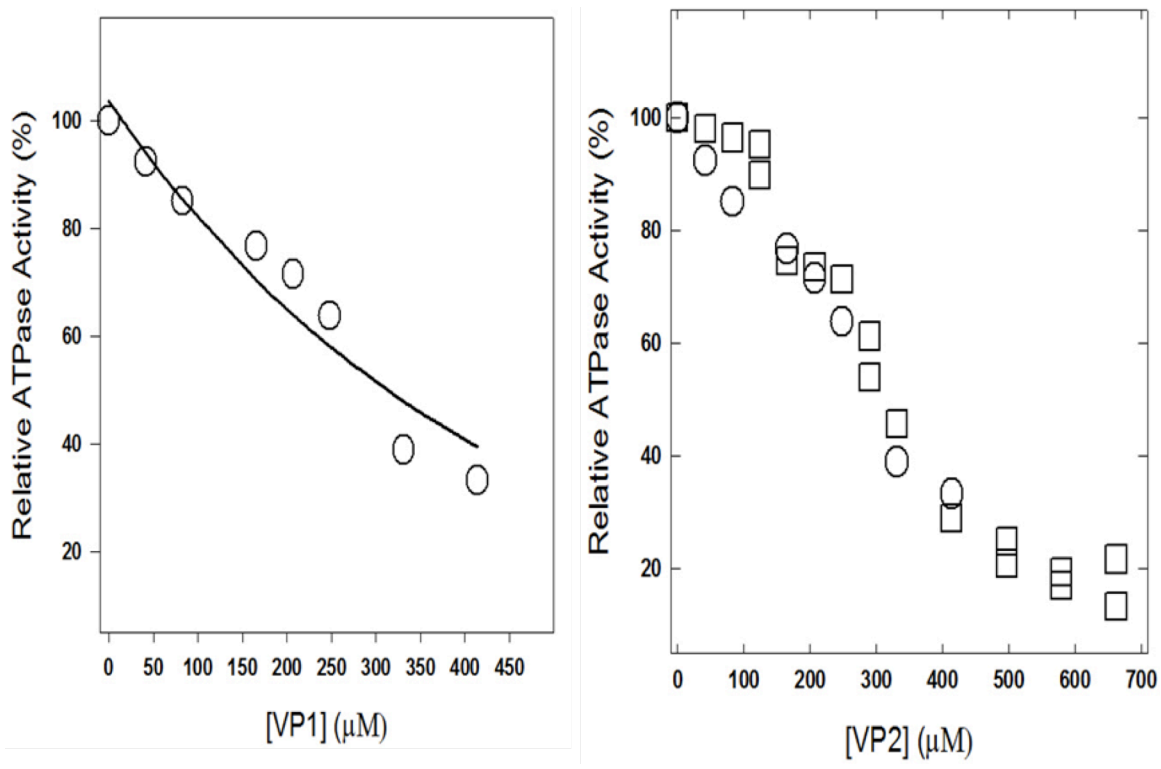

Figure 4. Venom peptides (VP1 and VP2) induced inhibition of ATP synthase.

Reversal of ATPase activity of membrane ATP synthase from the venom peptide inhibition

To test for the reversibility of venom peptide induced inhibition, membrane bound ATP synthase was treated with the maximum inhibitory concentrations of venom peptides for 1 hour

at 37 ^{o}C as shown in Figure 4. Then, the samples were diluted to non-inhibitory concentrations by adding T8 buffer, and ATPase activity was measured at OD_{700}. Inhibition by both venom peptides (VP1 and VP2) was found to be reversible (Figure 5).

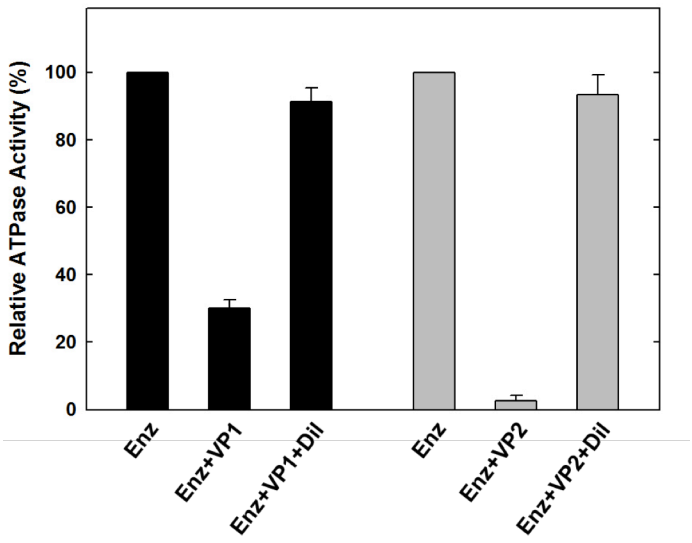

Figure 5 Reversal of VP1 and VP2 induced inhibition of membrane bound *E. coli* ATP synthase enzyme. (Enzyme:Enz ; Dilution:Dil)

CHAPTER 4

DISCUSSION

The purpose of this study was to examine whether or not the antimicrobial properties of peptides in general and venom peptides in particular, may be linked to the inhibition of ATP synthase. The results of this study demonstrate that both the studied venom peptides, VP1 and VP2, do bind and inhibit bacterial ATP synthase. VP2 was found to be a more potent inhibitor causing nearly 100% inhibition while VP1 caused about 70% inhibition. Previously, a number of α-helical cationic peptides were shown to inhibit ATP synthase (9). These include melittin, a 26- residue long honeybee venom peptide, the bacterial/chloroplast ε subunit, and several AMPs from amphibian origin that are the most important α-helical peptide inhibitors of ATP synthase (6,9,29-33).

Peptides bind at the β-subunit residues 380-386 of the βDELSEED- motif as shown in Figure 3. It has been demonstrated that the positively charged peptide residues exert their inhibitory effect through electrostatic interactions with the negatively charged βDELSEED-motif of ATP synthase (34). Similarly, it seems that that the direct electrostatic interactions between the venom peptides and acidic residues of the βDELSEED-motif are responsible for the inhibition of membrane bound *E. coli* ATP synthase.

The presence of 15 positively charged residues clustered in groups in a 34-residue long VP1 venom peptide may be the reason for partial inhibition of ATP synthase. Too many positive charges may cause steric hindrance for proper binding and inhibition of ATP synthase. Potent inhibition by venom peptide VP2 may be attributed to its 12 positively charged residues spread

widely in a 30-residue long peptide. It can be concluded that such dispersion not only decreases the repulsion among positively charged residues, but also helps in the proper orientation of the peptide for tighter binding and inhibition.

The process of inhibition was also found to be fully reversible. Membrane bound ATP synthase regained activity once it was brought back to non-inhibitory venom peptide concentrations by dilution with T8 buffer (Figure 5). This reversible process of inhibition indicates a non-covalent binding of venom peptides VP1 and VP2 at the βDELSEED-motif of wild-type *E. coli* ATP synthase (9).

Peptides are known to have pharmacological uses as antimicrobial (35,36) and anticancer agents (37). Many mechanisms have been postulated for their mode of action including membrane penetration (38,39) and cytolytic membrane disintegration (40). Most peptides are known to affect gram-negative and gram-positive bacteria, fungi, viruses, eukaryotic parasites, and cancer cells (37,41). Programmed cell death through the mitochondrial pathway inhibiting ATP synthase as a molecular target by several natural and synthetic inhibitors has been previously established (42-45). Additive effects of different α-helical AMPs have also been observed (46). These observations suggest that the combined effects of two or more inhibitors, or functional group modulation of inhibitors, may increase the extent and potency of inhibition.

Lately, the role of ATP synthase in the pathophysiology of many human disease conditions has become clearer (8). Due to this, the identification and characterization of potent inhibitors of ATP synthase on molar scale is extremely important. We conclude that further

identification and characterization of venom peptides is a promising avenue for understanding ATP synthase as a potential drug target for peptides.

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