IMPROVING THE STABILTY OF BIOACTIVE PEPTIDES

USING PROTEIN-BASED MOTIFS

by

JENNIFER RENEE WALKER

(Under the Direction of Juergen Wiegel)

ABSTRACT

Peptide instability and poor delivery in humans hinders the development of effective peptide drugs. To search for novel protective motifs for stabilizing peptides, an *in vivo* screen of randomized inhibitor peptides was developed and two protective motifs were identified. The first motif was generated by the fusion of the inhibitory peptides to the small stable protein, Rop. The second motif was generated by the placement of one or more prolines at the terminal ends of peptides. Repeating the *in vivo* screen with random peptides fused to either Rop or encoding terminal proline residues revealed an increase in the frequency of identifiable inhibitor peptides. Next, an *in vitro* method was developed to further investigate the novel proline protective motif. Randomized synthetic peptides beginning and ending with one proline, two proline or an alanine and two proline residues were tested for increased half-life in various eukaryotic and prokaryotic cell extracts. Peptides protected at the amino terminus with an alanine and two proline residues (APP) exhibited the longest

half-lives. Molecular modeling methods suggested that the APP motif exhibited two possible conformers with one possessing two right angles along the peptide backbone which may cause steric hindrance at the terminus. This increased steric strain within the APP motif may be responsible for increased resistance to peptidases. Then, APP was modified in search for more protective motifs. The alanine residue was replaced with amino acids having an increased tendency to form a *cis* bond, which has been proven to resist some peptidases. Five modified motifs were tested for increased peptide half-life in rat serum. The three proline residues (PPP) substituted motif displayed the longest half-life. This motif may provide a level of protection surpassing that of other known protective motifs.

While extended stability is one of many factors in peptide efficacy, uptake by the target cell is also important. A discovery was made that indicated biotinylated peptides could be transported across the membranes of Gram-negative bacteria via the biotin uptake pathway. Biotinylation may thus provide an alternative pathway for antibiotic peptide uptake.

INDEX WORDS: Peptide, Proline, Degradation, Stability, Uptake, Biotin, Antibiotic

IMPROVING THE STABILITY OF BIOACTIVE PEPTIDES USING PROTEIN-BASED MOTIFS

by

JENNIFER RENEE WALKER

B.S., The University of Georgia, 1996

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2002

IMPROVING THE STABILITY OF BIOACTIVE PEPTIDES

USING PROTEIN-BASED MOTIFS

by

JENNIFER RENEE WALKER

Approved:

Major Professor: Juergen Wiegel

Committee: William Whitman
Robert Maier
Timothy Hoover
Jeffrey Dean
Elliot Altman

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2002

DEDICATION

I eagerly expect and hope that I will in no way be ashamed, but will have sufficient courage so that now as always Christ will be exalted in my body whether by life or by death. For to me to live is Christ and to die is gain.

Phillippians 1:20-21

ACKNOWLEDGEMENTS

Without the emotional and spiritual support of my husband, John, none of this work was possible. His willingness to move to Athens, his patience with late night visits to the lab, and continual support afforded me strength and endurance. He provides me love and appreciation without expectation - what a gift! To my family, I thank Dad, for his pride in this endeavor, Mom, for her consistent encouragement, Jason and Jared, for being comic relief, and Jacqueline for being the surprise. To my church family at Beech Haven Baptist Church, I thank everyone for understanding my responsibilities during graduate school and relieving me of many pressures. Finally, I thank Dr. Juergen Wiegel for being an incredible source of support as my major professor; Dr. Jeff Dean, a wonderful mentor, who provided advice and insight throughout my undergraduate and graduate career; and Dr. Elliot Altman for suggesting graduate school in the first place and guiding me every step of the way.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Characteristics and Application of Naturally Occurring Peptides

Peptides are small polymers of less than 50 amino acids. Despite their small size, they display a wide range of biological activities. Peptides perform a variety of cellular functions in prokaryotic and eukaryotic organisms, from regulating blood flow diuresis, and vascular permeability in mammals (Venugopal, 2001); to signaling wound response in plants (Lindsey, *et al.*, 2002); to inhibiting competing bacteria through antibiotic activities (von Döhren, 1995). Peptides in general act as signal molecules, transport molecules, and antimicrobial agents. They are also used for many commercial applications, such as preservatives, antibiotics, and hormone supplements (Naider and Goodman, 2002). More recent peptide research has investigated peptide inhibitors that target specific intracellular proteins (Lam, 1997). The small size of peptides, as well as their inhibitory ability, makes them attractive to researchers trying to find new avenues for drug discovery.

In addition to novel drug discovery, there is also the application of peptides as delivery agents, vaccines, and hormone supplements. Drugs that are unable to penetrate mammalian cells may be attached to peptide carriers that can be translocated across cell membranes by specific dipeptide transporters (Fischer,

et al., 2001). Specified radiolabeled peptides can be used for tumor imaging because they bind to tumor cell surface receptors (Warner and O'Dorisio, 2002). Peptides cloned from infectious organisms such as the rubella virus can be developed into subunit vaccines (Demotz, *et al.*, 2001). Through genetic engineering, peptide drugs such as insulin for diabetic patients have been cloned and overproduced in *Escherichia coli* (Chance, *et al.,* 1981). All of these examples give evidence to the increasing utility of natural and novel peptides.

Due to the growing number of drug resistant pathogenic organisms, peptides from bacterial and mammalian cells are continually being isolated and tested for antimicrobial activity (Perschel and Collins, 2001; Epand and Vogel, 1999; Hancock and Scott, 2000). These peptides are often grouped according to structure to gain insight into their function. The α-helical structure group is one of the larger and better studied classes of antimicrobial peptides and is characterized by the cationic, amphipathic helix. The opposing hydrophobic and hydrophilic surfaces of α-helical peptides enable them to enter membranes and form pores that cause leakage of essential metabolites (Epand and Vogel, 1999; Hwang and Vogel, 1998; Tossi, *et al.*, 2000). α-Helical peptides, such as magainin (Bechinger, *et al.,* 1993; Matsuzaki, *et al.*, 1997) and buforin (Yi, *et al.,* 1996), have been studied for their antibiotic properties.

The β-sheet group of natural antibiotic peptides is a second group that includes cystine-rich peptides that form disulfide bonds creating a structure of several β-sheets. Examples include defensin peptides found in human

neutrophils and other mammalian cells (Thennarasu and Nagaraj, 1999; Pardi, *et al.,* 1992; Cole and Ganz, 2000; Selested, *et al.,* 1984; Eisenhauer, *et al.,* 1989). Defensins are cationic peptides that target cellular membranes (Epand and Vogel, 1999; Hwang and Vogel, 1998; Hancock and Diamond, 2000). The "loop" group is differentiated from the β-sheet group in that they only form a single disulfide bond creating a hairpin loop (Hancock, 1997). Examples include tachyplesin isolated from horseshoe crab (Kawano, *et al.,* 1990), and protegrin from porcine leukocytes (Fahrner, *et al.,* 1996).

Finally, all other known antibiotic peptides are loosely grouped as peptides with unusual amino acid composition or those that have no obvious structure. Nisin, produced by *Lactococcus lactis,* has several unusual amino acids such as 3 methyllanthionine and dehydroalanine. This peptide is used in the canning industries to prevent Gram-positive contamination (Hwang and Vogel, 1998). Other peptides like histatin or tritripticin are extremely rich in histidine or tryptophan residues, respectively (Raj, *et al.,* 1998; Lawyer, *et al.,* 1996). Many cathelicidins are proline-rich and are thought to lack specific secondary structure (Hwang and Vogel, 1998).

Synthesis and Application of Novel Peptides

As stated previously, biologically active peptides are present in all living cells. Purification of biologically important peptides has been a tedious task, so researchers investigated the possibility of creating bioactive peptides by chemical synthesis. The ability to make peptides synthetically enables scientists to

produce pure peptides and additionally investigate alternative structures that may increase stability or efficacy. Emil Fischer prepared the first synthetic peptide, glycylglycine, in the late 1890's (Fisher and Fourneau, 1901) but peptide chemistry did not begin rapid development until the 1950's when peptide bond formation methods were improved (Naider and Goodman, 2002). In 1953, the first synthetic peptide created was oxytocin, by duVigneaud, with a yield of <1% purified peptide (du Vigneaud, *et al.,* 1953).

Peptide synthesis in the 1950's required months of work due to the number of chemical reactions involved and produced racemic mixtures. Solid phase synthesis, invented independently by Merrifield (Merrifield, 1963) and Letsinger (Letsinger and Kornet, 1963) in the 1960's, gave peptide chemists a new tool to create peptides at a higher purity and yield. This method involved attaching a single amino acid to a copolymer and then chemically adding additional amino acids. Eventually, Merrifield's method was adopted by most scientists due to the simpler addition of protected amino acids through their carboxyl terminal. With the advent of high pressure liquid chromatography in the 1970's, peptide synthesis was improved by the ability to rapidly purify the products.

Alternative methods to Merrifield's copolymer peptide synthesis were also created in order to simultaneously generate large numbers of peptides each encoding a unique amino acid sequence, termed combinatorial peptide libraries (Naider and Goodman, 2002). The combinatorial peptide library approach to

peptide construction is based on a model similar to Merrifield's: the initial amino acid is attached to a solid structure and amino acids are sequentially added to the free amine end. One method of synthesis is the attachment of amino acids to a fixed structure, such as a pin, small enough to fit into a microtiter well. Separate solutions of single amino acids are aliquotted into individual wells. Pins are then sequentially dipped into a series of microtiter wells to attach amino acids onto the growing peptide chain. Selection of each amino acid can either be done randomly or purposefully with a specific sequence in mind (Geysen, *et al.,* 1984). The pin technique can produce half a micromole of peptide per pin (Al-Obeidi, *et al.,* 1998). In the teabag method, developed by Houghten, *et al.*, 1985, resin beads in polypropylene bags are sequentially immersed in different amino acid solutions to synthesize a peptide. Synthesis of random peptides was proposed by Furka, *et al.,* 1991, and later developed by Lam, *et al.,* 1991, resulting in the split-couple-mix method capable of making 10^5-10^6 randomized peptides at once. Figure 1.1a and 1.1b shows a summary of the available techniques.

A different approach to peptide synthesis developed in the 1980's uses recombinant DNA libraries containing randomly selected nucleotide sequences or sequences slightly modified from a known peptide. The DNA library is ligated into an expression vector and transformed into *Escherichia coli*. Transformed cells are then infected with filamentous phage. This technique, called phage display, allows the encoded peptide to be coupled to the pIII sequence and expressed at the tip of newly assembled phage. The end result is a

library of peptides displayed on one end of phage particles where they are available for protein interaction (Figure 1.2).

Once peptides are created synthetically, they are identified for their ability to bind to a specific protein target. To screen for bead bound peptides that successfully bind, the protein target is coupled to alkaline phosphatase and mixed with the peptide-bead library. Color generating reagents are added to react with peptide-bound proteins, thus staining the corresponding bead. Colored beads can be selected manually by a dissecting microscope (Schultz and Schultz, 1996). Selection of a random phage displayed peptides for a target protein is accomplished by adding a library of phage to protein bound in microtiter wells, a method called biopanning. After binding occurs, excess phage are washed off and the remaining phage are sequenced for the peptide oligonucleotide insert. Both techniques of solid-phase synthesis and phage display provide quick screening methods enabling researchers to isolate a few peptide sequences out of thousands (Smith, 1985; Parmley and Smith, 1988; Scott and Smith, 1990). However, the capacity of peptide inhibition is not known until the peptide is actually delivered to a cell system.

Combinatorial libraries generated either by solid-phase synthesis or phage display have opened up a host of opportunities. Random libraries created through solid phase synthesis are used to identify peptides that mimic binding sites of human enzymes, such as the tumor necrosis factor (Chirinos-Rojas, *et al.,* 1997) or T cell epitopes (Gundlach, *et al.,* 1996). Opioid-like peptides from

randomized libraries are identified with opioid receptor assays (Dooley and Houghten, 1999). Phage display has been used for identification of epitopes of protein antigens (Yao, *et al.,* 1995) and screening of tyrosine kinase mutants (Ting, *et al.,* 2001). Other areas of research using phage display include identification of fully human antibody fragments for treatment of autoimmune diseases (Ellmark, *et al.,* 2002) and development of modified enzymes for commercial industries (Verheart, *et al.,* 2002).

In spite of developments in peptide synthesis and screening that identify potential peptides for medicinal use, these peptides often exhibit short half-lives due to cellular proteases and peptidases or show low binding constants to target proteins. Peptidomimetics, a broad term used to describe peptide compound discovery by library screening or biological manipulation, can be utilized to modify natural peptides to possess enhanced bioactivity (Hruby and Balse, 2000; Hruby, *et al.,* 1997). Peptidomimetic discovery incorporates the synthesis and screening of novel peptides modified from peptides found as natural products in microbial broths, fungal metabolites, or in chemically assembled peptide collections (Hirschmann, 1991; Al-Obeidi, *et al.,* 1998). Peptidomimetics seeks to generate peptides that possess longer half-lives, improved receptor/acceptor selectivity, potency, good biodistribution and bioavailability.

Computers are playing a greater part in the discovery of sequences that can act as improved versions of natural peptides or are novel peptides that can bind to a unique target site. Based on protein structure-activity relationships and

computational chemistry, computer programs can be used to generate hypothetical peptide libraries. As virtual novel peptides are created, they can be "docked" to a binding site on a computer generated protein target (Blaney and Martin, 1997). Programs such as SYBYL, DISCOVER and QUANTA are software packages available to assist such studies (Nikiforovich and Marshall, 2001). Another program, GrowMol, was used to generate a cyclic peptide inhibitor of aspartic proteases (Ripka and Rich, 1998). Newer programs like Generate (Bultinck, *et al.,* 2002) and REBEL (Totrov and Abagyan, 2001) were created to address unique peptide modeling methods. Once peptides are identified through modeling methods, they can be synthesized and tested *in vitro* with the specific protein target.

Characteristics of Peptidases

A significant disadvantage to the use of synthetic or natural peptides as therapeutics is that they are highly susceptible to degradation by a variety of peptidases and proteases in whole animal systems (Lee, 1988; Sood and Panchagnula, 2000). Like peptides, proteases and peptidases are found in every cell system where their function is to cleave proteins and peptides into smaller fragments. Proteases are generally characterized as only cleaving large proteins; however, many proteases have been shown to cleave peptides as well as proteins (Woodley, 1994). Thus the terms protease and peptidase should not be associated with substrate size, but rather substrate specificity. As commonly used, the two names are irrelevant to function. The term "peptidase" should be

used to describe any enzyme that cleaves a peptide bond, which includes enzymes that are generally classified as proteases. (Dixon and Webb, 1964; Bergman and Ross, 1936; McDonald and Barrett, 1986; Woodley, 1994). There are exceptions of course - a few peptidases exist that only cleave tri- and di-peptides. But, for accuracy and simplicity sake, the term "peptidase" hereafter will be used to designate all enzymes, both proteases and peptidases that hydrolyze bonds in peptides.

Peptidases activate enzymes, such as mitochondrial proteins, by cleaving peptide extensions (Ito, 1999), or modify compounds, like neuropeptides, with cleavage of one or two terminal amino acids (Isaac, *et al.*, 2000). Peptidase activities range from a broad number of substrates to only a few. Lon (Goldberg, *et al.,* 1994), Clp (Thompson and Maurizi, 1994; Porankiewicz, *et al.,* 1999) and proteasomes (Ciechanover, 1998 and Baumeister, *et al.,* 1998) function in general to degrade proteins and peptides. A peptide degraded by one or several peptidases eventually is left as free amino acids available for cell metabolism (Christensen, *et al.*, 1999; Gonzales and Robert-Baudouy, 1996; Taylor, 1993). Peptidases that recognize specific substrates include that of aminopeptidase A, which prefers to cleave acidic N-terminal amino acids (Bausback, *et al.,* 1988), and dipeptidyl peptidase II which removes dipeptides from a free N-terminus, particularly if a proline residue is present (McDonald, *et al.,* 1968a; McDonald, *et al.*, 1968b).

Peptidases are found in a wide variety of locations within eukaryotic and prokaryotic cells. For example there are membrane-bound peptidases, like aminopeptidase M of the mammalian brain (Brownlees and William, 1993) and cytoplasmic peptidases like methionine aminopeptidases in *E. coli* (Gonzales and Robert-Badouy, 1996). Peptidases can be found in the cytoplasm, lysosomes, membranes and the extracellular environment. Because peptidases are so widespread among organisms and function similarly in both eukaryotes and prokaryotes, studies of peptide stability in either cell systems can provide similar results.

All peptidases fall into one of two major classes, depending on the cleavage site of the peptide or protein (Bai, *et al*, 1992; Bownlees and Williams 1993; Miller, 1996). Peptidases that require a free terminus as substrate are broadly classified as exopeptidases, and can be further classified into two subgroupings. Exopeptidases that act on the amino terminus of a peptide are termed aminopeptidases, while those that act on the carboxy terminus are called carboxypeptidases. A comparison of reviews listing all sequenced peptidases in both prokaryotic and eukaryotic systems indicated that more aminopeptidases than carboxypeptidases have been identified (Bai and Amidon, 1992; Bai *et al.*, 1995; Brownlees and Williams, 1993; Miller, 1996; Ryan, 1989). The second class of peptidases, endopeptidases, contains those that act on internal peptide bonds (Miller, 1996; Rawlings and Barrett, 1993; Lendeckel, *et al.*, 2000). Figure 1.3

shows where in general endo- and exopeptidases cleave by giving example of peptidases that attack at various peptide bonds.

Peptidases present a major obstacle to developing therapeutic peptides. While some peptidases target specific peptides, the focus of stable peptide synthesis is to thwart hydrolysis by broad spectrum peptidases. The design of stable or protected motifs for peptides is an important element in the development of novel therapeutic peptides (Hruby and Balse, 2000).

Protective Motifs for Peptides

As peptide synthesis improved in the 1970's, it became apparent that novel biologically active peptides needed to be redesigned to increase *in vivo* stability (Naider and Goodman, 2002). Peptides are degraded in the liver, blood, kidney, intestinal lumen or vascular endothelium (Lee, 1988; Moss, 1995). Some protective motifs applied to synthetic peptides were first observed in natural peptides. As natural peptides were isolated, unusual characteristics of peptide modification were found. For example, in Ehrlich ascite cells of mice, 80% of the proteins were N-α-acetylated (Brown and Roberts, 1976) which led to the suggestion that motifs like acetylation may be acting as a mechanism for stabilization in the cellular environment (Brown, 1979). Other peptide modifications observed in naturally occurring peptides include that of peptaibols isolated from *Stibella flavipes* characterized with a high number of α aminoisobutyric acid and C-terminal bonded amino alcohols (Jaworski and Brückner, 2001). D-amino acids are thought to form peptide bonds that are

resistant to peptidase cleavage (Hruby, *et al.,* 1991). D-amino acids have been discovered in natural antimicrobial peptides, such as contryphan-Vn from snail venom (Massilia, *et al.,* 2001). Cyclization by disulfide bonds was also discovered in many antimicrobial peptides, such as bactenecin, tachyplesins or protegrins, where the constrained structure of cyclized peptides appears to protect against peptidase degradation (Sitaram and Nagaraj, 1999). Cyclization is also thought to contribute to function. Gramicidin and polymyxin, both antimicrobial peptides, have highly stable cyclic structures and carry out their function of membrane destabilization while hiding one of their free termini from peptidase degradation (Franklin and Snow, 1989).

Novel as well as natural peptides with known structure and biological activity can be redesigned for improved stability and activity. Masking the free amino or carboxyl terminus is a common initial step in modification of peptide drugs (Wang, *et al.,* 1999). Protective ends are added to peptide drugs ranging from hormones to antiviral peptides. N-terminal modifications, such as methylation, amidation, or removal of the terminal amide group, resulted in longer half-lives of glucose-like peptide (GLP-1) analogues (Wettergren, *et al,* 1998; Gallwitz, *et al.,* 2000). Substitution of a terminal amino acid with threonine, glycine and serine on GLP-1, also resulted in longer metabolic stability (Deacon, *et al.,* 1998). Employing strategies of acetylation and amino acid substitution with proline, the half-life of thymus hormone, thymopeotin, was improved from 1 minute to 40 minutes. When thymopeotin was acetylated and amidated, no

degradation was measured within 30 minutes (Heavner, *et al.,* 1986). Even more dramatic was the amidation of the anti-HIV peptide, T140, whose half-life increased from 9.6 hours to 2 days (Tamamura, *et al.,* 2001).

Substitution of amino acids, whose peptide bonds are more difficult to cleave, offers another method of peptide stabilization. The amino acid proline possesses a more sterically hindered peptide bond than the other naturally occurring L-amino acids. This steric strain results from the α-nitrogen atom being part of the pyrrolidine ring structure. Proline is found in many α-helical structures and can introduce a kink of 20° or more in peptide chains (Vanhoof, *et al.,* 1995). More notably, proline can resist the action of many peptidases (Yaron and Naider, 1993). Hormones and neuropeptides such as interleukin II and substance P have X-Pro bonds that cannot be cleaved by general proteases. The prolyl bond has been shown to resist the specific actions of trypsin, chymotrypsin, thermolysin and carboxypeptidases A and B (Mentlein, 1988).

While the majority of peptide bonds are found in the *trans* conformation, 10-30% of prolyl amide bonds can exist as *cis* isomers. This incidence of *cis* bonds is remarkably high. Only one in 10^{-3} peptide bonds of other amino acid residues occur in the *cis* conformation (MacArthur and Thorton, 1991; Williamson, 1994). The side chain of the neighboring residue to the proline residue is also implicated in the formation of a *cis* proline peptide bond. Generally, the bulkier the side chain, the more likely the prolyl residue will assume *cis* conformation (Grathwohl and Wüthrich, 1976; MacArthur and Thornton, 1991; Williamson,

1994; Reimer, *et al.,* 1998). The *cis* conformation of proline plays a part in resisting peptidase activity (Yaron and Naider, 1993). This ability to form *cis* bonds allows prolyl bonds to resist even proline specific peptidases. As an example, dipeptidyl peptidase IV can only cleave at the *trans* arrangement of a proline residue and not the *cis* (Fisher, *et al.,* 1983). Thus proline appears to be a good candidate as a modifier to increase peptide stability.

In addition to the substitution or modification of single amino acids for improved peptide stability, the peptide backbone itself can be modified if a specific internal cleavage site of the peptide is identified. One method is the exchange of isoteric or isoelectronic units in place of NH-CO units. Groups, such as CH_2 or O, can also be attached as extensions. The result is greater resistance to internal peptide bond cleavage by endopeptidases (Hirschmann, 1991; Hruby and Balse, 2000). Another type of change in the peptide backbone is the use of D-amino acids. Incorporating D-amino acids in analogues of cecropin, a naturally occurring insect antimicrobial peptide, resulted in longer peptide stability (Merrifield, *et al.,* 1994).

Cyclization of the peptide by bridging cysteine side chains is also used to resist peptidase attack. Opioid peptides, such as enkephalins, were modified with internal sulfide side chains which induce a disulfide cyclization. The result was a highly selective analogue for δ opioid receptors that exhibited high restraint to enzyme degradation (Bonner, *et al.,* 1997; Hruby *et al.,* 2002). Figure

1.4 gives an overview of the many techniques used to modify peptides' terminal ends, backbone or overall structure for increased stability.

In addition to altering peptide structure, administering peptides that inhibit protease activity can be implemented to increase peptide drug stability (Woodley, 1994; Sood and Panchagnula, 2000). Aprotinin is one such inhibitor that can be delivered orally (Bai, *et al.,* 1995). Proline-specific peptidase inhibitors such as thioxo amino acid pyrrolidides and thiazolidides co-administered with peptide drugs help neuropeptides such as substance P and vasopressin evade degradation (Stöckel-Maschek, *et al.,* 2000). Other peptidases like the proline-specific serine dipeptidase can be effectively inhibited by DP-IV inhibitors, thus increasing the blood circulation of the drug GLP-1 (Moller, 2001).

Finding the right protective motif or structure is essential for creating bioactive peptides. Effectiveness of motifs varies from peptide to peptide and many different approaches must be taken to determine the best protective motif. Improving half-life, however, is just one of many aspects which needs to be considered in peptide drug development.

Development of Efficient Peptide Delivery

In addition to the challenge of peptide stability, the development of peptide drugs and antibiotics must take into account the delivery mechanism for uptake by target cells. Peptides can be transported across membranes by passive or active diffusion or enter by endocytosis. Endocytosis by eukaryotic cells can occur regardless of peptide size but are usually receptor-site specific. Diffusion

is limited by size exclusion for all cell membrane types (Lee, 1988). As peptide drugs vary from hormones to subunit vaccines to antibiotics, so do their ability to be absorbed by either eukaryotic or prokaryotic cells.

Several routes exist for peptide drug delivery. Peptide drugs delivered orally provide low cost for administration but high cost for development due to the presence of several barriers including chemical (low pH), enzymatic (peptidases and proteases), and physical barriers (tight junctions between epithelial cells). Creating peptide drugs able to survive the harsh environment of the stomach, resist cleavage by lumen peptidases, and are easily absorbed through the intestinal tract is difficult (Sood and Panchagnula, 2000). Other routes of delivery used to avoid these barriers include parenteral and intranasal routes. Even in bypassing the intestinal tract, peptide drugs must still maintain stability in blood and liver and must resist cell membrane peptidases. Several methods are being developed with a few currently available that enable the uptake of peptide drugs (Sood and Panchagnula, 2000).

Among the methods used to make peptide drugs bioavailable is chemical alteration of the peptide itself. Most drugs are hydrophilic, which decreases their association with cell membranes. As the lipophilicity of the drug increases so does its ability to penetrate membranes. The lipophilicity and permeability of the peptide may be increased by altering residue side chains to reduce hydrogen bonding with the solvent. Cyclization of the peptide also decreases interaction between solvent and side chains of the peptide, which can improve permeability

(Sood and Panchagnula, 2000). Of course, the process of changing amino acids or secondary structure risks altering peptide bioactivity but other measures to improve delivery can be used that do not alter the peptide drug activity.

Conjugation to transport molecules and delivery vectors is a method that allows for transport of peptide drugs across cellular membranes without significantly changing the peptide itself. Attachment to vectors, such as OX26, an antibody that binds to cell transport receptors, is one option. This system was shown to deliver neuropeptide drugs across the blood brain barrier (Pardridge, 1992; Gozes, 2001). Covalently linking peptides with polymers of water- and fat-soluble elements such as polyethylene glycol (PEG) can assist in absorption (Sood and Panchagnula, 2000). There are absorption enhancers, like sodium glyocholate, that temporarily disrupt cell barriers in the intestinal lining (Bai, *et al.*, 1995; Sood and Panchagnula, 2000). Transport molecules and hormones that bind to specific host cell receptors can be attached to the peptide and endocytosis of the peptide conjugate can then occur (Bai, *et al.*, 1995; Leone-Bay, *et al.*, 2000). Vitamin B_{12} is readily absorbed in the intestinal lumen by receptor-mediated endocytosis. Erythropoietin (EPO), a drug used to stimulate maturation of erythrocytes, was conjugated to B_{12} and shown to be biologically active after being co-transported across CaCo-2 membranes (Russel-Jones, *et al.*, 1995). Some methods, such as B_{12} conjugation, are still being developed to help potential peptide drugs while other methods like the use of the OX26 antibody are currently being tested for human application.

Antibiotic peptide drugs have a unique problem with respect to delivery. For internal infections in the host, peptide antibiotics must bypass two barriers if taken orally: translocation across the intestinal lining and then across the bacterial membrane - if the drug target is intracellular. The intestinal lumen has specific receptors that many antibiotics can access. For example, β-lactam drugs are transported through intestinal brush border receptors having broad specificity (Bai, *et al.,* 1995). There are also di- and tri-peptide transporters in the lumen that allow uptake of antibiotic peptides. When the antibiotic molecules reach the bacterial cell wall, many destabilize the membrane with their amphipathic structure and thus do not need a transport mechanism to cross the membrane. Cationic peptides are one group of antibiotics that bind to lipopolysaccharides and permeabilize the bacterial outer membrane (Hancock and Diamond, 2000). However, other types of peptides must either be actively transported across or passively diffuse through these membranes. For example, quinolones passively diffuse due to their small size, while aminoglycosides utilize the electron transport system of the cell membrane to cross the cell envelope (Chopra, 1988; Bryan, 1985). Effective intracellular targeting of antibiotics for Gram-negative cells requires that these agents must be able to cross the outer and cytoplasmic membranes by carrier-mediated transport systems (Chopra, 1988). What becomes the most difficult for intracellular targeting antibiotic peptides is crossing Gram-negative outer membranes.

The outer membrane of Gram-negative bacteria functions as a molecular sieve that allows few molecules to diffuse passively. (Nikaido, 1992; Nikaido, 1996; Decad and Nikaido, 1976; Payne and Smith, 1994). Porins in the outer membrane may be specific or non-specific in their molecular recognition (Nikaido and Nakae, 1979; Nikaido, 1992). Non-specific porins such as OmpF, OmpC and PhoE, allow rapid passage of small hydrophilic molecules (Nikaido and Rosenberg, 1983; Nikaido, 1996). Other membrane structures, such as the oligopeptide permease (Opp), have specificity for oligopeptides two or three amino acids in length are readily transported across the outer membrane (Payne and Gilvarg, 1968; Barak and Gilvarg, 1975; Naider and Becker, 1975). The size exclusion limit of the *E. coli* cell membrane was measured at 650 daltons or approximately a 6 amino acid oligopeptide (Payne and Gilvarg, 1968; Decad and Nikaido, 1976; Smith *et al.*, 1970), while other reports show the size limit to be between 600 and 800 daltons (Nikaido and Rosenberg, 1983; Nikaido and Vaara, 1985). In contrast to the Gram-negative outer membrane, the cell membrane of Gram-positive bacteria is much more permeable to large molecules. For example, *Lactococcus lactis* was able to take up peptides of over 18 residues (2140 daltons) in length (Detmers, *et al.*, 1998), while *Bacillus megaterium* could import molecules up to 10,000 daltons in mass (Scherrer and Gerhardt, 1971).

In general, Gram-negative bacterial transport of peptides, particularly antibiotic peptides that target intracellular proteins, varies with the drug's charge, size, hydrophilicity and the number of pores in the cell wall (Livermore,

1991). Intracellular targeting of antibiotic peptides and molecules too large for Gram-negative bacteria porins are rendered useless without a specific transport system. Therefore, a method of antibiotic delivery across Gram-negative cell membranes for larger peptides would benefit antibiotic peptide applications.

Basis of This Study

Peptide drugs that have become a mainstay in the pharmaceutical industry are constantly being improved for efficacy and stability. Improvement of binding constants or specificity is useless without the ability to deliver the peptide intact to its target. Several protective motifs are being used to improve stability but not all are applicable for every peptide, nor does one motif always improve the stability of every peptide. Drug delivery is often a limiting factor due to the inability of peptides to cross cell membranes and as a consequence many peptide antibiotics are rendered useless due to lack of uptake by the bacteria. With the rise of antibiotic resistance, there is a need for drugs that affect a broader scope of species and attack new molecular targets. This study investigated stabilizing motifs for the improvement of peptide half-lives, and examined the uptake of peptides by *E. coli* and other Gram-negative bacteria.

References

Al-Obeidi, F., Hruby, V. J., and Sawyer, T. K. (1998). Peptide and peptidomimetics. Mol Biotech 9, 205-223.

Atherton, F. R.; Hall, M. J.; Hassal, C. H.; Holmes, S. W.; Lambert, R. W.; Lloyd, W. J.; and Ringrose, P. S. (1980). Phosphonopeptide antibacterial agents related to alafosfalin: Design, synthesis, and structure-activity relationships. Antimicrob Agents Chemother 18, 897-905.

Bai, J. P. F. and Amidon, G. L. (1992). Structural specificity of mucosal cell transport and metabolism of amino acids and peptide drugs: implication for oral peptide delivery. Pharm Res 9, 969-978.

Bai, J. P. F., Chang, L-L., and Guo, J-H. (1995). Targeting of peptide and protein drugs to specific sites in the oral route. Crit Rev Ther Drug Carrier Sys 12, 339-371.

Bausback, H. H., Churchill, L. and Ward, P. E. (1988). Angiotensin metabolism by cerebral microvascular aminopeptidase A. Biochem Pharmacol 15, 155-160.

Barak, Z. and Gilvarg, C. (1975). Specialized peptide transport system in *Escherichia coli*. J Bacteriol 122, 1200-1207.

Bechinger, B., Zasloff, M., and Opella, S. J. (1993). Structure and orientation of the antibiotic peptides magainin in membranes by solid-state nuclear magnetic resonance spectroscopy. Prot Sci 2, 2077-2084.

Bergman, M. and Ross, W. F. (1936). On proteolytic enzymes X: The enzymes of papain and their activities. J Biol Chem 114, 717.

Blaney, J. M., and Martin, E. J. (1997). Computational approaches for combinatorial library design and molecular diversity analysis. Curr Opin Chem Biol 1:54-59.

Brown, J. L. (1979). A comparison of the turnover of α-N-acetylated and nonacetylated mouse L-cell proteins. J Bio Chem 254, 1447-1449.

Brown, J. L. and Roberts, W. K. (1976). Evidence that approximately eighty per cent of the soluble proteins from Ehrlich ascites cells are Nα-acetylated. J Biol Chem 251, 1009-1014.

Brownlees, J. and Williams, C.H. (1993). Peptidases, peptides at the mammalian blood-brain barrier. J Neurochem 60, 793-803.

Bryan, L. E. (1985). Antibiotic uptake and cytoplasmic membrane. Antibiot Chemother 36, 103-110.

Bonner, G. G., Davis, P., Stropova, D., Ferguson, R., Yamamura, H. I., Porreca, F., and Hruby, V. J. (1997). Opoid peptides: Simultaneous δ agonism and μ antagonism in somatostatin analogues. Peptides 18, 93-100.

Bultinck, P., Augustynen, S., Hilbers, H. W., Moret, E. E., and Tollenaere, J. P. (2002). Generate: a program for 3-D structure generation and conformational analysis of peptides and peptidomimetics. J Comput Chem 23, 746-754.

Chance, R. E., Kroeff, E. P., Hoffmann, J. A., and Frank, B. H. (1981). Chemical, physical, and biologic properties of biosynthetic human insulin. Diabetes Care 4:147-154.

Chinos-Rojas, C. L., Steward, M. W., and Partidos, C. D. (1997). Use of solid-phase random peptide library to identify inhibitors of TNF-alpha mediated cytotoxicity in vitro. Cytokine 9, 226-232.

Christensen, J. E., Dudley, E. G., Pederson, J. A., Steele, J. L. (1999). Peptidases and amino acid catabolism in lactic acid bacteria. Antonie Van Leeuwenhoek 76, 217-246.

Chopra, I. (1988). Molecular mechanisms involved in the transport of antibiotics into bacteria. Parasitology 96, S25-S44.

Cole, A. M., and Ganz, T. (2000). Human antimicrobial peptides: analysis and application. Biotechniques 29, 822-831.

Deacon, C. F., Dnudsen, L. B., Madsen, K., Wiberg, F. C., and Holst, J. J. (1998). Dipeptidyl peptidase IV resistant analogues of glucagon-like peptide-1 which have extended metabolic stability and improved biological activity. Diabetes 41, 271-278.

Decade, G. M. and Nikaido, H. (1976). Outer membrane of gram-negative bacteria. J Bactiol 128, 325-336.

Demotz, S., Moulon, C., Roggero, M. A., Fasel, N., and Slavica, M. (2001). Native-like long synthetic peptides as components of sub-unit vaccines: practical

and theoretical considerations for their use in humans. Mol Immunol 38, 415-422.

Detmers, F. J. M., Kunji, E. R. S., Lanfermeijer, F. C., Poolman, B., and Konings, W. N. (1998). Kinetics and specificity of peptide uptake by the oligopeptide transport system of *Lactococcus lactis.* Biochem 37, 16671-16679.

Diddens, H., Dorgerloh, M. and Zähner, H. (1979). Metabolic products of microorganisms on the transport of small peptide antibiotics in bacteria. J Antibiot 32, 87-90.

Dixon, M. and Webb, E. C. (1964). Enzymes. (Academic Press, Inc.: New York), pp. 193-194.

Dooley, C. T. and Houghten, R. A. (1999). New opioid peptides, peptidomimetics, and heterocyclic compounds from combinatorial libraries. Biopolymers 51, 379-390.

duVigneaud, V., Ressler, C., Swan, J. M., Roberts, C. W., Katsoyannis, P. G., and Gordon, S. (1953). The synthesis of an octapeptide amide with the hormone activity of oxytocin. J Am Chem Soc 75, 4879.

Eisenhauer, P. B., Harwig, S. S. L., Szklarek, D., Ganz, T., Selsted, M. E., and Lehrer, R. I. (1989). Purification and antimicrobial properties of three defensins from rat neutrophils. Inf Immun 57, 2021-2027.

Ellmark, P., Ottosson, C., Borrebaeck, C. A., Molmborg Hager, A. C., amd Furebring, C. (2002). Modulation of the CD-40-CD40 ligand interaction using human anti-CD40 singe-chain antibody fragments obtained from the n-CoDeR phage library. Immunology 106, 456-463.

Epand, R. M. and Vogel, H. J. (1999). Diversity of antimicrobial pepides and their mechanisms of action. Biochim Biophys Acta 1462, 11-28.

Fahrner, R. L., Dieckmann, T., Harwig, S. S., Lehrer, R. I., Eisenberg, D., and Feigon, J. (1996). Solution structure of protegrin-1, a broad-spectrum antimicrobial peptide from porcine leukocytes. Chem Biol 3, 543-50.

Fischer, E. and Fourneau, E. (1901). Ber Dtsch Chem Ges 34, 2868.

Fisher, G., Heins, J. and Barth, A. (1983). The conformation around the peptide bond between the P_1- and P_2-positions is important for catalytic activity of some proline-specific proteases. Biochem Biophys Acta 742, 452-462.

Fischer, P. M., Krausz, E. and Lane, D. P. (2001). Cellular delivery of impermeable effector molecules in the form of conjugates with peptides capable of mediating membrane translocation. Bioconjug Chem 12, 825-841.

Franklin T. J. and G. A. Snow. (1998). Biochemistry and Molecular Biology of Antimicrobial Drug Action, Fifth edition. (Kluwer Academic Publishers: London), pp. 46-49.

Furka, A., Sebestyen, F., Asgedom, M., and Dibo, G. (1991). General method for rapid synthesis of muti-component peptide mixtures. Int J Pep Protein Res 37, 487-493.

Gallwitz, B., Ropeter, T., Morys-Wortmann, C., Mentlein, R., Siegel, E. G., and Schmidt, W. E. (2000). GLP-1 analogues resistant to degradation by dipeptidyl-peptidase IV in vitro. Regul Pept 86, 103-111.

Geysen, H. M., Meloen, R. H. and Barteling, S. J. (1984). Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc Natl Acad Sci USA 81, 3998-4002.

Goldberg, A. L., Moerschell, R. P., Chung, C. H., and Maurizi, M. R. (1994). ATP-dependent protease La (Lon) from *Escherichia coli*. Meth Enzymol 244, 350-375.

Kawano, K., Yoneya, T., Miyata, T., Yoshikawa, K., Tokunaga, F., Terada, Y., and Iwanaga, S. (1990). Antimicrobial peptide, tachyplesin I, isolated from hemocytes of the horseshoe crab (Tachypleus tridentatus). NMR determination of the beta-sheet structure. J Biol Chem 265, 15365-7.

Gonzales, T., and Robert-Baudouy, J. (1996). Bacterial aminopeptidases: properties and functions. FEMS Micro Rev 18, 319-344.

Gozes, I. (2001). Neroprotective peptide drug delivery and development: potential new therapeutics. Trends Neurosci 24, 700-705.

Grathwohl, C. and Wüthrich, K. (1976). The X-Pro peptide bond as an NMR probe for conformational studies of flexible linear peptides. Biopolymers 15, 2025-2041.

Gundlach, B. R., Wiesmüller, K-H., Junt, T., Kienle, S., Jung, G. and Walden, P. (1996). Determination of T cell epitopes with random peptide libraries. J Immun Meth 192, 149-155.

Hancock, R. E. W. (1997). Peptide antibiotics. Lancet 349, 418-422.

Hancock, R. E. W. and Diamond, G. (2000). The role of cationic antimicrobial peptides in innate host defenses. Trends Microbiol 8, 402-410.

Hancock, R. E. W. and Scott, M. G. (2000). The role of antimicrobial peptides in animal defenses. Proc Natl Acad Sci USA 97, 8856-8861.

Heavner, G. A., Kroon, D. J., Audhya, T., and Goldstein, G. (1986). Biologically active analogs of thymopentin with enhanced enzymatic stability. Peptides 7, 1015-1019.

Hirschmann, R. (1991). Medicinal chemistry in the golden age of biology: lessons from steroid and peptide research. Angew Chem Int Ed Engl 30, 1278-1301.

Hruby, V. J., Agnes, R. S., and Cai, C. (2002). Design of peptide agonists. Methods Enzymol 343, 73-91.

Hruby, V. J., and Balse, P. M. (2000). Conformational and topographical considerations in designing agonist peptidomimetics from peptide leads. Curr Med Chem 7, 945-970.

Hruby, V. J., Kazmeirski, W., Kawasaki, A. M., and Matsunaga, T. O. (1991). Synthetic chemistry and the design of peptide-based drugs. In Peptide Pharmaceuticals, Approaches to the Design of Novel Drugs. Ed., Ward, D. J. (Elsevier: New York), pp. 135-161.

Hruby, V. J., Li, G., Haskell-Luevano., C., and Shenderovich, M. (1997). Design of peptides, proteins and peptidomimetics in chi space. Biopolymers 43, 219-266.

Houghten, R. A. (1985). General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. Proc Natl Acad Sci USA 82, 5131-5.

Hwang, P. M. and Vogel, H. J. (1998). Structure-function relationships of antimicrobial peptides. Biochem Cell Biol 76, 235-246.

Isaac, R. E., Siviter, R. J., Stancombe, P., Coates, D., and Shirras, A. D. (2000). Conserved roles for peptidases in the processing of invertebrate neuropeptides. Biochem Soc Trans 28, 460-464.

Ito, A. (1999). Mitochondrial processing peptidase: multiple-site recognition of precursor proteins. Biochem Biophys Res Comm 265, 611-616.

Jaworski, A. and Brückner, H. (2001). Sequences of polypeptide antibiotics stilboflavins, natural peptaibol libraries of the mold *Stilbella flavipes*. J Peptide Sci 7, 433-447.

Lam, K. (1997). Application of combinatorial library methods in cancer research and drug discovery. Anti-Cancer Drug Des 12, 145-167.

Lam, K. S., Salmon, S. E., Hersh, E. M., Hruby, V. J., Kazmierski, W. M., and Knapp, R. J. (1991). A new type of synthetic peptide library for identifying ligand-binding activity. Nature 354, 82-84.

Lawyer, C., Pai, S., Watabe, M., Borgia, P., Mashimo, T., Eagleton, L., and Watabe, K. (1996). Antimicrobial activity of a 13 amino acid tryptophan-rich peptide derived from a putative porcine precursor protein of a novel family of antibacterial peptides. FEBS Lett 390, 95-8.

Lee, V. H. (1988). Enzymatic barriers to peptide and protein absorption. Crit Rev Ther Drug Carr Sys 5, 69-97.

Lendeckel, U., Kahne, T., Riemann, D., Neubert, K., Arndt, M., and Reinhold, D. (2000). Review: the role of membrane peptidases in immune functions. Adv Exp Med Biol 477, 1-24.

Leone-Bay, A., Paton, D. R., and Weidner, J. J. (2000). The development and delivery agents that facilitate the oral absorption of macromolecular drugs. Med Res Rev 20, 169-186.

Letsinger, R. L. and Kornet, M. J. (1963). Popcorn polymer as a support in multistep synthesis. J Am Chem Soc 85, 3045.

Lindsey, K., Casson, S., and Chilley, P. (2002). Peptides: new signaling molecules in plants. Trends Plant Sci 7, 78-83.

Livermore, D. M. (1991). Antibiotic uptake and transport by bacteria. Scand J Infect Dis Suppl 74, 15-22.

MacArthur, M. W. and Thorton, J. M. (1991). Influence of proline residues on protein conformation. J Mol Biol 218, 397-412.

Massilia, G. R., Schininá, M. E., Ascenzi, P., and Polticelli, F. (2001). Contryphan-Vn: a novel peptide from the venom of the mediterranean snail *Conus ventricosus*. Biochem Biophys Res Comm 288, 908-913.

Matsuzaki, K., Sugishita, K., Harada, M., Fujii, N., and Miyajima, K. (1997). Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria. Biochim Biophys Acta 1327, 119-30.

McDonald, J. K. and Barrett, A. J. (1986). Mammalian Proteases: A glossary and bibliography, Vol. 2, Exopeptidases (Harcourt Brace Jovanovich: London), pp. 1-6.

McDonald J. K., Leibach, F. H., Grindeland, R. E., and Ellis S. (1968). Purification of dipeptidyl aminopeptidase II (dipeptidyl arylamidase II) of the anterior pituitary gland. Peptidase and dipeptide esterase activities. J Biol Chem 243, 4143-50.

McDonald, J. K., Reilly, T. J., Zeitman, B. B., and Ellis S. (1968). Dipeptidyl arylamidase II of the pituitary. Properties of lysylalanyl-beta-naphthylamide hydrolysis: inhibition by cations, distribution in tissues, and subcellular localization. J Biol Chem 243, 2028-37.

Mentlein, R. (1988). Proline residues in the maturation and degradation of peptide hormones and neuropeptides. FEBS Lett 234, 251-256.

Merrifield, R. B. (1963). Solid phase peptide synthesis I. The syntheis of a tetrapeptide. J Am Chem Soc 85, 2149.

Merrifield, R. B., Merrifield, E. L., Juvvadi, P., Adnreu, D., and Boman, H. G. (1994). Design and synthesis of antimicrobial peptides. Antimicrobial Peptides, Ciba Foundation Symposium 186, 5-26.

Miller, C. G. (1996). Protein degradation and proteolytic modification. In *Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology, 2nd edition, Ed. Neidhardt, F.C. (ASM Press: Washington, D.C..), pp. 938-954.

Moller, D. E. (2001). New drug targets for type 2 diabetes and the metabolic syndrome. Nature 414, 821-827.

Moss, J. (1995). Peptide prodrugs designed to limit metabolism. *In* Peptide-based Drug Design. Eds. Taylor, M. D., and Amidon, G. L. (American Chemical Society: Washington D.C .), pp. 423-424.

Naider, F. and Becker, J. M. (1975). Multiplicity of oligopeptide transport systems in *Escherichia coli*. J Bacteriol 122, 1208-1215.

Naider F. and Goodman, M. (2002). Scope of the volumes, 1.1 Historical aspects: 100 years of peptide synthesis. Vol. E22, Ed. M. Goodman. Synthesis of Peptides and Peptidomimetics. Supplementary volume to Methods of Organic Chemistry, 4th edition (George Thieme Verlag Stuttgart: New York), pp. 1-14.

Nikaido, H. (1992). Porins and specific channels of bacterial out membranes. Mol Microbiol 6, 435-442.

Nikaido, H. (1996). Outer membrane. In *Escherichia coli* and *Salmonella* Cellular and Molecular Biology. 2nd edition. Ed. Neidhardt F. C. (ASM Press: Washington D. C.), pp. 29-47.

Nikaido, H. and Nakae, T. (1979). The outer membrane of gram-negative bacteria. Adv Microb Physiol 20, 163-250.

Nikaido, H., and Rosenberg, E. Y. (1983). Porin channels in *Escherichia coli*: Studies with liposomes reconstituted from purified proteins. J. Bact 153, 241-252.

Nikiforovich, G. V., and Marshall, G. R. 2001. Editorial: current developments in computational studies of peptides. Biopolymers 60, 77-78.

Nikaido, H. and Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. Microbiol Rev 49, 1032.

Pardi, A., Zhang, X. L., Selsted, M. E., Skalicky, J. J., and Yip, P. F. (1992). NMR studies of defensin antimircobial peptides. 2. Three-dimensional structures of rabbit NP-2 and human HNP-1. Biochemistry 31, 11357-11364.

Pardridge, M. (1992). Recent developments in peptide drug delivery to the brain. Pharmacol Toxicol 71, 3-10.

Parmley, S. F. and Smith, G. P. (1988). Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. Gene 73, 305-318.

Payne, J. W. and Gilvarg, C. (1968). Size restriction on peptide utilization in *Escherichia coli*. J Biol Chem 243, 6219-6299.

Payne, J. W. and Smith, M. W. (1994). Peptide transport by micro-organisms. Adv Microb Phys 36, 2-80.

Perschel, A., and Collins, L. V. (2001). Staphylococcal resistance to antimicrobial peptides of mammalian and bacterial origin. Peptides 22, 1651-1659.

Porankiewicz, J., Wang, J., and Clarke, A. K. (1999). New insights into the ATP-dependent Clp protease: *Escherichia coli* and beyond. Mol Microbiol 32, 449-458.

Raj, P. A., Marcus, E., and Sukumaran, D. K. (1998). Structure of human salivary histatin 5 in aqueous and nonaqueous solutions. Biopolymers 45, 51-67.

Rawlings, N. D., and Barrett, A. J. (1993). Evolutionary family of peptidases. Biochem J 290, 205-218.

Reimer, U., Scherer, G., Drewello, M., Kruber, S., Schutkowski, M., and Fischer, G. (1998). Side-chain effects on peptidyl-prolyl *cis/trans* isomerization. J Mol Biol 279, 449-460.

Ripka, A. S., and Rich, D. H. (1998). Peptidomimetic design. Curr Opin Chem Biol 2, 441-452.

Russell-Jones, G. J., Westwood, S. W., and Habberfield, A. D. (1995). Vitamin B_{12} mediated oral delivery systems for graulocyte-colony stimulating factor and erythropoietin. Bioconj Chem 6, 459-465.

Ryan, J. W. (1998). Peptidase enzymes of the pulmonary vascular surface. Am J Physiol 257, L53-L60.

Scherrer, R. and Gerhardt, P. (1971). Molecular sieving by the *Bacillus megaterium* cell wall and protoplast. J Bactiol 107, 718-735.

Schultz, J. S. and Schultz, J. S. (1996). The combinatorial library: a multifunctional resource. Biotechnol Prog 12, 729-743.

Scott, J. K. and Smith, G. P. (1990). Searching for peptide ligands with an epitope library. Science 249, 386-249.

Selsted, M. E., Szklarek, D., and Lehrer, R. I. (1984). Purification and antibacterial acitivity of antimicrobial peptides of rabbit granulocytes. Infect Immun 45, 150-154.

Sitaram, N. and Nagaraj, R. (1999). Interaction of antimicrobial peptides with biological and model membranes: structural and charge requirements for activity. Biochim Biophys Acta 1462, 29-54.

Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228, 1315-1316.

Smith, R. L., Archer, E. G., and Dunn, F. W. (1970). Uptake of [^{14}C]-labeled tri-, tetra-, and pentapeptides of phenylalanine and glycine by *Escherichia coli*. J Bacteriol 245, 2967-2971.

Sood, A. and Panchagnula, R. (2001). Peroral route: an opportunity for protein and peptide drug delivery. Chem Rev 101, 3275-3303.

Staskawicz, B. J. and Panopoulos, N. J. (1980). Phaseolotoxin transport in *Escherichia coli* and *Salmonella typhimurium* via the oligopeptide permease. J Bacteriol 142, 474-479.

Stöckel-Maschek, A., Mrestani-Klaus, C., Stiebitz, B., Demuth, H-U., and Neubert, K. (2000). Thioxo amino acid pyrrolidides and thiazolidides: new inhibitors of proline specific peptidases. Biochim Biophys Acta 1479, 15-31.

Tamamura, H., Omagari, A., Hiramatsu, K,. Gotoh, K., Kanamoto, T., Xu, Y., Kodama, E., Matsuoka, M., Hattori, T., Yamamoto, N., Nakashima, H., Otaka, A., and Fujii. N. (2001). Development of specific CXCR4 inhibitors possessing high selectivity indexes as well as complete stability in serum based on anti-HIV peptide Ti40. Bioorg Med Chem Lett 11, 1897-1902.

Taylor, A. (1993). Aminopeptidases: towards a mechanism of action. Trends Biochem. Sci. 18, 167-171.

Thennarasu, S. and Nagaraj, R. (1999). Synthetic peptides corresponding to the beta-hairpin loop of rabbit defensin NP-2 show antimicrobial activity. Biochem Biophys Res Commun 254, 281-283.

Thomson, M. W. and Maurizi, M. R. (1994). Activity and specificity of *Escherichia coli* ClpAP protease in cleaving model peptide substrates. J Biol Chem 268, 18201-18208.

Ting, A. Y., Witte, K., Shah, K., Kraybill, B., Shokat, K. M., and Schultz, P. G. (2001). Phage-display evolution of tyrosine kinases with altered nucleotide specificity. Biopolymers 60, 220-228.

Tomkinson, B. (1999). Tripeptidyl peptidases: enzymes that can count. Trends Biochem. Sci. 24, 355-359.

Tossi, A., Sandri, L. and Giangaspero, A. (2000). Amphipathic, α-helical antimicrobial peptides. Biopolymers 55, 4-30.

Totrov, M. and Abagyan, R. (2001). Rapid boundary element solvation electrostatics calculations in folding simulations: successful folding of a 23-residue peptide. Biopolymers 60, 124-133.

Vanhoof, G., Gossens, F., De Meester, I., Hendriks, D., and Scharpe, S. (1995). Proline motifs in peptides and their biological processing. FASEB J 9, 736-44.

Venugopal, J. (2001). Cardiac natriutetic peptides - hope or hype? J Clinic Pharm Ther 26, 15-31.

Verheart, R. M. D., Beekwilder, J., Olsthoorn, R., van Duin, J., and Quax, W. J. (2002). Phage display selects for amylases with improved low pH start-binding. J Biotech 96, 103-118.

von Döhren, H. (1995). Peptides. Biotechnology 28, 129-171.

Wang, W., Jiang, J., Ballard, C. E., and Wang, B. (1999). Prodrug approaches to the improved delivery of peptide drugs. Curr Pharm Des 5, 265-287.

Warner, R. R. P. and O'Dorisio, T. M. (2002). Radiolabeled peptides in diagnosis and tumor imaging: clinical overview. Sem Nucl Med 32, 79-83.

Wettergren, A., Pridal, L., Wøjdemann, M., and Holst, J. J. (1998). Amidated and non-amidated glucagon-like peptide-1 (GLP-1): non-pancreatic effects (cepahlic phase acid secretion) and stability in plasma in human. Regul Pept 77, 83-87.

Williamson, M. P. (1994). The structure and function of proline-rich regions in proteins. Biochem J 287, 249-260.

Woodley, J. F. (1994). Enzymatic barriers for GI peptide and protein delivery. Crit Rev Ther Drug Carrier Sys 11, 61-95.

Yao, Z-J., Kao, M. C. C., and Chung, M. C. M. (1995). Epitope identification by polyclonal antibody from phage-display random peptide library. J Protein Chem 14, 161-166.

Yaron, A. and Naider, F. (1993). Proline-dependent structural and biological properties of peptides and proteins. Crit Rev Biochem Mol Biol 28, 31-81.

Yi, G. S., Park, C. B., Kim, S. C., and Cheong, C. (1996). Solution structure of an antimicrobial peptide buforin II. FEBS Lett 398, 87-90.

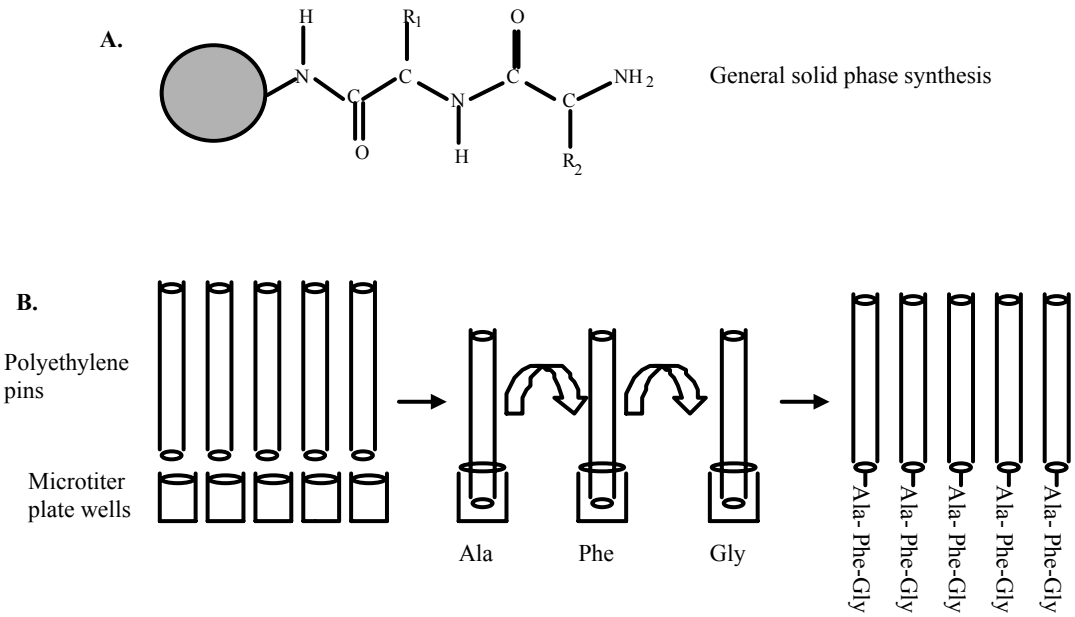


Figure 1.1a Solid phase synthesis. A. Synthesis of the amino acid chain on a solid support, such as a bead, begins with attachment of the first amino acid by its carboxyl end. **B.** An amino acid is assembled onto a polystyrene bead or pin, additional amino acids are then added by sequential reactions. Multiple pins are dipped into wells each containing a different single amino acid solution to create a peptide. The amino acid reactions can be done in predetermined order to synthesize a peptide of specific sequence.

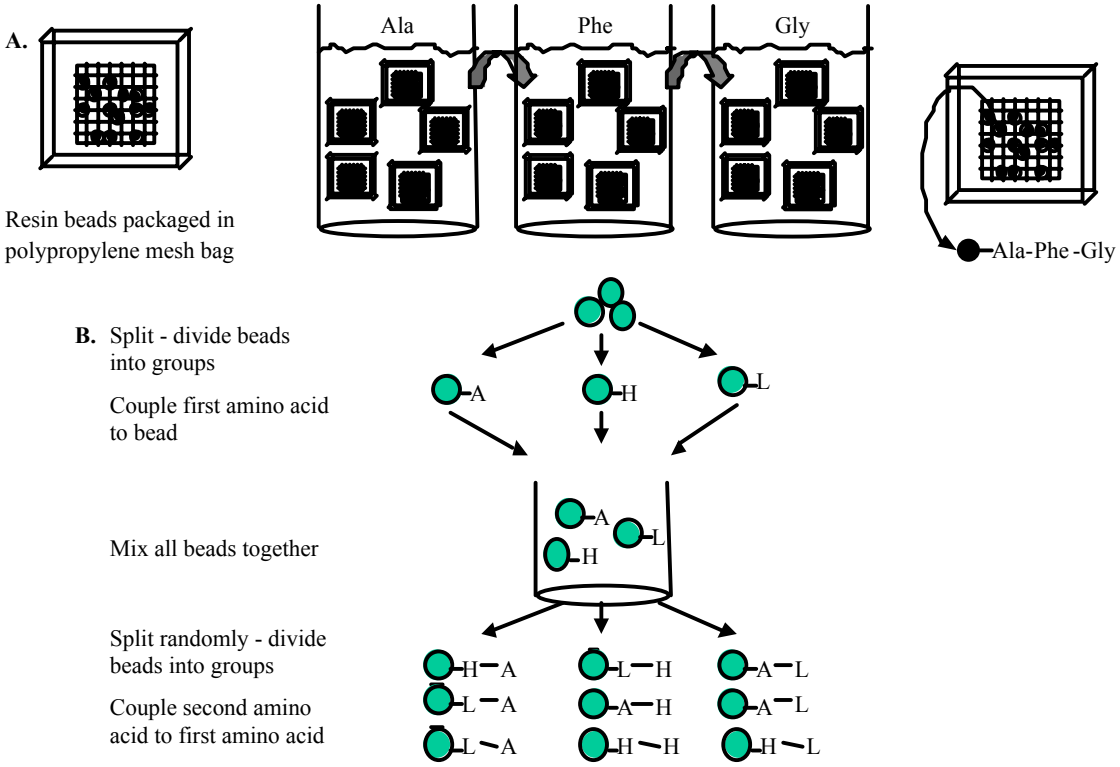


Figure 1.1b Solid phase synthesis continued. A. Houghten's tea-bag method. Beads are packaged into bags that can be dropped into amino acid solutions. This method can be used to make a known or randomized sequence. **B.** The split-couple-mix method provides random peptide assembly. Beads are separated into groups where different amino acids are coupled to the beads. Then, all beads are mixed, divided randomly into new groups and added to new single amino acid solutions. This split-couple-mix method can be used to generate a variety of peptide sequences.

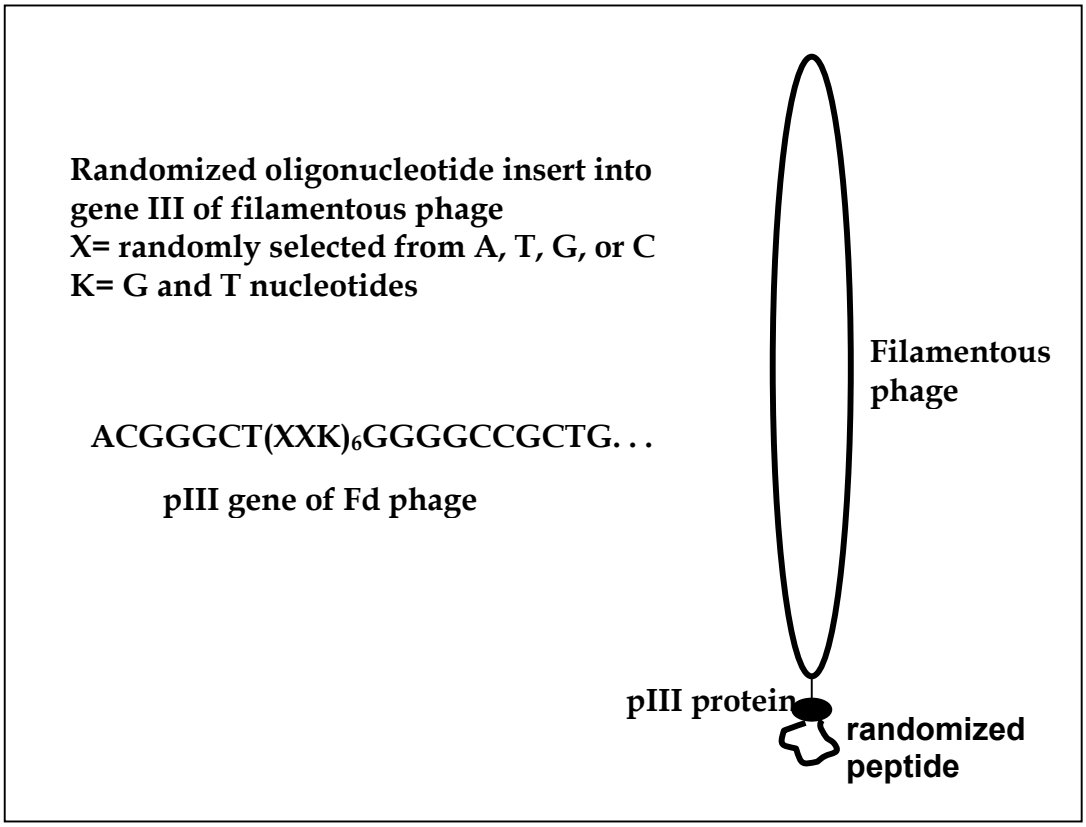


Figure 1.2 Phage display. Naturally assembled peptides are made by creating a randomized oligonucleotide where X is selected from a mixture of G, A, T, and C, and K is selected from a mixture of G and C. The oligo is inserted into the filamentous phage plasmid encoding the pIII gene followed by transformation of *E. coli*. Upon infection of filamentous phage, new phage are assembled in *E. coli* cells with expression of the pIII with the fused randomized peptide at the tip of the phage, a position that allows for peptide binding to fixed protein targets.

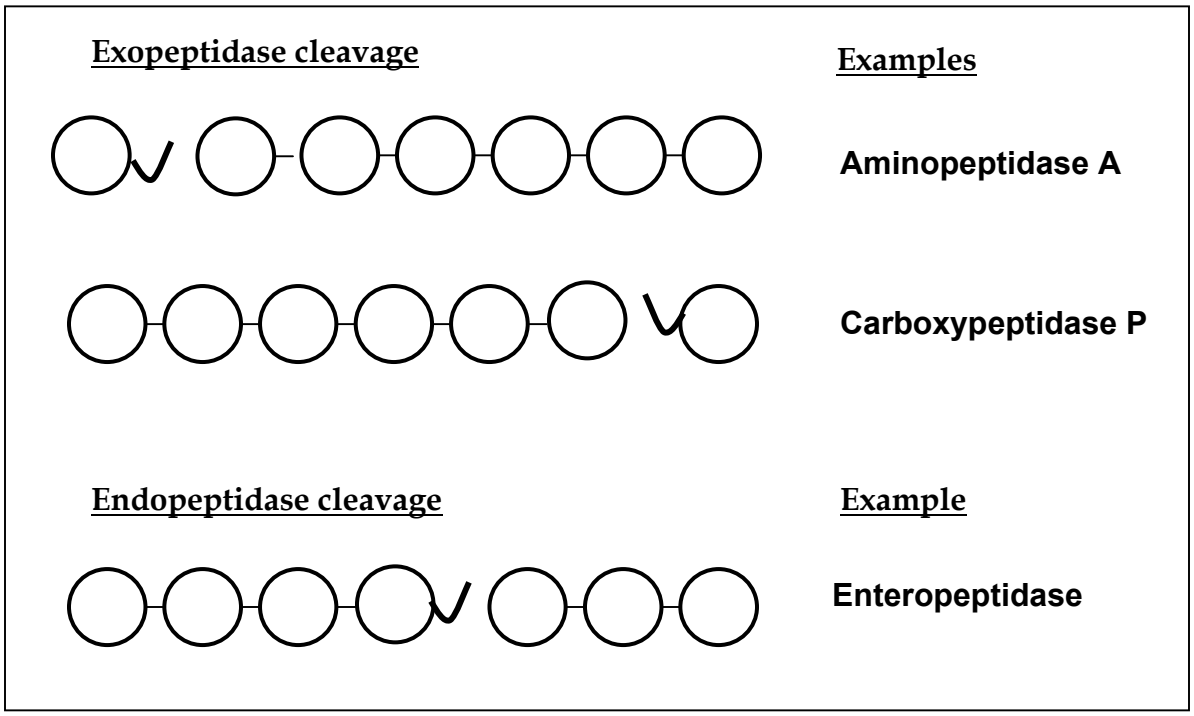


Figure 1.3 Peptidase cleavage sites. Exopeptidases can cleave one or two amino acids from either the carboxyl or amino terminus. Endopeptidases cleave at sites in the middle of a peptide sequence. Examples of peptidases for each type of activity are given.

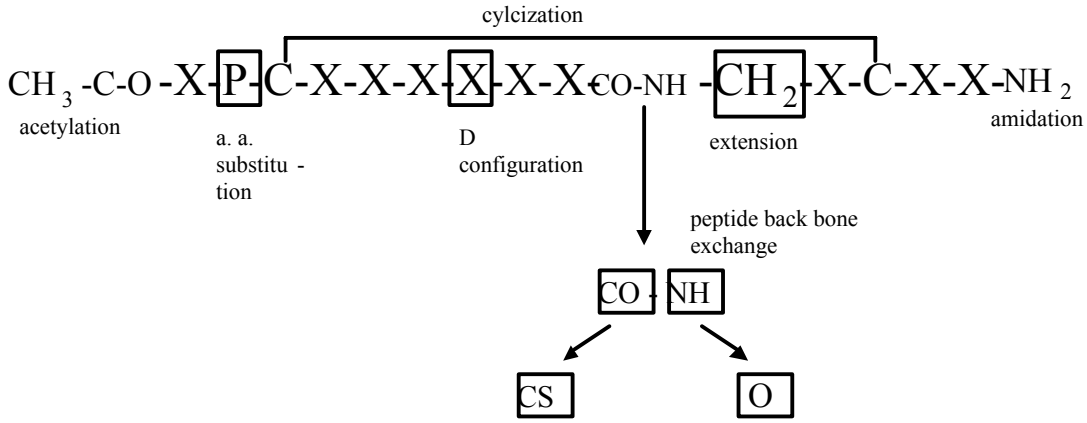


Figure 1.4 Peptide modifications for peptidase protection. This diagram indicates where peptide modification can occur. Terminal ends are modified by acetylation at the amino terminus or by amidation at the carboxyl terminus. Modification along the peptide backbone is represented by amino acid substitution, amide bond extension and amide bond replacement. Steric strain on the peptide bonds can be achieved by replacing the L-amino acids with a D-amino acid or by cyclization with a disulfide bond.

CHAPTER 2

AN *IN VIVO* STUDY OF NOVEL BIOACTIVE PEPTIDES THAT INHIBIT THE GROWTH OF *ESCHERICHIA COLI*[1]

[1] Walker, J. R., J. R. Roth, and E. Altman. (2001). J Pep Res 58:380-388. Reprinted here with permission of publisher.

Abstract

We have created a system in which synthetically produced novel bioactive peptides can be expressed *in vivo* in *Escherichia coli*. 20,000 of these peptides were screened and 21 inhibitors were found which could inhibit the growth of *E. coli* on minimal media. The inhibitors could be placed into one of two groups, one day inhibitors which were partially inhibitory, and two day inhibitors which were completely inhibitory. Sequence analysis showed that two of the most potent inhibitors were actually peptide-protein chimeras where the peptides had become fused to the 63 amino acid Rop protein which was also contained in the expression vector that was used in this study. Given that Rop is known to form an incredibly stable structure, it could be serving as a stabilizing motif for these peptides. Sequence analysis of the predicted coding regions from the next 10 most inhibitory peptides showed that four out of the 10 peptides contained one or more proline residues either at or very near the carboxyl-terminal end of the peptide which could act to prevent degradation by peptidases. Collectively, based on what we observed in our screen of synthetic bioactive peptides that could prevent the growth of *E. coli* and what has been learned from structural studies of naturally occurring bioactive peptides, the presence of a stabilizing motif seems to be important for small peptides, if they are to be biologically active.

Introduction

Bioactive peptides are small peptides that elicit a biological activity. Over 500 of these peptides which average 20 amino acids in size have now been identified and characterized (Rivier, *et al.*, 1990; Smith and Rivier, 1992; Wieland and Bodanszky, 1991). They have been isolated in a variety of systems, exhibit a wide range of actions, and have been utilized as therapeutic agents in the field of medicine, as well as diagnostic tools in both basic and applied research. Some of the better known peptides that are employed as therapeutic agents include calcitonin, gastrin, glucagon, luteinizing hormone releasing factor, oxytocin, secretin, somatostatin, and vasopressin (Wearley, 1991).

Where the mode of action of these peptides has been determined, it has been found to be due to the interaction of the bioactive peptide with a specific protein target (Siddle and Hutton, 1990). In most of the cases, the bioactive peptide acts by binding to and inactivating its protein target with incredibly high specificity. Binding constants of these peptides for their protein targets typically have been determined to be in the nM range (Rivier and Marshall, 1990; Smith and Rivier, 1992) with binding constants as high as 10^{-12} M having been reported (Bozou, *et al.*, 1986; Le-Nguyen, *et al.*, 1990).

There has been an increasing interest in employing synthetically derived bioactive peptides as novel pharmaceutical agents due to the inherent ability of these peptides to bind to and inhibit specific protein targets (Eichler, *et al.*, 1995; Lam, 1996; Lowman, 1997). Three obvious areas in which synthetically produced

peptides could be utilized are in the development of new antibacterial, antiviral, and anticancer agents. To develop antibacterial or antiviral agents, synthetically derived peptides would be isolated which could either bind to and prevent bacterial or viral surface proteins from interacting with their host cell receptors or else prevent the action of specific toxin or protease proteins. To develop anticancer agents, synthetically derived peptides would be isolated which could bind to and prevent the action of specific oncogenic proteins.

Researchers have been engineering novel bioactive peptides through the use of two different *in vitro* approaches. The first approach involves the chemical synthesis of a randomized library of 6-10 amino acid peptides (Eichler, *et al.*, 1995; Lam, 1996; Lebl and Krchnak, 1997). In the second approach, a randomized oligonucleotide library is cloned into a Ff filamentous phage gene which allows peptides that are 6-38 amino acids in length to be expressed on the surface of the bacteriophage (Lowman, 1997; Smith and Scott, 1993). The resulting peptide libraries are then usually mixed with a matrix-bound protein target. Peptides that bind are eluted and their sequences are determined. From this information new peptides are synthesized and their inhibitory properties are determined. Although there has been some limited success using this *in vitro* approach and a few inhibitor peptides have been developed, the use of synthetically derived peptides has not yet become a mainstay in the pharmaceutical industry.

We wanted to develop an *in vivo* approach which would allow us to isolate numerous inhibitor peptides and rationalized that anything we learned

about what makes these peptides function could then be utilized in the more applied *in vitro* systems in order to develop new pharmaceutical agents. To maximize our chances of isolating inhibitor peptides, we did not want to focus our efforts on only one target and thus decided to pursue intracellularly produced peptides which could inhibit the growth of *Escherichia coli*. Any intracellular protein that was necessary for the normal cellular growth of *E. coli* would be a target in this approach.

To implement this *in vivo* approach, an oligonucleotide library that is capable of encoding up to 20 amino acid peptides is cloned into an expression vector which allows the peptides to either be turned off or overproduced in the cytoplasm of *E. coli*. This pool of clones is transformed into *E. coli* under repressed conditions and the resulting bacterial transformants are then screened for any that can not grow on minimal media when the peptides are overproduced. In this scheme, any transformant bacterial colony which overproduces a peptide that inhibits a protein necessary for growth on minimal media will be identified.

Experimental Procedures

Media

Rich LB and minimal M9 media used in this study was prepared as described by Miller (Miller, 1972). Ampicillin was used in rich media at a final concentration of 100 ug/ml and in minimal media at a final concentration of 50

ug/ml. Isopropyl β-D-thiogalactoside (IPTG) was added to media at a final concentration of 1 mM.

Bacterial strains and plasmids

ALS225 which is MC1061/F'*lacI*^{q1}*Z+Y+A+* was the *E. coli* strain used in this study (Warren, *et al.,* 2000). The genotype for MC1061 is *araD139 Δ(araABOIC-leu)7679 Δ(lac)X74 galU galK rpsL hsr- hsm+* (Casadaban and Cohen, 1980). pLAC11 is the highly regulable expression vector that was used in this work (Warren, *et al.,* 2000).

Generating the randomized peptide library

The 93 base oligonucleotide 5' TAC TAT AGA TCT ATG $(XXX)_{20}$ TAA TAA GAA TTC TCG ACA 3', where X denotes an equimolar mixture of the nucleotides A, C, G, or T, was synthesized with the trityl group on and subsequently purified using an OPC cartridge. The complementary strand of the 93 base oligonucleotide was generated by a fill-in reaction with Klenow using an equimolar amount of the 18 base oligonucleotide primer 5' TGT CGA GAA TTC TTA TTA 3'. After extension, the resulting ds-DNA was purified using a Promega DNA clean-up kit and restricted with *Bgl* II and *Eco*R I. The digested DNA was again purified using a Promega DNA clean-up kit and ligated to the pLAC11 vector which had been digested with the same two restriction enzymes. The resulting library was then transformed into electrocompetent ALS225 cells.

Sequencing the coding regions of the inhibitor peptide clones

The forward primer 5' TCA TTA ATG CAG CTG GCA CG 3' and the reverse primer 5' TTC ATA CAC GGT GCC TGA CT 3' were used to sequence both strands of the inhibitor peptide clones. If an error-free consensus sequence could not be deduced from these two sequencing runs, both strands of the inhibitor peptide clones in question were resequenced using the forward primer 5' TAG CTC ACT CAT TAG GCA CC 3' and the reverse primer 5' GAT GAC GAT GAG CGC ATT GT 3'. The second set of primers were designed to anneal downstream of the first set of primers in the pLAC11 vector.

Generating antisense derivatives of the inhibitor clones

Oligonucleotides were synthesized which duplicated the DNA insert contained between the *Bgl* II and *Eco*R I restriction sites for five of the anchorless inhibitor peptides with one major nucleotide change. The "T" of the ATG start codon was changed to a "C" which resulted in an ACG which can not be used as a start codon. The oligonucleotides were extended using the same 18 base oligonucleotide primer that was used to construct the original peptide library. The resulting ds-DNA was then restricted, and cloned into pLAC11 exactly as described in the section "Generating the randomized peptide library" in Materials and Methods. The antisense oligonucleotides that were used are as follows: pPep1(antisense), 5' TAC TAT AGA TCT ACG GTC ACT GAA TTT TGT GGC TTG TTG GAC CAA CTG CCT TAG TAA TAG TGG AAG GCT GAA ATT AAT AAG AAT TCT CGA CA 3'; pPep5(antisense), 5' TAC TAT AGA TCT

ACG TGG CGG GAC TCA TGG ATT AAG GGT AGG GAC GTG GGG TTT ATG GGT TAA AAT AGT TTG ATA ATA AGA ATT CTC GAC A 3'; pPep12(antisense), 5' TAC TAT AGA TCT ACG AAC GGC CGA ACC AAA CGA ATC CGG GAC CCA CCA GCC GCC TAA ACA GCT ACC AGC TGT GGT AAT AAG AAT TCT CGA CA 3'; pPep13(antisense), 5' TAC TAT AGA TCT ACG GAC CGT GAA GTG ATG TGT GCG GCA AAA CAG GAA TGG AAG GAA CGA ACG CCA TAG GCC GCG TAA TAA GAA TTC TCG ACA 3'; pPep19(antisense), 5' TAC TAT AGA TCT ACG AGG GGC GCC AAC TAA GGG GGG GGG AAG GTA TTT GTC CCG TGC ATA ATC TCG GGT GTT GTC TAA TAA GAA TTC TCG ACA 3'.

Secondary structure predictions

The propensity for the inhibitor peptides to form α-helixes or β-sheets was initially considered using both Chou and Fasman (Chou, 1990; Chou and Fasman, 1978) and Garnier, Osguthorpe, and Robson analysis (Garnier, *et al.,* 1978; Gibrat, *et al.,* 1987). The peptide sequences were then analyzed using the PepTool protein structure software package from BioTools Incorporated. The membrane spanning domain in pPep6 was analyzed using AMPHI (Jahnig, 1990), SOAP (Kyte and Doolittle, 1982), SOSUI (Hirokawa, *et al.,* 1998), and TMpredict (Hoffman and Stoffel, 1993) algorithms, as well as the predictive rules of Klein, Kanehisa, and DeLisi (Klein, *et al.,* 1985).

Chemicals and reagents

Extension reactions were carried out using Klenow from New England Biolabs while ligation reactions were performed using T4 DNA Ligase from Life Sciences. Isopropyl β-D-thiogalactoside (IPTG) was obtained from Diagnostic Chemicals Limited.

Results

Construction of the peptide library

In order to isolate potential inhibitor peptides, a totally randomized oligonucleotide library that encoded up to 20 amino acid peptides was cloned into the pLAC11 expression vector (Warren *et al.,* 2000) as shown in Figure 2.1 and transformed into *E. coli* under repressed conditions. Most of the routinely employed expression vectors such as pKK223-3, pKK233-2, pTrc99A, and the pET series produce significant amounts of protein from cloned genes even when grown under repressed conditions (Balbás and Bolivar, 1990; Brosius, 1988). We were concerned that we might not be able to clone potent inhibitor peptides unless we were able to isolate them under completely repressed conditions. For this reason, we chose to use the highly regulable pLAC11 expression vector for our studies. Because encoded peptides that are expressed using pLAC11 are under the control of the wild-type *lac* operon, their expression can be turned off or on by the presence or absence of the gratuitous inducer, IPTG.

Researchers employing the fusion-phage technology which also utilizes oligonucleotides to encode randomized amino acids in synthesized peptides

have been using **[**XX(G,T)**]** or **[**XX(G,C)**]** codons instead of **[**XXX**]** codons to eliminate two out of the three stop codons, thus increasing the amount of full-length peptides that can be synthesized without a stop codon (Smith and Scott, 1993). We opted to use the **[**XXX**]** codon design instead for the following two reasons. First, both the **[**XX(G,T)**]** and **[**XX(G,C)**]** oligonucleotide codon schemes eliminate half of the codons and thus biases the distribution of amino acids that are generated. Second, the **[**XX(G,T)**]** and **[**XX(G,C)**]** codon schemes drastically affect the preferential codon usage of highly expressed genes and removes a number of the codons which are utilized by the abundant tRNAs that are present in *E. coli* (Grosjean and Fiers, 1982; Ikemura, 1981). Interestingly, when synthesizing a twenty amino acid randomized peptide using the **[**XX(G,T)**]** or **[**XX(G,C)**]** codon design, in theory, 53% of the peptides will not contain a stop codon. This percentage is derived via the following calculation: $(31 \text{ encoding codons}/32 \text{ total codons})^{20 \text{ amino acids}}$. Using the **[**XXX**]** codon design, 38% of the peptides will not contain a stop codon: $(61 \text{ encoding codons}/64 \text{ total codons})^{20 \text{ amino acids}}$. We didn't feel that the 15% increase that results from the **[**XX(G,T)**]** or **[**XX(G,C)**]** codon design was worth the possible risks of introducing codon bias and nonpreferred codons into the oligonucleotides and thus chose to utilize the **[**XXX**]** motifs in our randomized libraries.

Identifying and characterizing inhibitor peptides from the library

Using a grid-patching technique where the clones were patched onto both rich repressing plates and minimal inducing plates, we screened through 20,000

potential candidates. Minimal media which imposes more stringent growth demands on the cell was chosen for the screen in order to maximize the number of inhibitors that could be found. It is well known that growth in minimal media puts more demands on a bacterial cell than growth in rich media, as evidenced by the drastically reduced growth rate, and thus a peptide that adversely affects cell growth would be more likely detected on minimal media. We isolated 21 IPTG-dependent inhibitors in this screen and found that they could be categorized into two classes based on their inhibitory properties on plates. One day inhibitors either showed no growth or very little growth after 24 hours, but showed significant growth after 48 hours. Two day inhibitors were completely inhibitory and showed no growth for a full 48 hours. Figure 2.2 shows a representative plate that differentiates between a one day and two day inhibitor.

To verify that all of the inhibitors were legitimate, we isolated plasmid DNA from each inhibitory clone, transformed them into a fresh background, and then checked that they were still inhibitory on plates and that their inhibition was dependent on the presence of the inducer, IPTG. This was accomplished by patching the retransformed clones onto both minimal glucose ampicillin repressing plates and minimal glycerol ampicillin IPTG inducing plates. In order to make a more accurate assessment of how inhibitory the inhibitors were, we subjected the inhibitors to a growth rate analysis in liquid media. To do this, minimal cultures containing either the inhibitor to be tested or pLAC11 as a control were diluted 1 to 100 into minimal media and induced with 1 mM IPTG.

OD_{550} readings were then taken hourly until the cultures had passed log phase. Figure 2.3 shows a comparison of the inhibition caused by a one day or two day inhibitor using the pLAC11 vector as a control. Growth rates were determined as the spectrophotometric change in OD_{550} per unit time within the log phase of growth. The inhibition of the growth rate was then calculated for the inhibitors using pLAC11 as a control. As indicated in Table 2.1, the one day inhibitors inhibited the bacterial growth rate at an average of 25% while the two day inhibitors inhibited the bacterial growth rate at an average of 75%.

The hypothetical data in Table 2.2 shows how a 25% or 75% inhibition of the growth rate affects the growth of a culture. When the control strain reaches an OD_{550} of 0.640, a strain which contains a model peptide that inhibits the growth rate at exactly 50% will have only reached an OD_{550} of 0.080. Thus the growth of the culture that is being inhibited by this model peptide will only be 12.5% (0.080/0.640 x 100) of that of the control strain at this point and the inhibitor peptide would have effectively inhibited the growth of the culture by 87.5% (100% - 12.5%). A one day inhibitor which inhibited the growth rate at 25% would have only reached an OD_{550} of 0.226 when the control strain reached an OD_{550} of 0.640. Thus the growth of the culture that is being inhibited by a one day inhibitor will only be 35.3% (0.226/0.640 x 100) of that of the control strain at this point and the inhibitor peptide would have effectively inhibited the growth of the culture by 64.7% (100% - 35.3%). A two day inhibitor which

inhibited the growth rate at 75% would have only reached an OD_{550} of 0.028 when the control strain reached an OD_{550} of 0.640. Thus the growth of the culture that is being inhibited by a two day inhibitor will only be 4.4% (0.028/0.640 x 100) of that of the control strain at this point and the inhibitor peptide would have effectively inhibited the growth of the culture by 95.6% (100% - 4.4%). These calculations are consistent with the fact that two day inhibitors prevent the growth of bacteria on plates for a full 48 hours while the one day inhibitors only prevent the growth of bacteria on plates for 24 hours.

In order to continue our analysis, we next wanted to verify that all of the candidates contained 66 bp inserts as expected (see Figure 2.1). While most of them did, two of our most potent two day inhibitors, pPep3 and pPep14, were found to contain a huge deletion. Sequence analysis of these clones revealed that the deletion had caused the carboxyl-terminal end of the inhibitor peptides to become fused to the amino-terminal end of the short 63 amino acid Rop protein (data not shown). The *rop* gene which is part of the ColE1 replicon is located downstream from where the oligonucleotide library is inserted into the pLAC11 vector (Warren *et al.,* 2000).

Sequence analysis of the top 10 anchorless inhibitor peptides

The top 10 anchorless inhibitor peptides were sequenced and their coding sequences were determined (see Table 2.3). Eight out of the 10 inhibitors are predicted to encode peptides which are terminated before the double TAA TAA termination site which was incorporated into the oligonucleotide. Two of the

inhibitors, pPep6 and pPep10, which contain deletions within the randomized portion of the oligonucleotide, are terminated beyond the *Eco*R I site. One of the inhibitors, pPep17, contains a termination signal just after the ATG start codon. However, just downstream is a Shine Dalgarno site and a GTG codon which should be used as the start codon. Interestingly, the start sites of several proteins such as Rop are identical to that proposed for the pPep17 peptide (Cesareni, *et al.*, 1982). The average and median length for the 8 peptides whose termination signals occurred before or at the double TAA TAA termination site was 13 amino acids.

The characteristics of the predicted coding regions of the inhibitor peptides proved to be quite interesting. Three out of the 10 peptides, pPep1, pPep13, and pPep17, contained a proline residue as their last amino acid. Additionally, one of the peptides, pPep12, contained two proline residues at the n-2 and n-3 positions. Thus there appears to be a bias for the placement of proline residues at or near the end of several of the inhibitory peptides. Secondary structure analysis predicted that three out of the 10 peptides contained a known motif that could potentially form a very stable structure. pPep13, is predicted to be 72% α-helical, pPep10 is predicted to be 45% β-sheet, and pPep6 is predicted to contain a hydrophobic membrane spanning domain. According to the algorithms that are commonly used to predict the presence of these motifs in proteins, a randomly generated oligonucleotide such as the one used in our studies would have had no better than a one in a 1000 chance of

generating the motifs that occurred in these peptides (Chou, 1990; Chou and Fasman, 1978; Garnier *et al.,* 1978; Gibrat, *et al.,* 1987; Hirokawa, *et al.,* 1998; Hoffman and Stoffel, 1993; Jahnig, 1990; Klein, *et al.,* 1985;Kyte and Doolittle, 1982; O'Neill and DeGrado, 1990).

Verifying that the inhibitory clones do not function as antisense

To verify that the inhibitory clones which we isolated functioned as expressed peptides and not as antisense RNA or DNA, the insert regions between the *Bgl* II and *Eco*R I sites for five of the inhibitors were recloned into the pLAC11 vector using oligonucleotides which converted the ATG start codon to an ACG codon thus abolishing the start site. In all five cases the new constructs were no longer inhibitory (see Table 2.4), thus confirming that it is the encoded peptides that causes the inhibition and not the DNA or transcribed mRNA.

Discussion

We have developed an *in vivo* approach by which novel synthetic bioactive peptides can be isolated that inhibit the growth of *E. coli*. In our initial screening of 20,000 potential inhibitory clones, 21 inhibitors were isolated which could be grouped into two classes; 18 one day inhibitors which were partially inhibitory and prevented the growth of bacteria on minimal plates for 24 hours, and 3 two day inhibitors which were completely inhibitory and prevented the growth of bacteria on minimal plates for 48 hours. The one day inhibitors were found to inhibit the bacterial growth rate at an average of 25%, and thus inhibited the overall growth of the bacteria by 65%. The two day inhibitors were

found to inhibit the bacterial growth rate at an average of 75% and thus inhibited the overall growth of the bacteria by 96%.

Two of the most potent two day inhibitors proved to be fusion peptides in which the carboxyl terminus of the peptides was fused to the amino terminus of the Rop protein. Given the fact that the Rop protein is known to form an incredibly stable structure (Eberle, *et al.,* 1990), Rop could be serving as a stable protein anchor for these two peptides. The characteristics of the predicted coding regions of the top 10 anchorless inhibitor peptides proved to be quite interesting as well. Three out of the 10 peptides (30%) contained a proline residue as their last amino acid. According to the genetic code, a randomly generated oligonucleotide such as the one used in our studies would only have a 6% chance of placing proline at any one position. This five-fold bias is striking when one considers the fact that proline has been argued to prevent the ability of peptidases to degrade peptides (Vanhoof, *et al.,* 1995; Yaron and Naider, 1993). Additionally, one of the peptides contained a proline residue at the n-2 and n-3 positions.

It has become increasingly clear that most naturally occurring bioactive peptides whose structures have been determined contain ordered structures which should help to stabilize them. For example, dermaseptin (Mor, *et al.,* 1994), endorphin (Blanc, *et al.,* 1983), glucagon (Bedarkar, *et al.,* 1977), magainins (Bechinger, *et al.,* 1993), mastoparan (Cachia, *et al.,* 1986), melittin (Terwilliger and Eisenberg, 1982), motilin (Khan, *et al.,* 1990), PKI 5-24 (Reed, *et al.,* 1987), and

secretin (Gronenborn, *et al.,* 1987) form α-helixes, while atrial natriuretic peptide (Misono, *et al.,* 1984), calcitonin (Barling, *et al.,* 1985), conotoxins (Olivera, *et al.,* 1991), defensins (Lehrer, *et al.,* 1988), EETI II (Heitz, *et al.,* 1989), oxytocin (Urry, *et al.,* 1968), somatostatin (Namboodiri, *et al.,* 1982), and vasopressin (Fong, *et al.,* 1964) contain disulfide bonds. Collectively, both our data from synthetic bioactive peptides that can inhibit the growth of *E. coli* and data from naturally occurring bioactive peptides strongly suggest that peptide stability is of paramount concern and that the presence of structural motifs are necessary to stabilize bioactive peptides if they are to be functional. Research on developing novel synthetic inhibitor peptides for use as potential therapeutic agents over the last few years has shown that peptide stability is a major problem that must be solved if designer synthetic peptides are to become a mainstay in the pharmaceutical industry (Bai, *et al.,* 1995; Egleton and Davis, 1997; Wearley, 1991). Based on what has been learned in this study, it is clear that methods need to be developed which can be used to make synthetically produced bioactive peptides more stable. Our laboratory is currently investigating whether specific motifs can be incorporated into synthetic inhibitor peptides in order to make them more stable and recent experiments have demonstrated that the deliberate incorporation of the Rop or proline motifs into synthetically engineered bioactive peptides dramatically increases the frequency at which potent two day inhibitors can be obtained (J.R. Walker, J.W. Warren, and E. Altman, personnel communication).

References

Bai, J. P. F., Chang, L.-L., and Guo, J.-H. (1995). Targeting of peptides and protein drugs to specific sites in the oral route. Crit Rev Ther Drug Carrier Syst 12, 339-371.

Balbás, P., and Bolivar, F. (1990). Design and construction of expression plasmid vectors in *Escherichia coli*. Methods Enzymol 185, 14-37.

Barling, P. M., Preston, J. R., Bibby, N. J., and Wilson, T. (1985). Indirect [3H]methyl exchange as a general method for labeling methionine residues: application to calcitonin. Anal Biochem 144, 542-552.

Bechinger, B., Zasloff, M., and Opella, S.J. (1993). Structure and orientation of the antibiotic peptide magainin in membranes by solid-state nuclear magnetic resonance spectroscopy. Protein Sci 2, 2077-2084.

Bedarkar, S., Blundell, T. L., Dockerill, S., Tickle, I. J., and Wood, S. P. (1977). Polypeptide hormone-receptor interactions: the structure and receptor binding of insulin and glucagon. Ciba Found Symp 60, 105-121.

Blanc, J. P., Taylor, J. W., Miller, R. J., and Kaiser, E. T. (1983). Examination of the requirement for an amphiphilic helical structure in beta-endorphin through the design, synthesis, and study of model peptides. J Biol Chem 258, 8277-8284.

Bozou, J. C.., Amar, S., Vincent, J. P., and Kitabgi, P. (1986). Neurotensin-mediated inhibition of cyclic AMP formation in neuroblastoma N1E115 cells: involvement of the inhibitory GTP-binding component of adenylate cyclase. Mol Pharmacol 29, 489-496.

Brosius, J. (1988). Expression vectors employing lambda-, trp-, lac-, and lpp-derived promoters. Biotechnology 10, 205-225.

Cachia, P. J., Van-Eyk, J., Ingraham, R.H., McCubbin, W. D., Kay, C. .M., and Hodges, R. S. (1986). Calmodulin and troponin C: a comparative study of the interaction of mastoparan and troponin I inhibitory peptide [104-115]. Biochemistry 25, 3553-3562.

Casadaban, M., and Cohen, S. N. (1980). Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J Mol Biol 138, 179-207.

Cesareni, G., Muesing, M. A., and Polisky, B. (1982). Control of ColE1 DNA replication: The rop gene product negatively affects transcription from the replication primer promoter. Proc Natl Acad Sci USA 79, 6313-6317.

Chou, P. Y. (1990). Prediction of protein structural classes from amino acid composition. In Prediction of Protein Structure and the Principles of Protein Conformation. Ed., Fasman, G.D. (New York, N.Y.: Plenum Press), pp. 549-586.

Chou, P. Y., and Fasman, G. D. (1978). Prediction of the secondary structure of proteins from their amino acid sequence. Adv Enzymol Relat Area Mol Biol 47, 45-148.

Eberle, W., Klaus, W., Cesareni, G., Sander, C., and Rosch, P. (1990). Proton nuclear magnetic resonance assignments and secondary structure determination of the ColE1 Rop protein. Biochemistry 29, 7402-7407.

Egleton, R. D., and Davis, T. P. (1997). Bioavailability and transport of peptides and peptide drugs into the brain. Peptides 18, 1431-1439.

Eichler, J., Appel, J. R., Blondelle, S. E., Dooley, C.. T., Dorner, B., Ostresh, J. M., Perez-Paya, E., Pinilla, C., and Houghten, R. A. (1995). Peptide, peptidomimetic, and organic synthetic combinatorial libraries. Med Res Rev 15, 481-496.

Fong, C.. T., Silver, L., and Louie, D. D. (1964). Necessity of the disulfide bond of vasopressin for antidiuretic activity. Biochem Biophys Res Comm 14, 302-306.

Garnier, J., Osguthorpe, D.J., and Robson, B. (1978). Analysis of the accuracy and implications of simple method for predicting the secondary structure of globular proteins. J Mol Biol 120, 97-120.

Gibrat, J.-F., Garnier, J., and Robson, B. (1987). Further developments of protein secondary structure prediction using information theory. New parameters and consideration of residue pairs. Mol Biol 198, 425-443.

Gronenborn, A. M., Boverman, G., and Clore, G. M. (1987). A 1H-NMR study of the solution conformation of secretin. Resonance assignment and secondary structure. FEBS Lett 215, 88-94.

Grosjean, H. and Fiers, W. (1982). Preferential codon usage in prokaryotic genes: the optimal codon-anticodon interaction energy and the selective codon usage in efficiently expressed genes. Gene 18, 199-209.

Heitz, A., Chiche, L., Le-Nguyen, D., and Castro, B. (1989). 1H 2D NMR and distance geometry study of the folding of Ecballium elaterium trypsin inhibitor, a member of the squash inhibitors family. Biochemistry 28, 2392-2398.

Hirokawa, T., Boon-Chieng, S., and Mitaku, S. (1998). SOSUI: Classification and secondary structure prediction system for membrane proteins. Bioinformatics 14, 378-379.

Hoffman, K., and Stoffel, W. (1993). TMbase - A database of membrane spanning proteins segments. Biol Chem Hoppe-Seyler 347, 166.

Ikemura, T. (1981). Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes: a proposal for a synonymous codon choice that is optimal for the *E. coli* translational system. J Mol Biol 151, 389-409.

Jahnig, F. (1990). Structure predictions of membrane proteins are not that bad. Trends Biochem Sci 15, 93-95.

Khan, N., Graslund, A., Ehrenberg, A., and Shriver, J. (1990). Sequence-specific 1H NMR assignments and secondary structure of porcine motilin. Biochemistry 29, 5743-5751.

Klein, P., Kanehisa, M., and DeLisi, C. (1985). The detection and classification of membrane-spanning proteins. Biochim et Biophys Acta 815, 468-476.

Kyte, J., and Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. J Mol Biol 157, 105-132.

Lam, K. S. (1996). Application of combinatorial library methods in cancer research and drug discovery. Anticancer Drug Des 12, 145-167.

Lebl, M., and Krchnak, V. (1997) Synthetic peptide libraries. Methods Enzymol 289, 336-392.

Lehrer, R.I., Ganz, T., Selsted, M. E., Babior, B. M., and Curnutte, J. T. (1988). Neutrophils and Host Defense. Ann Intern Med 109, 127-142.

Le-Nguyen, D., Heitz, A., Chiche, L., Castro, B., Boigegrain, R. A., Favel, A., and Coletti-Previero, M. A. (1990). Molecular recognition between serine proteases and new bioactive microproteins with a knotted structure. Biochimie 72, 431-435.

Lowman, H. B. (1997). Bacteriophage display and discovery of peptide leads for drug development. Annu Rev Biophys Biomol Struct 26, 401-424.

Miller, J. H. (1972). Experiments in molecular genetics. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.)

Misono, K. S., Fukumi, H., Grammer, R. T., and Inagami, T. (1984). Rat atrial natriuretic factor: complete amino acid sequence and disulfide linkage essential for biological activity. Biochem Biophys Res Comm 119, 524-529.

Mor, A., Amiche, M., and Nicolas, P. (1994). Structure, synthesis, and activity of dermaseptin b, a novel vertebrate defensive peptide from frog skin: relationship with adenoregulin. Biochemistry 33, 6642-6650.

Namboodiri, M. A, Favilla, J. T., and Klein, D. C. (1982). Activation of pineal acetyl coenzyme A hydrolase by disulfide peptides. J Biol Chem 257, 10030-10032.

Olivera, B. M., Rivier, J., Scott, J. K., Hillyard, D. R., and Cruz, L. J. (1991). Conotoxins. J Biol Chem 266, 22067-22070.

O'Neill, K. T., and DeGrado, W. F. (1990). A thermodynamic scale for the helix-forming tendencies of the commonly occurring amino acids. Science 250, 646-651.

Reed, J., Kinzel,V., Cheng, H. C., and Walsh, D. A. (1987). Circular dichroic investigations of secondary structure in synthetic peptide inhibitors of cAMP-dependent protein kinase: a model for inhibitory potential. Biochemistry 26, 7641-7647.

Rivier, J. E. and Marshall, G. R., Eds. (1990). Peptides: Chemistry, Structure, and Biology. (The Netherlands: ESCOM Science Publishers BV).

Siddle, K., and Hutton, J. C., Eds. (1990). Peptide Hormone Action. (Oxford, England: IRL Press at Oxford University Press).

Smith, G. P., and Scott, J. K. (1993). Libraries of peptides and proteins displayed on filamentous phage. Methods Enzymol 217, 228-257.

Smith, J. A., and Rivier, J. E., Eds. (1992). Peptides: Chemistry and Biology. (The Netherlands: ESCOM Science Publishers BV).

Terwilliger, T. C., and Eisenberg, D. (1982). The structure of melittin. I: structure determination and partial refinement. J Biol Chem 257, 6010-6015.

Urry, D. W., Quadrifoglio, F., Walter, R., and Schwartz, I. L. (1968). Conformational studies on neurohypophyseal hormones: the disulfide bridge of oxytocin. Proc Natl Acad Sci USA 60, 967-974.

Vanhoof, G., Goossens, F., De Meester, I, Hendriks, D., and Scharpe, S. (1995). Proline motifs in peptides and their biological processing. FASEB J 9, 736-44.

Warren, J. W., Walker, J. R., Roth, J. R., and Altman, E. (2000). Construction and characterization of a highly regulable expression vector, pLAC11, and its multipurpose derivatives, pLAC22 and pLAC33. Plasmid 44, 138-151.

Wearley L. L. (1991). Recent progress in protein and peptide delivery by noninvasive routes. *Crit Rev Ther Drug Carrier Syst* 8, 331-394.

Wieland, T., and Bodanszky, M. (1991.*)* The World of Peptides: a brief history of peptide chemistry. (Berlin: Springer-Verlag).

Williams, R. W., and Weaver, J. L. (1990). Secondary structure of substance P bound to liposomes in organic solvents and in solution from Raman and CD spectroscopy. J Biol Chem 265, 2505-2513.

Xu, T., Telser, E., Troxler, R. F., and Oppenheim, F. G. (1990). Primary structure and anticandidal activity of the major histatin from parotid secretion of the subhuman primate, *Macaca fascicularis*. J Dent Res 69, 1717-1723.

Yaron, A., and Naider, F. (1993). Proline-dependent structural and biological properties of peptides and proteins. Crit Rev Biochem Mol Biol 28, 31-81.

Randomized Oliognucleotide (total length = 93 bases)

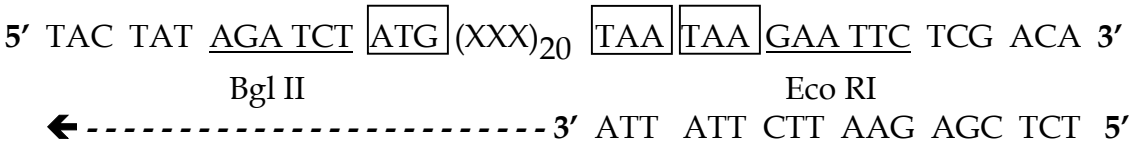


Figure 2.1 Scheme for generating the randomized 20 amino acid peptide library *in vivo*. The complementary strand of the 93 base randomized oligonucleotide is generated by filling-in with Klenow using the 18 base oligonucleotide primer. The resulting ds-DNA is digested with *Bgl* II and *Eco*R I, and then ligated into the pLAC11 expression vector which has also been digested with the same two restriction enzymes. Wherever an "X" occurs in the oligonucleotide, a random mixture of all four of the nucleotides, A, C, G, and T, is used.

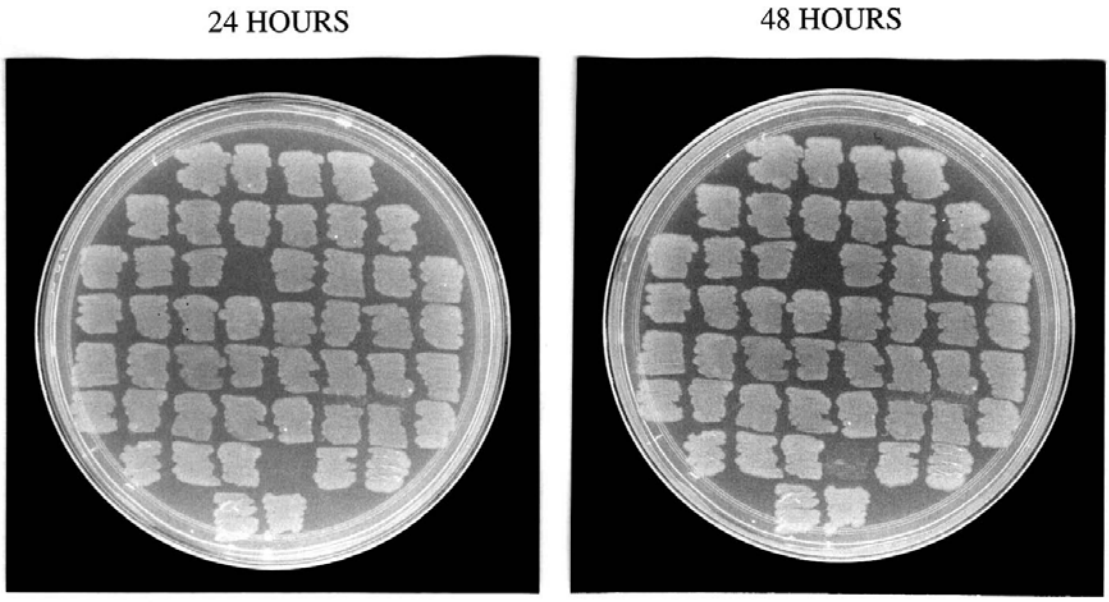


Figure 2.2 A minimal inducing plate showing the difference between a 1- and 2-day inhibitor. Shown here is a minimal inducing plate that contains both a 1-day and a 2-day inhibitor, photographed at 24 and 48 hours. The 2-day inhibitor on row three from the top is completely inhibitory and does not grow at either 24 or 48 h. The 1-day inhibitor on row seven from the top of the plate does not grow at 24 h but does grow at 48 h.

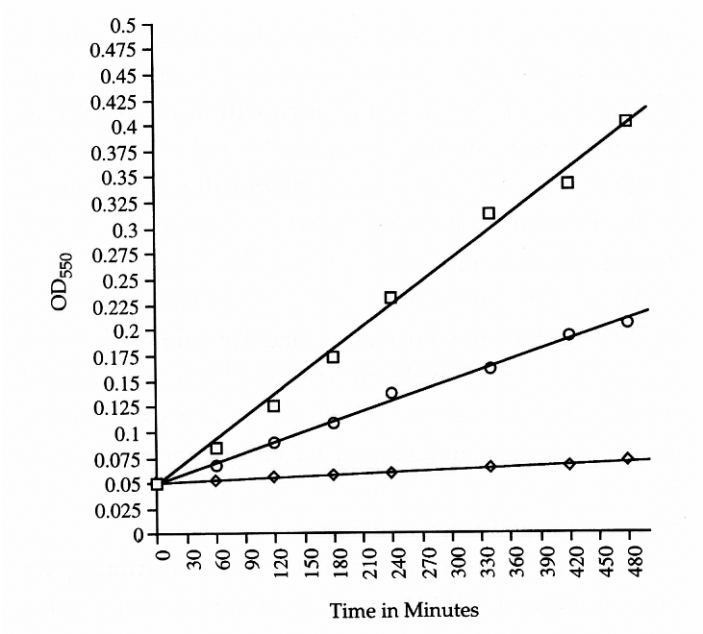


Figure 2.3 Growth curves showing the inhibitory effects of a 1-day versus 2-day inhibitor. ALS225 cells containing the pLAC11 vector (control), and the 1-day inhibitor pPep11, or the 2-day inhibitor pPep12, were grown in minimal M9 glycerol media with IPTG added to 1 mM. At ≈ 1-h intervals A_{550} readings were taken. Data points for pLAC11, pPep11, and pPep12, are indicated by squares, circles, and diamonds, respectively.

Table 2.1 Ability of the inhibitor peptides to inhibit cell growth

Inhibitor	Type	% inhibition of the growth rate	Inhibitor	Type	% inhibition of the growth rate
pLAC11 (control)	---	0	pPep11	1-day	22
pPep1	1-day	25	pPep12	2-day	82
pPep2	1-day	23	pPep13	1-day	28
pPep3	2-day	80	pPep14	2-day	71
pPep4	1-day	21	pPep15	1-day	23
pPep5	1-day	24	pPep16	1-day	24
pPep6	1-day	27	pPep17	1-day	28
pPep7	1-day	26	pPep18	1-day	24
pPep8	1-day	29	pPep19	1-day	29
pPep9	1-day	22	pPep20	1-day	19
pPep10	1-day	24	pPep21	1-day	23

Growth rates for cells containing the induced inhibitors were determined as described in the text and then the percentage inhibition was calculated by comparing these values to the growth rate of cells that contained the induced pLAC11 vector. The averaged values of three independent determinations are shown.

Table 2.2 Hypothetical data from peptides that inhibit the growth rate at 25%, 50%, or 75%.

Time in hours	A_{550} readings on a control culture that contains pLAC11	A_{550} readings on a culture that contains a peptide that inhibits the growth rate at		
		25%	50%	75%
0	0.010	0.010	0.010	0.010
2.5	0.020	0.017	0.015	0.012
5	0.040	0.028	0.020	0.014
7.5	0.080	0.047	0.030	0.017
10	0.160	0.079	0.040	0.020
12.5	0.320	0.133	0.060	0.024
15	0.640	0.226	0.080	0.028

Table 2.3 Sequence analysis of the insert region from the top 10 anchorless inhibitory clones and the peptides that they are predicted to encode

pPep1 - 13 aa

```
CAG GAA AGA TCT ATG GTC ACT GAA TTT TGT GGC TTG TTG GAC CAA CTG CCT TAG TAA TAG TGG AAG GCT GAA ATT
                 M   V   T   E   F   C   G   L   L   D   Q   L   P   *   *   *
AAT AAG AAT TC
```

pPep5 - 16 aa

```
CAG GAA AGA TCT ATG TGG CGG GAC TCA TGG ATT AAG GGT AGG GAC GTG GGG TTT ATG GGT TAA AAT AGT TTG ATA
                 M   W   R   D   S   W   I   K   G   R   D   V   G   F   M   G   *
ATA AGA
```

pPep6 - 42 aa - last 25 aa could form a hydrophobic membrane-spanning domain

```
CAG GAA AGA TCT ATG TCA GGG GGA CAT GTG ACG AGG GAG TGC AAG TCG GCG ATG TCC AAT CGT TGG ATC TAC GTA
                 M   S   G   G   H   V   T   R   E   C   K   S   A   M   S   N   R   W   I   Y   V
ATA AGA ATT CTC ATG TTT GAC AGC TTA TCA TCG ATA AGC TTT AAT GCG GTA GTT TAT CAC AGT TAA
 I   R   I   L   M   F   D   S   L   S   S   I   S   F   N   A   V   V   Y   H   S   *
```

pPep7 - 6 aa

```
CAG GAA AGA TCT ATG TAT TTG TTC ATC GGA TAA TAC TTA ATG GTC CGC TGG AGA ACT TCA GTT TAA TAA GAA TTC
                 M   Y   L   F   I   G   *
```

pPep8 - 21 aa

```
CAG GAA AGA TCT ATG CTT CTA TTT GGG GGG GAC TGC GGG CAG AAA GCC GGA TAC TTT ACT GTG CTA CCG TCA AGG
                 M   L   L   F   G   G   D   C   G   Q   K   A   G   Y   F   T   V   L   P   S   R
TAA TAA GAA TTC
*   *
```

pPep10 - 20 aa - predicted to be 45% β-sheet - amino acids 6-14

```
CAG GAA AGA TCT ATG ATT GGG GGA TCG TTG AGC TTC GCC TGG GCA ATA GTT TGT AAT AAG AAT TCT CAT GTT TGA
                 M   I   G   G   S   L   S   F   A   W   A   I   V   C   N   K   N   S   H   V   *
```

pPep12 - 14 aa

```
CAG GAA AGA TCT ATG AAC GGC CGA ACC AAA CGA ATC CGG GAC CCA CCA GCC GCC TAA ACA GCT ACC AGC TGT GGT
                 M   N   G   R   T   K   R   I   R   D   P   P   A   A   *
AAT AAG AAT TC
```

pPep13 -18 aa - predicted to be 72% α-helical - amino acids 3-15

```
CAG GAA AGA TCT ATG GAC CGT GAA GTG ATG TGT GCG GCA AAA CAG GAA TGG AAG GAA CGA ACG CCA TAG GCC GCG
                 M   D   R   E   V   M   C   A   A   K   Q   E   W   K   E   R   T   P   *
TAA TAA GAA TTC
```

pPep17 - 12 aa

```
CAG GAA AGA TCT ATG TAG CCC AAT GCA CTG GGA GCA CGC GTG TTA GGT CTA GAA GCC ACG TAC CCA TTT AAT CCA
                 M   *                               M*  L   G   L   E   A   T   Y   P   F   N   P
TAA TAA GAA TTC
 *   *
```

pPep19 - 5 aa

```
CAG GAA AGA TCT ATG AGG GGC GCC AAC TAA GGG GGG GGG AAG GTA TTT GTC CCG TGC ATA ATC TCG GGT GTT GTC
M   R   G   A   N   *
TAA TAA GAA TTC
```

The landmark *Bgl* II and *Eco*R I restriction sites for the insert region are underlined. Because the ends of the oligonucleotide from which these inhibitors were constructed contained these restriction sites, the oligonucleotide was not gel isolated when the libraries were prepared in order to maximize our oligonucleotide yields. Because of this, several of the inhibitory clones were found to contain one (n-1) or two (n-2) base deletions in the randomized portion of the oligonucleotide.

Table 2.4 Antisense test of five of the anchorless inhibitory peptides

Inhibitory Peptide	% inhibition of the growth rate	Antisense Construct	% inhibition of the growth rate
pPep1	26	pPep1-anti	0
pPep5	23	pPep5-anti	0
pPep12	80	pPep12-anti	0
pPep13	28	pPep13-anti	0
pPep19	29	pPep19-anti	0

Growth rates for cells containing the induced inhibitors or antisense constructs were determined and then the % inhibition was calculated by comparing these values to the growth rate of cells that contained the induced pLAC11 vector.

CHAPTER 3

STABILIZATION OF PEPTIDES USING PROTEIN-BASED MOTIFS[1]

[1] Walker, J. R., R. K. Altman, J. W. Warren, and E. Altman. Submitted to *Cell* 10/15/02.

Abstract

Methods by which peptides can be stabilized using protein-based motifs in order to inhibit peptidases have been investigated. Using an *in vivo* approach developed to screen for synthetic peptides which can inhibit the growth of *Escherichia coli*, the protecting of amino or carboxyl peptide terminus via fusion to the very stable Rop protein or the incorporation of two proline residues was found to increase the frequency at which potent inhibitor peptides can be isolated. Using an *in vitro* degradation assay in which extracts from several different cell types were tested, peptides stabilized with multiple proline residues were demonstrated to be more resistant to degradation than peptides stabilized by amidation or acetylation, two approaches that are routinely utilized to improve the stability of peptide drugs.

Introduction

Over the past several years there has been an increasing interest in using novel synthetic peptides as a means to generate new therapeutic agents for the pharmaceutical industry (Eichler, *et al.*, 1995; Lam, 1997; Lowman, 1997). The premise behind this new area of research stems from what has been learned from studying naturally occurring peptides that possess biological activities. These bioactive peptides for the large part appear to act by binding to a specific protein target (Siddle and Hutton, 1990) and in many cases it has been shown that the peptides inactivate the protein target with incredible specificity. Binding constants for the naturally occurring bioactive peptides that have been studied

tend to be in the nM range (Rivier and Marshall, 1990; Smith and Rivier, 1992) with binding constants as high as 10^{-12}M having been reported (Bozou, *et al.*, 1986; Le-Nguyen, *et al.*, 1990).

Two major strategies have been employed by researchers trying to engineer novel synthetic peptides. The first involves the use of chemically synthesized combinatorial peptide libraries of up to 10 amino acids in length (Eichler, *et al.*, 1995; Lam, 1997; Lebl and Krchnak, 1997). The second strategy involves the use of fusion phage libraries where randomly encoded peptides are fused to a coat protein of a Ff phage which allows the randomized peptide to be displayed on the outside surface of the phage (Lowman, 1997; Smith and Scott, 1993). The libraries that are generated via either chemical synthesis or as fusion phage are then usually mixed with a matrix bound protein target and peptides that can bind tightly to the protein target are selected. New peptides are generated; either based on individual peptide sequence data or collective consensus data from multiple peptides, and tested for their inhibitory potential. Peptides are initially tested *in vitro* by measuring their ability to inhibit the enzymatic activity of the protein target and then promising peptides are tested further in clinical trials.

Although there is enormous potential for the development of synthetic inhibitor peptides using the approaches described above, this technology has not become a mainstay in the pharmaceutical industry due to the problem of peptide stability and the unwanted degradation of the potential peptide drug by

peptidases in the host cells (Lauta, 2000). Approaches to solve this problem have included acetylating the amino terminus of the peptide, amidating the carboxyl terminus of the peptide, the addition of D-amino acids or modified amino acids as opposed to the naturally occurring L-amino acids, cyclized peptides, as well as the development of enhanced delivery systems which would protect the peptides from premature degradation (Hruby and Balse, 2000; Pinilla, *et al.*, 1995; Sanders, 1990). Modifying peptides through the use of protein-based stabilizing motifs that could circumvent the problem of peptide degradation was investigated in this study.

There are three major classes of peptidases which can degrade larger peptides, the amino and carboxy exopeptidases, which act at either the amino or the carboxyl terminal end of the peptide, respectively, and the endopeptidases which act on the internal peptide. Aminopeptidases, carboxypeptidases, and endopeptidaess have been identified in both prokaryotic and eukaryotic cells and where they have been extensively characterized, most of these peptidases have been found to function similarly (Rawlings and Barrett, 1993). Interestingly, when the abundance of exopeptidases has been examined in either prokaryotic or eukaryotic systems, more aminopeptidases have been identified than carboxypeptidases (Bai and Amidon, 1992; Bai, *et al.*, 1995, Brownlees and Williams, 1993; Miller, 1996; Ryan, 1989).

An *in vivo* approach was developed by which novel bioactive peptides can be identified as inhibiting the growth of *Escherichia coli* (Walker, *et al.*, 2001). In

the initial study, two potential protein-based stabilizing motifs were discovered. The first motif involved the use of a small stable protein anchor while the second motif involved the use of proline residues which have been shown to be more resistant to degradation by peptidases (Vanhoof, *et al.*, 1995). In this study, an *in vivo* system was used to investigate whether these protein-based stabilizing motifs can more effectively create inhibitor peptides. Additionally, an *in vitro* assay was developed to further characterize the stabilizing motifs. Because the actions of peptidases appear to be quite similar in both prokaryotic and eukaryotic cells, what was learned in these studies can be directly applicable to designing more effective inhibitor peptides for use as novel drugs.

Material and Methods

Bacterial strains and plasmids

The bacterial strains used in this study are listed in Table 3.1. MG1655 *clpP*::cam was constructed by transducing MG1655 to chloramphenicol resistance using a P1 lysate that was prepared from SG22098. The highly regulable pLAC11 expression vector was used to make the p-Rop(C) and p(N)Rop- fusion vectors as well as the randomized peptide libraries, which were protected by two proline residues at both the amino and carboxyl termini (Warren, *et al.*, 2000).

Media for the in vivo studies

Rich LB and minimal M9 media were prepared as described by Miller, 1972. Ampicillin was used in rich medium at a final concentration of 100 ug/ml

and in minimal medium at a final concentration of 50 ug/ml. Isopropyl β-D-thiogalactoside (IPTG) was added to media at a final concentration of 1 mM.

Construction of the p-Rop(C) fusion vector

The forward primer 5' TAC TAT AGA TCT ATG ACC AAA CAG GAA AAA ACC GCC 3' and the reverse primer 5' TAT ACG TAT TCA GTT GCT CAC ATG TTC TTT CCT GCG 3' were used to amplify a 558 bp DNA fragment using pBR322 as a PCR template. This fragment contained a *Bgl* II restriction site incorporated into the forward primer, followed by an ATG start codon and the Rop coding region. The fragment extended beyond the Rop stop codon and through the *Afl* III restriction site of pBR322. The amplified ds-DNA was gel isolated, restricted with *Bgl* II and *Afl* III, and ligated into the pLAC11 expression vector digested with the same two restriction enzymes. The resulting p-Rop(C) fusion vector was 2623 bp in size.

Construction of the p(N)Rop- fusion vector

The forward primer 5' AAT TCA TAC TAT AGA TCT ATG ACC AAA CAG GAA AAA ACC GC 3' and the reverse primer 5' TAT ATA ATA CAT GTC AGA ATT CGA GGT TTT CAC CGT CAT CAC 3' were used to amplify a 201 bp DNA fragment using pBR322 as a PCR template. This fragment contained a *Bgl* II restriction site incorporated into the forward primer followed by an ATG start codon and the Rop coding region. The reverse primer placed an *Eco*R I restriction site just before the Rop TGA stop codon and an *Afl* III restriction site immediately after the Rop TGA stop codon. The amplified ds-DNA was gel

isolated, restricted with *Bgl* II and *Afl* III, and then ligated into the pLAC11 expression vector digested with the same two restriction enzymes. The resulting p(N)Rop- fusion vector was 2262 bp in size.

Generating the randomized peptide libraries

All of the peptide libraries used in this study were constructed as described in Walker, *et al.*, 2001. To prepare the randomized peptide libraries for use with the p-Rop(C) fusion vector, the oligonucleotides 5' TAC TAT AGA TCT ATG $(XXX)_{20}$ CAT AGA TCT GCG TGC TGT GAT 3' and 5' ATC ACA GCA CGC AGA TCT ATG 3' were used. After extension, the resulting ds-DNA was digested with *Bgl* II and ligated into the p-Rop(C) fusion vector digested with the same restriction enzyme and subsequently dephosphorylated using alkaline phosphatase. To prepare the randomized peptide libraries for use with the p(N)Rop- fusion vector, the oligonucleotides 5' TAC TAT GAA TTC $(XXX)_{20}$ GAA TTC TGC CAC CAC TAC TAT 3' and 5' ATA GTA GTG GTG GCA GAA TTC 3' were used. After extension the resulting ds-DNA was digested with *Eco*R I and ligated into the p(N)Rop- fusion vector digested with the same restriction enzyme and subsequently dephosphorylated using alkaline phosphatase. To construct randomized 20 amino acid peptide libraries containing two proline residues at the amino and carboxyl terminal ends of the peptides, the oligonucleotides 5' TAC TAT AGA TCT ATG CCG CCG $(XXX)_{16}$ CCG CCG TAA TAA GAA TTC GTA CAT 3' and 5' ATG TAC GAA TTC TTA TTA CGG CGG 3' were used. After extension the resulting ds-DNA was digested with *Bgl*

II and *Eco*R I and ligated into the pLAC11 expression vector digested with the same two restriction enzymes. In the randomized oligonucleotides an X denotes that an equimolar mixture of the nucleotides A, C, G, or T was used.

Preparation of the cell extracts for the in vitro degradation assay

Bacterial cells were grown in rich LB media (Miller, 1972), which yeast cells were grown in 1.0% yeast extract, 2.0% peptone, 2.0% glucose. Human colon CCD-18Co cells (ATCC CRL-1459) were grown in Minimal Essential Medium Eagle (ATCC 30-2003) with Earle's balanced salt solution, 0.1 mM non-essential amino acids, 2.0 mM L-glutamine, 1.0 mM sodium pyruvate, 1.5 g/L $NaHCO_3$, and 10% fetal bovine serum. Human small intestine FHs74 Int cells (ATCC CCL-241) were grown in Hybri-Care media (ATCC 46-X) with 1.5 g/L $NaHCO_3$ and 10% fetal bovine serum. For bacteria and yeast, 500 mL of cells were grown to an OD_{550} of 0.5, centrifuged, washed twice with $T_{10}E_{0.1}$ (10.0 mM Tris; pH 8.0, 0.1 mM EDTA; pH 8.0) and resuspended in 2.0 mL of 10.0 mM Tris; pH 8.0. For human cells, 10-50-75 cm^2 T flasks were seeded and allowed to grow to 95% confluency in a 37^0C incubator with 5% CO_2 atmosphere. Each flask was then washed with HBSS (0.4 g/L KCl, 0.06 g/L KH_2PO_4, 8 g/L NaCl, 0.35 g/L $NaHCO_3$, 0.048 g/L Na_2HPO_4, 1.0 g/L glucose) containing 0.125 mM EDTA; pH 8.0. To liberate the cells, the flasks were treated with 1.5 mL of HBSS containing 0.25% trypsin and 0.5 mM EDTA; pH 8.0. The trypsin was neutralized by adding 5 mL of media containing 10% fetal bovine serum to each flask. The cells were centrifuged, washed with HBSS containing 0.125 mM EDTA; pH 8.0, washed

twice with HBSS lacking glucose and ETDA, and resuspended in 2.0 mL of 10.0 mM Tris; pH 8.0. All cell suspensions were lysed using three passes at 15,000 psi through a French Pressure cell maintained at 4^0C. The lysates were then centrifuged at 15,000 rpm, 4^0C, for 10 minutes to pellet debris and unlysed cells and the supernatant was saved as cell extract. To prepare rat serum, one 300g Sprague-Dawley rat was euthanized with CO_2 and a heart puncture was performed to draw the blood which was immediately transferred to a tube and centrifuged at 4^0C, 10,000 rpm, for 10 minutes. The cleared serum was removed using a pipet.

Peptide synthesis

The randomized biotinylated peptides XXXXXX[KBtn]XXXXXA (unprotected), PXXXX[KBtn]XXXXP (P at both ends), PPXXXX[KBtn]XXXXPP (PP at both ends), APPXXXX[KBtn]XXXXPPA (APP at both ends), APPXXXX[K-Btn]XXXXA (APP amino), AXXXX[K-Btn]XXXXPPA (APP carboxyl), (Ac)AXXXXX[KBtn]XXXXXA (acetylated), XXXXXX[KBtn]XXXXXA(NH_2) (amidated), and CXXXXXX[KBtn]XXXXXXC (cyclized) were synthesized by Sigma Genosys, The Woodlands, Texas, United States, where A denotes the L-amino acid alanine, P denotes the L-amino acid proline, X denotes an equimolar mixture of the 20 natural L-amino acids except for proline, and KBtn denotes the L-amino acid lysine to which biotin has been attached. To ensure that the length of the randomized portion of the peptides did not affect the degradation profiles, the unprotected peptides XXXX[KBtn]XXXXA and AXXXX[KBtn]XXXXA was

also tested. Their half-lives were determined to be within 5% of the XXXXXX[KBtn]XXXXXA peptide used as the control for these studies.

In vitro degradation assay

All cell extracts were used at a final concentration of 10 mg/mL, except for the *S. typhimurium* extracts, which were used at a final concentration of 25 mg/mL. 50 uL of cell extract mixed with 50 uL of peptide at a concentration of 1 mg/mL in 10 mM Tris; pH 8.0 was incubated at 37^0C. 10 uL aliquots were removed at 15, 30, 60, 90, 120, or 240 minute intervals, placed in 90 uL of SDS-PAGE gradient gel buffer, boiled for 5 minutes, and run on a 10–16% tricine gradient gel (Schagger and Von Jagow, 1987). The gel was blotted onto a nitrocellulose membrane and the resulting Western blot was treated with NeutrAvidin Horseradish Peroxidase Conjugate and SuperSignal West Dura Extended Duration Chemiluminescent Substrate from Pierce, Rockford, Illinois, United States. The biotinylated peptides were then visualized by exposing the blots to autoradiography film and the resulting bands were quantified using the AlphaEase 5.5 Densitometry Program from Alpha Innotech, SanLeandro, California, United States. The half-lives that are reported in Tables 5.8 are the average of three independent experiments where the average deviation was less than 10%.

Chemicals and reagents

Extension reactions were carried out using Klenow from New England Biolabs, Beverly, Massachusetts, United States, while ligation reactions were

performed using T4 DNA ligase also from New England Biolabs. Alkaline phosphatase (calf intestinal mucosa) from Pharmacia, Piscataway, New Jersey, United States, was used for dephosphorylation. IPTG was obtained from Diagnostic Chemicals Limited, Oxford, Connecticut, United States. NeutrAvidin Horseradish Peroxidase Conjugate and SuperSignal West Dura Extended Duration Chemiluminescent Substrate were purchased from Pierce.

Results

Isolation and characterization of inhibitor peptides protected at their carboxyl terminal end via fusion to the Rop protein

During initial studies with inhibitor peptides, a completely randomized oligonucleotide library was used to direct the synthesis of peptides containing up to 20 amino acid peptides in the highly-regulable expression vector, pLAC11 (Walker, *et al.*, 2001). In a screen of 20,000 peptides, 21 that could inhibit the growth of *E. coli* were found. While most of the inhibitors encoded peptides of up to 20 amino acid as expected, two of the most potent inhibitors turned out to be fusion peptides in which the carboxyl terminus of the peptide had become fused to the amino terminus of the Rop protein (the *rop* gene is located downstream from where the oligonucleotides are inserted into the pLAC11 expression vector). Because the Rop protein is known to form a very stable structure (Eberle, *et al.*, 1991; Steif, *et al.*, 1993), the Rop protein was likely serving as a stable protein anchor which protected the carboxyl terminal end of the two inhibitor peptides. Rop is a small 63 amino acid dimeric four-helix-bundle

protein whose monomer consists of two antiparallel α-helices that are connected by a sharp hairpin loop. It is a dispensable part of the ColE1 replicon carried on many plasmids, such as pBR322, and it can be deleted without affecting replication, partitioning, or copy numbers of plasmids containing a ColE1 ori (Soberon, *et al.*, 1980).

To test whether peptides could be stabilized by fusing the carboxyl terminus of the peptides to the amino terminus of the Rop protein, the p-Rop(C) fusion vector, a derivative of pLAC11, was constructed as described. To isolate potential inhibitor peptides which were protected at their carboxyl terminal end, a totally randomized oligonucleotide library that encoded up to 20 amino acid peptides was cloned into the p-Rop(C) fusion vector as shown in Figure 3.1 and transformed into *E. coli* under repressed conditions. Initial studies of 20,000 anchorless peptides, found only a single potent inhibitor that could inhibit the growth of *E. coli* for two days on plates. This frequency was used as a basis by which to judge whether protecting the carboxyl terminus of peptides with the Rop protein would increase the number of inhibitor peptides that could be isolated.

Peptides protected at their carboxyl terminal end were screened using the previously described grid-patching technique and 16 potent two-day inhibitors were isolated from a screen of 10,000. To verify that all of the inhibitors were legitimate, plasmid DNA was isolated from each inhibitory clone, transformed into a fresh background, and transformants were checked for IPTG-inducible

inhibition on plates. In order to accurately assess how inhibitory the inhibitors were, the first ten were subjected to a growth rate analysis in liquid media. Cultures containing either the inhibitors or a p-Rop(C) control were diluted 1:100 into new media containing 1 mM IPTG. OD_{550} readings were then taken hourly until the control culture had passed log phase. Growth rates were determined as the spectrophotometric change in OD_{550} per unit time during the log phase of growth. The relative decrease in growth rate was then calculated for each inhibitor compared to the p-Rop(C) control. As indicated in Table 3.2, the peptides inhibited bacterial growth rate an average of 92%.

Isolation and characterization of inhibitor peptides protected at their amino terminal end via fusion to the Rop protein

In addition to screening inhibitor peptides protected at their carboxyl terminal end by Rop, protecting the amino terminal end of the peptides was also tested. Where exopeptidases have been extensively characterized in either prokaryotic or eukaryotic systems, more aminopeptidases have been identified than carboxypeptidases. Thus, an argument can be made that stabilizing the amino terminal end of a peptide might be more effective at preventing degradation by peptidases than stabilizing the carboxyl terminal end. The p(N)Rop- fusion vector was used to test whether peptides could be stabilized by fusing the amino terminus of the peptides to the carboxyl terminus of the Rop protein. To isolate potential inhibitor peptides protected at their amino-terminal ends, a randomized oligonucleotide library that encoded peptides up to 20

amino acid in length was cloned into the p(N)Rop- fusion vector as shown in Figure 3.2 and transformed into *E. coli* under repressive conditions. Rop fusion peptides (6,000) were screened using the grid-patching technique, and 14 two-day inhibitors were isolated. The inhibitors were verified as described in the previous section and the first 10 inhibitors were subjected to growth rate analysis comparing to the p(N)Rop- control. As indicated in Table 3.3, the peptides inhibited the bacterial growth rate average of 93%.

Isolation and characterization of anchorless inhibitor peptides protected at their amino terminal and/or carboxyl terminal ends by two proline residues

The results of the Rop-peptide fusion studies suggested that peptides could be stabilized by protecting either their amino or carboxyl terminal ends. In previous studies with anchorless inhibitor peptides, a significant number of the inhibitor peptides containing one or more proline residues at or near their carboxyl terminal ends was found (Walker, *et al.*, 2001). It has been proposed that peptidases have a difficult time cleaving prolyl bonds (Vanhoof, *et al.*, 1995; Yaron and Naider, 1993). Therefore anchorless peptides protected by two proline residues placed at both the amino terminal and carboxyl terminal ends of the peptide were tested. To isolate potential inhibitor peptides protected at both ends by two proline residues, a totally randomized oligonucleotide library that encoded peptides of 20 amino acids was cloned into the pLAC11 expression vector (Warren, *et al.*, 2000) as shown in Figure 3.3 and transformed into *E. coli* under repressed conditions. Peptides (7,500) were screened using the grid-

patching technique and 12 two-day inhibitors were isolated. The inhibitors were verified as previously described for the Rop-peptide fusion studies and the first 10 inhibitors were characterized by growth rate analysis using pLAC11 as a control. As indicated in Table 3.4, the peptides inhibited bacterial growth rates at levels averaging 53% and thus did not inhibit the growth rate to the same degree as the Rop fusion peptides. However, as demonstrated in Walker, *et al.*, (2001), these inhibitors were still very potent. A peptide that inhibited growth rates by 53% would have an overall potency of 89% as compared to Rop fusion peptides, which had an overall potency of 98%. The sequences of the 10 inhibitor peptides are shown in Table 3.5. Only four of the inhibitors contained two proline residues at both their amino and carboxyl terminus. Five of the inhibitors contained two proline residues at only the amino terminus and one inhibitor contained two proline residues at only the carboxyl terminus.

Using an in vitro degradation assay to study the impact of proline stabilizing motifs on peptides

While the Rop fusion studies showed that stable inhibitor peptides could be obtained by fusing Rop to either the amino or carboxyl terminus of the peptide, the studies with double proline residues strongly suggested that at least in the case of smaller protective motifs, stability might be achieved by simply protecting the amino terminal end of the peptides. To extend these *in vivo* studies, an *in vitro* system was developed in which peptide stability could be directly assessed by mixing peptides with a cellular extract containing proteases

and peptidases. Randomized peptides were chosen in order to avoid the possibility that a single peptide might contain amino acid sequences susceptible to degradation by specific peptidases. By using a library of peptides, the overall effect of the peptidases could be normalized.

To validate this approach, the stability or half-life of a randomized peptide was measured in bacterial extracts that were deficient for known proteases or peptidases. In *E. coli* and *Salmonella typhimurium*, the known proteases and peptidases have been well characterized. The two main proteases having a role in peptide degradation in *E. coli* are Lon and ClpP, which are encoded respectively by the *lon* and *clpP* genes. In *S. typhimurium*, where peptidases have been the most thoroughly characterized, numerous peptidases have been identified, and strains have been constructed that delete several of the peptidases. Using extracts prepared from *E. coli* strains containing *lon* or *clpP* deletions and a *S. typhimurium* strain from which nine peptidase genes were deleted, half-lives were determined for an unprotected randomized peptide. As shown in Table 3.6, deletion of the Lon protease caused the peptide's half-life to increase by 647%, deletion of the Clp protease caused the peptide's half-life to increase by 84%, and deletion of multiple peptidases caused the peptide's half-life to increase by 711%. These results proved that the *in vitro* system acted as expected and suggested it to be a good method by which to assess peptide stability.

To address whether a single proline residue would provide the same level of protection as two proline residues, and whether the protective effect of the two proline residues could be enhanced by moving them to an internal position, three randomized peptides that contained P, PP, or APP residues at both their amino and carboxyl terminal ends were tested. Cellular extracts for degradation assays were prepared from *E. coli, Saccharomyces cerevisiae*, human intestine and human colon cells, as well as serum prepared from a Sprague-Dawley rat. The human intestine and colon cells were chosen because significant degradation of peptides has been demonstrated to occur in the intestine and colon. As shown in Table 3.7, APP offered better protection than PP, which offered better protection than a single proline in all of the extracts tested. The ability of proline residues to protect against degradation varied with the extract tested. The APP motif, for example, increased the stability of peptides in *E. coli* by 55%, but increased the stability of peptides in rat serum by 186% and in *S. cerevisiae* by 570%. An example of the data obtained in these degradation studies is shown in Figure 3.4.

Because APP offered the greatest protection, the effect of placing APP at only the amino or carboxyl terminus of the peptide was tested. As shown in Table 3.8, APP at only the amino terminus offered better protection than APP at only the carboxyl terminus for all the extracts that were tested. With the exception of *S. cerevisiae* extracts, APP at only the amino terminus of the peptide offered better protection than when APP was placed at both ends of the peptide. To test how the level of protection conferred by the APP motif compared to other

available stabilization motifs, randomized peptide that were acetylated at the amino terminus, amidated at the carboxyl terminus, or cyclized which completely prevents the access of exopeptidases to a free end of the peptide were synthesized and subjected to degradation assays. As shown in Table 3.9, APP at the amino terminus offered better protection than acetylation, or amidation, and offered almost the same level of protection as cyclizing the peptide in the eukaryotic extracts. With *E. coli* extracts, cyclization did not offer much protection against degradation and thus APP at the amino terminus of the peptide offered better protection than acetylation, amidation, or cyclization.

Discussion

Initial studies of novel bioactive peptides that inhibited the growth of *E. coli*, discovered two protective motifs that appeared to enhance the stability of peptides: a small stable protein anchor Rop and the incorporation of terminal proline residues. Results of the implementation of these motifs in the *in vivo* screen are summarized in Table 3.10. Protecting either the amino or carboxyl terminal end of the peptide by fusing the peptide to the stable Rop protein increased the frequency at which potent inhibitors could be isolated by as much as 47-fold. The incorporation of two proline residues at the terminal end of the peptides also significantly increased the frequency at which potent inhibitor peptides could be isolated. However, the resulting peptides were not as potent as those that could be isolated using the Rop protein anchor. The simplest explanation for these findings which show that protecting the terminal ends of

peptides via fusion to a small stable protein or the incorporation of two proline residues is that this prevents the action of exopeptidases that act on the terminal ends of peptides. The fact that Rop appears to be a better stabilizing anchor than two proline residues could be explained by their size difference. The significantly larger Rop protein may very well act by sterically hindering the ability of peptidases to gain access to the protected peptide.

An *in vitro* degradation assay was developed to better assess the ability of prolines to protect against peptide degradation. Initial experiments examined peptides that were protected at both their amino and carboxyl terminal ends by a single proline (P), two prolines (PP), or two prolines with an alanine residue as the terminal amino acid (APP). APP conferred better protection than PP which conferred better protection than P. This protection may be conferred by proline's unique bent structure that results in peptide bonds that are more difficult to cleave by peptidases. In addition, it is known that most exopeptidases act at or near the last peptide bond of the peptide and bind one or two of the terminal amino acids in order to cleave (Cunningham and O'Connor, 1997; Taylor, 1993; Woodley, 1994). Thus two proline residues should offer better protection than a single proline residue since the steric hindrance afforded by the proline residue can fully come into play. APP should offer better protection than PP, since by not placing the last proline residue at the terminal end of the peptide, the terminal proline is not sacrificed to exopeptidases that bind to the last one or two terminal amino acids. Also, peptide bonds involving a proline have been shown

to be more restrictive in conformation and are 10-30% more likely to exist in their *cis* conformation, which is more difficult to cleave than the *trans* conformation (Brandts, *et al.*, 1975; Reimer, *et al.*, 1998; Yao, *et al.*, 1994). In contrast, peptide bonds involving amino acids other than proline have been calculated to form *cis* bonds at a frequency of less than 0.1% (Ramachandran and Mitra, 1976). For example, dipeptidyl peptidase IV, a peptidase that specifically cleaves proline bonds, has been shown to only be able to cleave the *trans* conformation of its substrate (Fisher, *et al.*, 1983). Finally, molecular modeling programs such as Sybyl (Clark, *et al.*, 1989), which determine protein structure, can be used to show that the bent structure caused by proline residues is more pronounced in PP than in P alone and that the presence of a terminal amino acid, such as alanine, in APP causes the steric hindrance in PP to be even more pronounced.

Because most of the peptides that were isolated in the *in vivo* screen involving two proline residues only contained prolines at the amino terminal ends, the difference between protecting peptides at only their amino or carboxyl end with proline residues was assessed. The data showed that placing the APP motif at the amino terminal end of the peptide increased peptide stability more than when the APP motif was placed at the carboxyl terminal end of the peptide. This finding is consistent with the fact that more aminopeptidases have been identified than carboxypeptidases in both prokaryotic and eukaryotic systems in studies where the general nature of exopeptidases have been characterized (Bai and Amidon, 1992; Bai, *et al.*, 1995; Brownlees and Williams, 1993; Miller, 1996;

Ryan, 1989). The stabilizing effects of the APP motif was also compared to other routinely used approaches to stabilize peptides, and was found in general to increased the stability of peptides better than amidation or acetylation and was almost as effective as cyclization.

The fact that incorporating proline residues at the terminal ends of peptides caused such a significant increase in the resistance of peptides to degradation by peptidases could be viewed as surprising given the existence of a class of proline-specific peptidases that degrade peptides containing proline residues (Cunningham and O'Connor, 1997; Walter, *et al.*, 1980). It has been argued that most peptidases have a difficult time degrading proline residues and this is why proline-specific peptidases have evolved. Since proline-specific peptidases were naturally present in all test extracts, it might have been expected that the proline stabilizing motifs should have caused no enhancement of half-lives because the proline-specific peptidases would have neutralized the proline stabilizing motifs. The data here indicates that degradation by peptidases has to be considered in terms of the sum effect of all the peptidases that are present. It may very well be that the proline stabilizing motifs have no effect on the proline-specific peptidases but have a huge effect on the rest of the peptidases. Because there are many more general peptidases than proline-specific peptidases, the incorporation of prolines does have a significant effect on protecting peptides from degradation by peptidases.

Numerous researchers have used amidation and acetylation as a means of increasing the stability of both natural and synthetic peptides that have potentially interesting pharmaceutical properties. Because the use of APP at the amino terminus was clearly superior to both amidation and acetylation in the direct comparisons conducted in this study, the use of the APP motif could provide another means to increase the stability of pharmaceutically promising peptides. While cyclization provided the best protection in general, its use is limited to newly engineered peptides where a cyclic motif could be incorporated into the peptide, since cyclization can impart structural changes to existing peptides and might adversely affect their bioactivity.

The use of APP or a small stabilizing protein motif such as Rop, represents new possibilities for the design of more effective peptide drugs that are not as susceptible to degradation by peptidases. Because the amino and carboxypeptidases that have been characterized appear to function quite similarly in all cell types (Rawlings and Barrett, 1993), incorporating protein-based stabilizing anchors into peptide drugs should significantly decrease the action of exopeptidases in host cells. Additionally, the use of protein-based motifs has another distinct advantage over existing technologies such as amidation, acetylation, or the use of modified or D-amino acids, which requires chemical modification. Peptide drugs that are stabilized using APP or a small stable protein anchor could be produced using recombinant protein synthesis in *E. coli* or human cells.

References

Bai, J. P. F. and Amidon, G. L. (1992). Structural specificity of mucosal cell transport and metabolism of amino acids and peptide drugs: implication for oral peptide delivery. Pharm. Res. *9*, 969-978.

Bai, J. P. F., Chang, L.-L., and Guo, J.-H. (1995). Targeting of peptides and protein drugs to specific sites in the oral route. Crit. Rev. Ther. Drug *12*, 339-371.

Bozou, J. C.., Amar, S., Vincent, J. P., and Kitabgi, P. (1986). Neurotensin-mediated inhibition of cyclic AMP formation in neuroblastoma N1E115 cells: involvement of the inhibitory GTP-binding component of adenylate cyclase. Mol. Pharmacol. *29*, 489-496.

Brandts, J. F., Halvorson, H. R., and Brennan, M. (1975). Consideration of the possibility that the slow step in protein denaturation reactions is due to cis-trans isomerism of proline residues. Biochemistry *14*, 4953-4963.

Brownlees, J. and Williams, C.. H. (1993). Peptidases, peptides at the mammalian blood-brain barrier. J. Neurochem. *60*, 793-803.

Clark, M., Cramer, III, R. D., and Van Opdenbosch, N. (1989). Validation of the general purpose Tripos 5.2 force field. J. Comp. Chem. *10*, 982-1012.

Cunningham, D.F. and O'Connor, B. (1997). Proline specific peptidases. Biochim. Biophys. Acta *1343*, 160-186.

Eberle, W., Pastore, A., Sander, C., and Rosch, P. (1991). The structure of ColE1 Rop in solution. J. Biomolecular NMR. *1*, 71-82.

Eichler, J., Appel, J. R., Blondelle, S. E., Dooley, C.. T., Dorner, B., Ostresh, J. M., Perez-Paya, E., Pinilla, C., and Houghten, R. A. (1995). Peptide, peptidomimetic, and organic synthetic combinatorial libraries. Med. Res. Rev. *15*, 481-496.

Fisher, G., Heins, J., and Barth, A. (1983). The conformation around the peptide bond between the P_1- and P_2- positions is important for catalytic activity of some proline-specific peptidases. Biochim. Biophys. Acta *742*, 452-462.

Hruby, V. J. and Balse, P. M. (2000). Conformational and topographical considerations in designing agonist peptidomimetics from peptide leads. Curr. Med. Chem. *7*, 945-970.

Lam, K. S. (1997). Application of combinatorial library methods in cancer research and drug discovery. Anticancer Drug Des. *12*, 145-167.

Lauta, V. M. (2000). Pharmacological elements in clinical application of synthetic peptides. Fundam. Clin. Pharmacol. *14*, 425-442.

Lebl, M. and Krchnak, V. (1997). Synthetic peptide libraries. Methods Enzymol. *289*, 336-392.

Le-Nguyen, D., Heitz, A., Chiche, L., Castro, B., Boigegrain, R. A., Favel, A., and Coletti-Previero, M. A. (1990). Molecular recognition between serine proteases and new bioactive microproteins with a knotted structure. Biochimie *72*, 431-435.

Lowman, H. B. (1997). Bacteriophage display and discovery of peptide leads for drug development. Annu. Rev. Biophys. Biomol. Struct. *26*, 401-424.

Miller, C. G. (1996). Protein Degradation and Proteolytic Modification. In *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology, 2nd Edition, F.C. Neidhardt, ed. (Washington, D.C.: ASM Press), pp. 938-954.

Miller, J.H. (1972). Experiments in Molecular Genetics (Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory).

Pinilla, C., Appel, J., Blondelle, S., Dooley, C., Dorner, B., Eichler, J., Ostresh, J., and Houghten, R. A. (1995). A review of the utility of soluble peptide combinatorial libraries. Biopolymers *37*, 221-240.

Ramachandran, G. N. and Mitra, A. K. (1976). An explanation for the rare occurrence of *cis* peptide units in proteins and polypeptides. J. Mol. Biol. *107*, 85-92.

Rawlings, N. D. and Barrett, A. J. (1993). Evolutionary families of peptidases. Biochem. J. *290*, 205-218.

Reimer, U., Scherer, G., Drewello, M., Kruber, S., Schutkowski, M., and Fischer, G. (1998). Side-chain effects on peptidyl-prolyl *cis/trans* isomerization. J. Mol. Biol. *279*, 449-460.

Rivier, J. E. and Marshall, G. R., eds. (1990). Peptides: Chemistry, Structure, and Biology (The Netherlands: ESCOM Science Publishers BV).

Ryan, J. W. (1989). Peptidase enzymes of the pulmonary vascular surface. Am. J. Physiol. *257*, L53-60.

Sanders, L. M. (1990). Drug delivery systems and routes of administration of peptide and protein drugs. Eur. J. Drug Metab. Pharmacokinet. *15*, 95-102.

Shagger, H. and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. *166*, 368-379.

Siddle, K., and Hutton, J. C., eds. (1990). Peptide Hormone Action (Oxford, England: IRL Press at Oxford University Press).

Smith, G. P. and Scott, J. K. (1993). Libraries of peptides and proteins displayed on filamentous phage. Meth. Enzymol. *217*, 228-257.

Smith, J. A., and Rivier, J. E., eds. (1992). Peptides: Chemistry and Biology (The Netherlands: ESCOM Science Publishers BV).

Soberon, X., Covarrubias, L., and Bolivar, F. (1980). Construction and characterization of new cloning vehicles: IV. Deletion derivatives of pBR322 and pBR325. Gene *9*, 287-305.

Steif, C., Weber, P., Hinz, H.-J., Flossdorf, J., Cesareni, G., and Kokkinidis, M. (1993). Subunit interactions provide a significant contribution to the stability of the dimeric four-α-helical-bundle protein Rop. Biochemistry *32*, 3867-3876.

Taylor, A. (1993). Aminopeptidases: towards a mechanism of action. Trends Biochem. Sci. *18*, 167-171.

Vanhoof, G., Goossens, F., De Meester, I., Hendriks, D., and Scharpe, S. (1995). Proline motifs in peptides and their biological processing. FASEB J. *9*, 736-44.

Walker, J. R., Roth, J. R., and Altman, E. (2001). An *in vivo* study of novel bioactive petpides that inhibit the growth of *Escherichia coli*. J. Peptide Res. *58,* 380-388.

Walter, R., Simmons, W.H., and Yoshimoto, T. (1980). Proline specific endo- and exopeptidases. Mol. Cell. Biochem. *30*, 111-127.

Warren, J., Walker, J. R., Roth, J. R., and Altman, E. (2000). Construction of a highly regulable expression vector, pLAC11, and its multipurpose derivatives, pLAC22 and pLAC33. Plasmid *44,* 138-151.

Woodley, J. F. (1994). Enzymatic barriers for GI peptide and protein delivery. Crit. Rev. Ther. Drug Carrier Sys. *11*, 61-95.

Yao, J., Feher, V. A., Espejo, F., Reymond, M. T., Wright, P. E., and Dyson, H. J. (1994). Stabilization of a type VI turn in a family of linear peptides in water solution. J. Mol. Biol. *243*, 736-753.

Yaron, A. and Naider, F. (1993). Proline-dependent structural and biological properties of peptides and proteins. Crit. Rev. Biochem. Mol. Biol. *28*, 31-81.

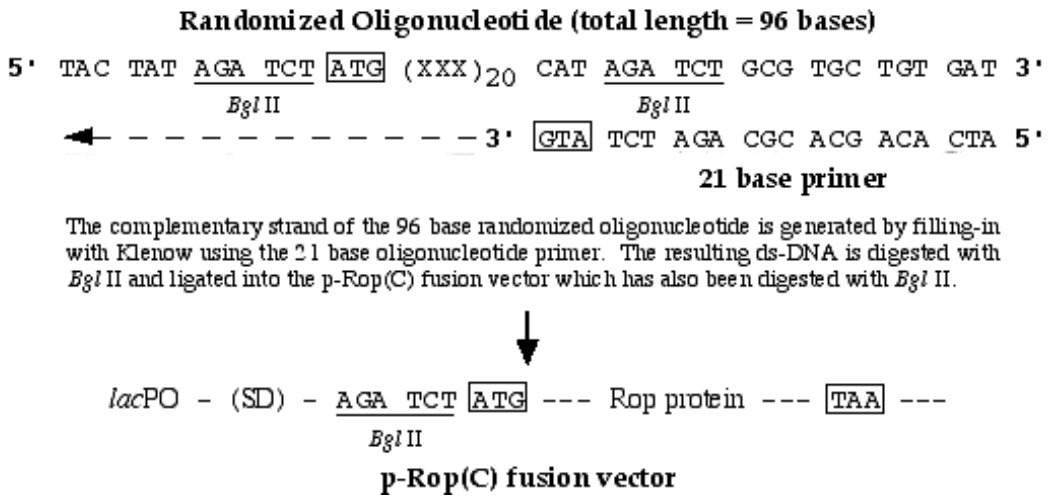


Figure 3.1 Scheme for generating the randomized 20 amino acid peptide library where the carboxyl terminal end of the peptides are fused to the amino terminal end of the Rop protein. Wherever an "X" occurs in the oligonucleotide, a random mixture of all four of the nucleotides, A, C, G, and T, are used. Because of the way the oligonucleotide library has been engineered, either orientation of the incoming digested ds-DNA fragment results in a fusion product.

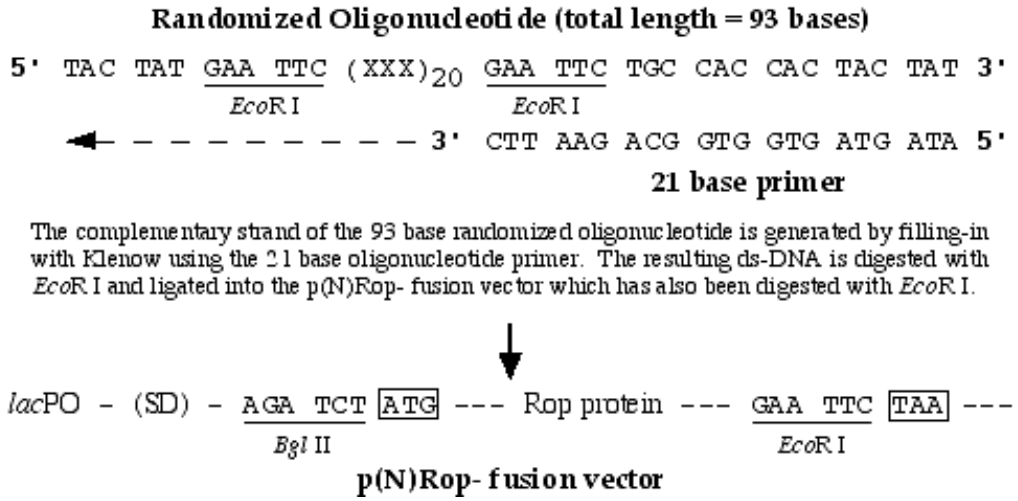


Figure 3.2 Scheme for generating the randomized 20 amino acid peptide library where the amino terminal end of the peptides are fused to the carboxyl terminal end of the Rop protein. Wherever an "X" occurs in the oligonucleotide, a random mixture of all four of the nucleotides, A, C, G, and T, are used. Because of the way the oligonucleotide library has been engineered, either orientation of the incoming digested ds-DNA fragment results in a fusion product.

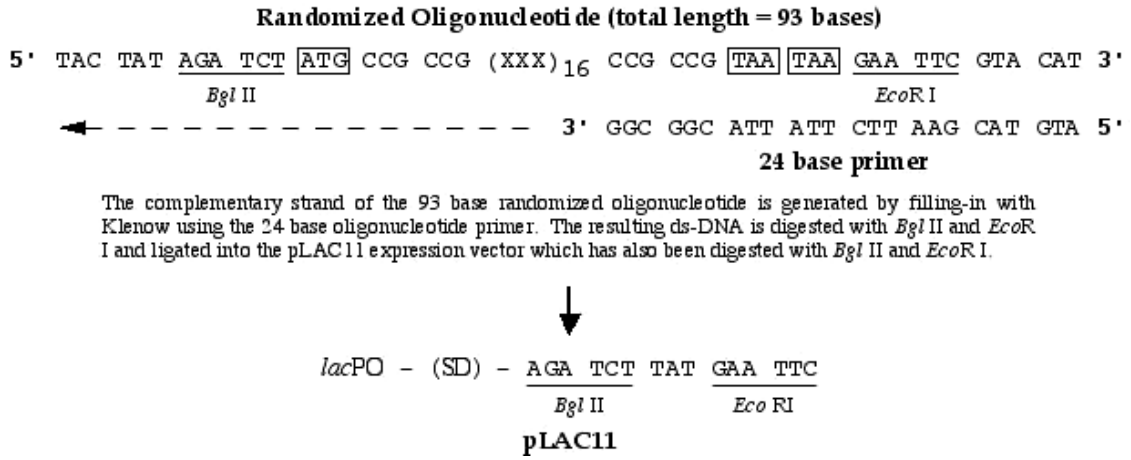


Figure 3.3 Scheme for generating the randomized 20 amino acid peptide library where the peptides are protected at both the amino terminal and carboxyl terminal ends by two proline residues. Wherever an "X" occurs in the oligonucleotide, a random mixture of all four of the nucleotides, A, C, G, and T, are used.

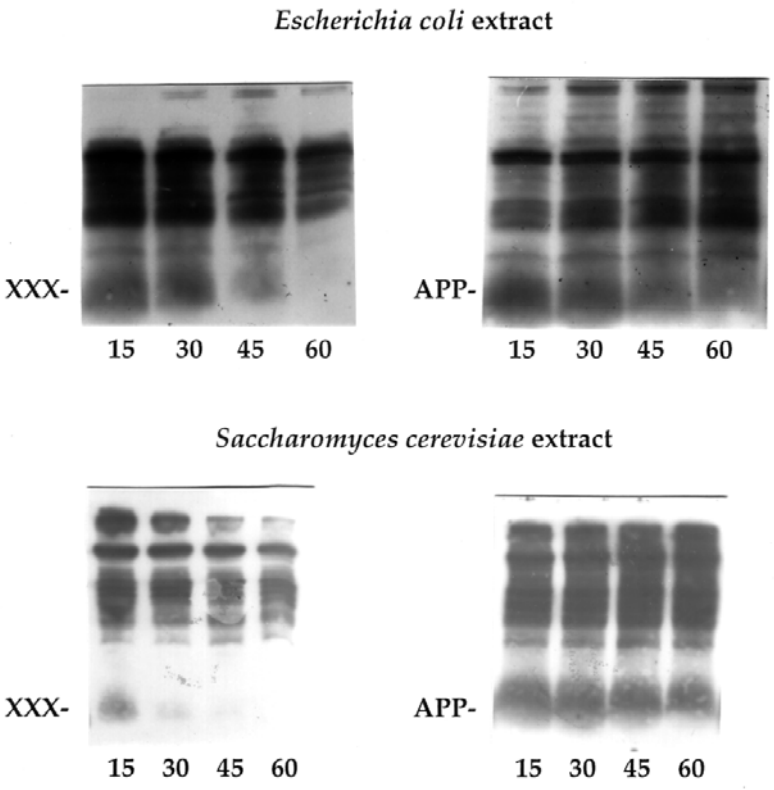


Figure 3.4 Degradation studies of XXXXXX[KBtn]XXXXXA (unprotected) and APPXXXX[KBtn]XXXXPPA (APP at both ends) using *E. coli* and *S. cerevisiae* extracts. The degradation experiments were performed as described in Experimental Procedures.

Table 3.1 Bacterial Strains

Strain	Genotype	Reference
E. coli strains		
ALS225	*araD139* Δ*(araABOIC-leu)7679* D(*lac)X74 galU galK rpsL hsr- hsm*+ F'*lacI*^{q1}*Z*+*Y*+*A*+	Warren, *et al.*, 2000
MG1655	F- λ-	Guyer, M. S., *et al.*, 1980
MG1655 *lon*::Tn10	F- λ- *lon*::Tn10	Carol Gross, University of California, San Francisco
MG1655 *clpP*::cam	F-λ- *clpP*::cam	This study
SG22098	F- λ- *araD*139 Δ(*lac*)U169 *rpsL*150 *thi f1bB*5301 *deoC7 ptsF*25 *clpP*::cam	Michael Maurizi, National Cancer Institute
S. typhimurium LT2 strains		
TN1379	*leuBCD*485	Charles Miller, University of Illinois
TN1727	*leuBCD*485 *pepA*16 *pepB*11 *pepN*90 *pepP*1 *pepQ*1 *pepT*1 Δ*supQ*302(*proAB pepD*) *optA*1 *dcp*-1 *zxx*848::Tn5 *zxx*845::Tn10	Charles Miller, University of Illinois

Table 3.2 Inhibitory Effects of Peptide Inhibitors Stabilized by Fusing the Carboxyl Terminal End of the Peptide to the Amino Terminal End of the Rop Protein

Inhibitor	% Inhibition of the Growth Rate
pRop(C)1	87
pRop(C)2	99
pRop(C)3	85
pRop(C)4	98
pRop(C)5	95
pRop(C)6	99
pRop(C)7	91
pRop(C)8	86
pRop(C)9	93
pRop(C)10	91

The inhibitory effects were determined as described in the text using p-Rop(C) as a control. The data is the average of duplicate experiments.

Table 3.3 Inhibitory Effects of Peptide Inhibitors Stabilized by Fusing the Amino Terminal End of the Peptide to the Carboxyl Terminal End of the Rop Protein

Inhibitor	% Inhibition of the Growth Rate
pRop(N)1	81
pRop(N)2	96
pRop(N)3	95
pRop(N)4	92
pRop(N)5	99
pRop(N)6	93
pRop(N)7	87
pRop(N)8	91
pRop(N)9	95
pRop(N)10	96

The inhibitory effects were determined as described in the text using p(N)-Rop as a control. The data is the average of duplicate experiments.

Table 3.4 Inhibitory Effects of Peptide Inhibitors Stabilized by Two Proline Residues at the Amino and/or Carboxyl Terminal Ends of the Peptide

Inhibitor	% Inhibition of the Growth Rate
pPro1	50
pPro2	49
pPro3	50
pPro4	59
pPro5	52
pPro6	93
pPro7	54
pPro8	42
pPro9	41
pPro10	42

The inhibitory effects were determined as described in the text using pLAC11 as a control. The data is the average of duplicate experiments.

Table 3.5 Sequence Analysis of the Insert Region from the Peptides Stabilized by Two Proline Residues

pPro1 - 21aa

AGA TCT ATG CCG CCG ATT CTA TGG GGC GAA GCG AGA AAG CGC TTG TGG GGT GGG GAT CAT ACA CCG CCG TAA TAA
M P P I L W G E A R K R L W G G D H T P P * *
GAA TTC

pPro2 - 27aa

AGA TCT ATG CCG CCG CCG TTG GAT ATT GTG TCG GGT ATT GAG GTA GGG GGG CAT TTG TGG TGC CGC CGT ATT AAG AAT TCT CAT
M P P P L D I V S G I E V G G H L W C R R I K N S H
GTT TGA
V *

pPro3 - 8aa

AGA TCT ATG CCG CCG GAC AAT CCG GTC CTG TGA TGA AGC GGA GGT CGA CCA AGG GGA TAT CAG CCG CCG TAA TAA
M P P D N P V L * *
GAA TTC

pPro4 - 9aa

AGA TCT ATG CCG CCG CTA TTG GAC GGA GAT GAC AAA TAG ATA TAT GCG TGG TTG TTT TTC TGT CCG CCG TAA TAA
M P P L L D G D D K *
GAA TTC

pPro5 - 10aa

AGA TCT ATG CCG CCG AGG TGG AAG ATG TTG ATA AGA CAG TGA CAG ATG CGT TCC ATT ACT CCC GCC GTA ATA AGA
M P P R W K M L I R Q *
ATT C

pPro6 - 7aa

AGA TCT ATG ATG AGA GTA GCG CCG CCG TAA TAA GAA TTC
M M R V A P P * *

pPro7 - 14aa

AGA TCT ATG CCG CCG TTG CGC GGG GCA TGC GAT GTA TAT GGG GTA AAT TGA ATG TCT TGT GGG CCG CCG TAA TAA
M P P L R G A C D V Y G V N *
GAA TTC

pPro8 - 21aa

AGA TCT ATG CCG CCG GGG AGA GGG GAA GCG GTG GGA GTG ACA TGC TTG AGC GCG AAC GTG TAC CCG CCG TAA TAA
M P P G R G E A V G V T C L S A N V Y P P * *
GAA TTC

pPro9 - 21aa

TCT ATG CCG CCG GGA AGG GTA GTG TTC TTT GTC GCT ATC TTT GTT TCC GCA ATA TGC CTC CCG CCG TAA TAA GAA
M P P G R V V F F V A I F V S A I C L P P * *
TTC

pPro10 - 21aa

AGA TCT ATG CCG CCG AGG TTC GCT CAT GAG AGT GTT AAA GGG CTG GGG GAC GTT ACA AAA GCT CCG CCG TAA TAA
M P P R F A H E S V K G L G D V T K A P P * *
GAA TTC

The landmark *Bgl* II and *Eco*R I restriction sites for the insert region are underlined. Since the ends of the oligonucleotide from which these inhibitors were constructed contained these restriction sites, the oligonucleotide was not gel isolated when the libraries were prepared in order to maximize oligonucleotide yields. Because of this, three of the inhibitory clones, pPro2, pPro5, and pPro6 were found to contain deletions in the randomized portion of the oligonucleotide. All of the inhibitory clones contained two proline residues at either their amino or carboxyl termini.

Table 3.6 Peptide Degradation in Protease and Peptidase Deficient Bacterial Extracts

Strain from which Extract was Prepared*	Peptide Half-Life in Minutes
E. coli Strains	
MG1655	44.9
MG1655 *lon*::Tn10	290.6
MG1655 *clpP*::cam	82.5
S. typhimurium Strains	
TN1379	42.0
TN1379 *dcp*-1 *optA*1 *pepA*16 *pepB*11 *pepD* *pepN*90 *pepP*1 *pepQ*1 *pepT*1	298.5

*Because of the decreased potency of *S. typhimurium* extracts relative to *E. coli* extracts, the *S. typhimurium* extracts were used at a concentration of 25 mg/mL.

Table 3.7 Degradation Studies on Peptides that Contain the P, PP, or APP Motif at Both Their Amino and Carboxyl Terminus

Extract	Peptide Half-Life in Minutes			
	Unprotected	P at both ends	PP at both ends	APP at both ends
E. coli	44.9	38.2	51.1	69.8
S. cerevisiae	23.3	44.4	99.0	156.0
Human Intestine	121.6	99.2	166.3	172.8
Human Colon	58.1	64.5	76.1	109.2
Rat Serum	54.1	80.7	85.3	154.5

Table 3.8 Degradation Studies on Peptides that Contain the APP Motif at Either Their Amino or Carboxyl Terminus

Extract	Peptide Half-Life in Minutes			
	Unprotected	APP at both ends	APP Amino	APP Carboxyl
E. coli	44.9	69.8	99.6	54.6
S. cerevisiae	23.3	156.0	86.0	44.4
Human Intestine	121.6	172.8	200.7	99.0
Human Colon	58.1	109.2	144.0	95.1
Rat Serum	54.1	154.5	165.3	121.2

Table 3.9 Degradation Studies on Peptides that Contain the APP Motif at Either Their Amino or Carboxyl Terminus as Compared to Peptides Amidated at Their Carboxyl Terminus, Acetylated at Their Amino Terminus, or Cyclized

Extract	Peptide Half-Life in Minutes					
	Un-protected	APP Amino	Acetylated	APP Carboxyl	Amidated	Cyclized
E. coli	44.9	99.6	34.9	54.6	46.7	52.3
S. cerevisiae	23.3	86.0	44.2	44.4	73.9	145.0
Rat Serum	54.1	165.3	67.3	121.2	75.7	217.2

Table 3.10 Summary of the Frequency and Potency at Which the Different Types of Inhibitor Peptides can be Isolated

Type of Inhibitor Peptide	Frequency at Which a Potent Two Day Inhibitor Peptide can be Isolated	Average Inhibition of Growth Rate	Average Potency*
Anchorless	1 in 20,000	25%	65%
Protected at the C-terminal end via Rop	1 in 625	92%	98%
Protected at the N-terminal end via Rop	1 in 429	93%	98%
Protected at the C-terminal and/or N-terminal end via two prolines	1 in 625	53%	89%

*The average potency was calculated as described in Walker, *et al.*, 2001

CHAPTER 4

CONFORMATIONAL ANALYSIS OF THE APP AND PP PROTECTIVE PEPTIDE MOTIF

Introduction

Historically, peptides developed for pharmaceutical application have come from natural resources. Yet with new production methods, synthetic peptides are now being utilized due to their more potent function. Once peptides are tested *in vitro,* they are then subjected to tests of *in vivo* efficacy. Here synthetic peptides face one major disadvantage over natural peptides; many prove to be highly susceptible to degradation by a variety of peptidases present in the mammalian body (Lee, 1988; Sood and Panchagnula, 2000). Peptide drugs that possess a high potential for efficacy in the human body, but are unstable cannot proceed to final tests. This barrier to synthetic peptide development may be overcome by peptide drug modifications that enhance stability. One method is applying peptidase inhibitors to accompany the drug (Bai, *et al.,* 1995). Alternatively, placing unnatural amino acids at potential peptidase cleavage sites or adding amide or acetyl groups to the carboxyl or amide ends respectively are other types of stabilizing modification (Hruby, *et al.,* 1991; Moss, 1995). Unfortunately, any one type of protection against peptidases is not universally effective, nor are all motifs compatible with every peptide.

In the pursuit of new protective motifs for peptides, an *in vivo* approach was developed (Walker, *et al.,* 2001). Intracellularly produced peptides were screened for the ability to avoid degradation. A library of randomly selected nucleotides that encoded peptides of 20 amino acids placed under control of the highly inducible expression vector, pLac 11 (Warren, *et al.,* 2000) was used to transform *Escherichia coli*. Once expressed in the cell, the randomly encoded peptides were monitored for growth inhibitory effects. This procedure was used to identify peptides inhibitory to cell function and growth that possessed some stabilizing motif to impede the action of peptidases. Oligonucleotide inserts in inhibited *E. coli* clones were sequenced and then analyzed for stabilizing structures. Sequence results indicated a pattern in four out of ten amino acid sequences having a motif of one or more proline residues at or near the carboxyl terminus, with the peptide of highest inhibitory capacity, Pep 12, possessing two proline residues near the carboxyl end (Walker, *et al.,* 2001). To test the hypothesis that this motif increased peptide stability, randomized synthetic peptides having a proline (P), proline-proline (PP) or alanine-proline-proline (APP) motif at either end were tested for increased half-life.

Proline itself is known to resist cleavage by peptidases (Mentlein, 1988; Williamson, 1994; Yaron and Naider, 1993; Cunningham and O'Connor, 1997). This unusual amino acid forms a pyrrolidine ring along the backbone of the peptide chain creating a sterically hindered structure (MacArthur and Thornton, 1991; Vanhoof, *et al.*,1995). With two proline peptide bonds at a terminal end, the

restrictive nature of the molecule is increased (Williamson, 1994). In previous studies, synthetic peptides subjected to a variety of eukaryotic and prokaryotic extracts revealed peptides protected with the APP motif possessed a three-fold increase in half-life over unprotected peptides. The PP motif provided little if any protection resulting in less than a one-fold increase in half-life. Furthermore, placing the APP motif at only one end of the peptides increased half-life almost two-fold over having APP both termini (Walker, *et al.*, submitted). There appeared to be a synergistic effect from the addition of alanine to the two prolines. Alanine added to the amide side of proline may contribute to this more sterically hindered conformation.

While the peptide bond of most amino acid residues prefer the planar *trans* configuration (Ramachandran and Sasisekharan, 1968), prolyl peptide bonds more frequently assume a *cis* configuration. Of the proteins in Brookhaven Protein Data bank containing double prolines, 6.5-11% were calculated to have a Pro-*cis*-Pro bond (Stewart, *et al.*, 1990; MacArthur and Thornton, 1991). A *cis* orientation makes the amide bond even more hindered, thus more resistant to peptidases. Therefore, a *cis* peptide bond occurring in the proline motifs could assist in explaining in part the protective nature of APP or PP. A computer modeling method was used to investigate the conformational space of APP and PP, and predict the likelihood that the bond between the two prolines prefers the *cis* orientation.

Materials and Methods

All conformational studies performed using with the Tripos Sybyl program. The APP and PP structures were created using the protein building feature of Sybyl. An N- methyl group (NME) was added to the carboxyl end of the second proline of both motifs to simulate the effect of a peptide chain. Kollman-all charges were loaded on each structure to account for attractive interaction between molecules. The structures were then subjected to an energy minimization routine using the Tripos force field (Clark, *et al.,* 1989) for 1000 iterations (calculations), a gradient of 0.5 kcal/mol and a dielectric constant of 4.0. The 4.0 dielectric constant was chosen in order to analyze the peptide in the absence of a solvent. Following minimization, each structure was analyzed for low energy conformers in view of rotation about the peptide backbone using the systematic search feature. The systematic search feature calculates relative energy and displays a physical representation of each structure as peptide bonds selected are rotated through increments of 20°. Figure 4.1 details the bonds chosen for rotational analysis. The Cα-N bond of each proline ring was excluded from the systematic search due to the complications introduced by the puckering of the proline ring and the rigidity of the torsional angle of this bond. Lowest energy conformers were only computed for a span of 10 kcal/mol since an energy minimum was desired. All other settings were left at default values. Lowest energy conformers for APP and PP were minimized again using the Kollman-all atom force field, which takes into account hydrogen bonding

(Weiner, *et al.,* 1986). Dihedral angles between the prolyl bonds of the two prolines were measured using the Spartan program.

Results

Conformers and measurements

Only motifs attached to the amino end of a peptide were analyzed since degradation results indicated that motifs were most effective at this terminal end (Walker, *et al*., submitted). An initial systematic search for lowest energy conformers based on the 20° incremental rotations around the peptide backbone bonds showed the APP sequence to have two distinct conformers differing in energy by 0.2 kcal/mol. Energy minimization of the two conformers using the Kollman-all atom force field resulted in an energy difference of 2.7 kcal/mol. These two low energy conformers both exhibited *trans* prolyl bonds (Figure 4.2). Figure 4.3 presents a magnified view of the prolines in each APP conformer and their dihedral angles. Conformer #1 exhibited an extended structure of the two proline and alanine residues in relation to the NME cap with a distance of 8.524 Å from the amino terminus of alanine and the amino terminus of the NME cap. In contrast, conformer #2 showed a highly "kinked" structure resulting in the distance between the amino end of alanine and the amino end of the methyl cap being reduced to 5.663 Å. The dihedral angle between the two proline rings showed a difference of 121.3° between the two conformers: -69.0° for conformer #1 and 52.3° for conformer #2. Figure 4.4 shows distances measured over selected atoms for each conformer, as well as the angles as seen from a top view

of each structure. The ten lowest energy APP conformers, all within a range of 0.3 kcal/mol in the Tripos force field, exhibited a consistent transition between the two conformers as potential energy decreased (data not shown).

The lowest energy conformer for the PP motif exhibited *trans* peptide bonds with an energy difference of 10.6 kcal/mol less than conformer #1 of APP. Figure 4.2 shows the PP conformer. Higher energy structures of PP, similar to the kinked APP configuration #2, all had energy differences greater than 10 kcal/mol. A high energy PP conformer having a right turn between the prolyl bonds was minimized with the Kollman-all atom force field which returned the structure to that of the lowest energy PP conformer. The dihedral angle of the proline rings was -79.3° and the measured distance between the amide end of proline and the methyl group of NME was 5.22 Å.

Discussion

Comparison of the conformers

The two APP conformers, being separated by 2.7 kcal/mol difference with Kollman's force field or 0.2 kcal/mol with the Tripos field, could possibly flip from one arrangement to another with relative ease. Conformer #1 had an extended structure with the second proline and alanine residues following the first proline residue in a linear arrangement. Somewhat surprising was the relatively small energy difference given the completely different special arrangement. Conformer #2 reveals a flip of the APP group around the NME cap with almost two right angles of 86.4° and 94° (Figure 4.3). With respect to

conformer #1, the three amino acids of conformer #2 turn almost 180° around the NME cap. The two prolines with right angle turns align the alanine carbon in a *cis*-like arrangement with the carbon of the NME cap. The alanine, although in *trans* configuration, may induce some interaction between its methyl group and the proline rings, facilitating the peptide backbone angles.

Although naturally occurring amide bonds of proline residues show a significant percentage of *cis* bonds the fact that no *cis* bonds were predicted between alanine and proline is not all surprising. Bulky amino acid residues such as aromatic residues have been indicated to facilitate the formation of *cis* bonds with proline (Reimer, 1998; Yao, *et al.,* 1994; Grathwohl and Kurtwüthrich, 1976). Although the two proline residues assume a *trans* bond in both the proline-proline and alanine-proline-proline motifs, there is a dramatic difference in arrangement with respect to each other. The PP *trans* conformer, which does not show a large degree of change as it approaches a minimum, assumes an arrangement almost identical to the two prolines in APP conformer #1. Since minimization to the high energy PP conformation returned to the low energy PP conformer shown in Figure 4.2, this is likely the only low energy conformation for this motif.

The potential flipping that occurred between the APP conformations as calculated energy decreased may indicate slow folding of the motif. Proline isomerization has been measured as the rate limiting step in renaturing peptides (Pal, *et al.,* 1999; Levitt, 1981) as shown in the slow folding of proteins

acylphosphatase and ribonuclease T_1 (Pertinez, *et al.,* 2000; Kiefhaber, *et al.,* 1990; Stein, 1993). The presence of two proline residues may decrease the folding rate of the APP motif. Additionally, the flipping of APP arrangement of conformer #1 to that of conformer #2 suggests that the APP conformer exists as a ratio of two distinct arrangements. Thus, the presence of the more highly "kinked" conformer #2 could present a steric deterrent to peptidase degradation.

Application

The ability to protect peptides for potential drug application is a consistent limitation for the discovery of novel medicinal peptides. Although some stabilizing motifs have been identified, such as amide protecting groups, not all motifs equally apply to all peptide drugs. A tendency for randomly encoded stable inhibitor peptides to maintain one or two prolines at terminal ends was noted in previous studies. *In vitro* results showed that the PP motif provided more stability than unprotected peptides, but less stability than the APP motif. Calculations performed by the protein modeling program, Sybyl, suggested APP to have one of two conformations with two right angles along its peptide backbone. The PP motif was predicted to preferentially assume an extended configuration that may be more susceptible to peptidase attack than the angled conformation of APP. The APP right-angled conformer with a more sterically hindered arrangement may prevent general or even proline-specific peptidases from attacking the peptide. This may help to explain the *in vitro* experimental results.

References

Bai, J. P. F., Chang, L-L., and Guo, J-H. (1995). Targeting of peptide and protein drugs to specific sites in the oral route. Crit Rev Ther Drug Carrier Sys 12, 339-371.

Clark, M., Cramer, III, R. D., Van Opdenbosch, N. (1989). Validation of the general purpose Tripos 5.2 force field. J Comp Chem 10, 982-1012

Cunningham, D. F. and O'Connor, B. (1997). Proline specific peptidases. Biochim Biophys Acta 1343, 160-186.

Grathwohl, C. and Wüthrich, K. (1976). The X-Pro peptide bond as an Nmr probe for conformational studies of flexible linear peptides. Biopolymers 15, 2025-2041.

Hruby, V. J., Kazmeirski, W., Kawasaki, A. M. and Matsunaga, T. O. (1991). Synthetic chemistry and the design of peptide-based drugs. In Peptide Pharmaceuticals, Approaches to the Design of Novel Drugs. Ed., Ward, D. J. (Elsevier: New York), pp.135-161.

Kiefhaber, T., Quaas, R., Hahn, U., and Schmid, F. X. (1990). Folding of ribonuclease T_1. 2. Kinetic models for the folding and unfolding reactions. Biochemistry 29, 3061-3070.

Lee, V. H. (1988). Enzymatic barriers to peptide and protein absorption. Crit Rev Ther Drug Carr Sys 5, 69-97.

Levitt, M. (1981). Effect of proline residues on protein folding. J Mol Biol 145, 251-63.

MacArthur, M. W. and Thorton, J. M. (1991). Influence of proline residues on protein conformation. J Mol Biol 218, 397-412.

Mentlein, R. (1988). Proline residues in the maturation and degradation of peptide hormones and neuropeptides. FEBS Lett 234, 251-256.

Moss, J. (1995). Peptide prodrugs designed to limit metabolism. In Peptide-based Drug Design. Eds. Taylor, M. D., and Amidon, G. L. (American Chemical Society: Washington D.C .) pp. 423-424.

Pal, D., Charkrabarti, P. J. (1999). *Cis* peptide bonds in proteins: residues involved, their conformations, interactions and locations. J Mol Biol 294, 271-88.

Pertinhez, T. A., Hamada, D., Smith, L. J., Ohiti, F., Taddei, N., Stefanit, M., and Dobson, C. M. (2000). Initial denaturing conditions influence the slow folding phase of acylphophatase associated with proline isomerization. Prot Sci 9, 1466-1473.

Ramachandran, G. N. and Sisekharan, V. (1968). Conformation of polypeptides and proteins. Adv Protein Chem 23, 283-438.

Reimer, U., Scherer, G., Drewello, M., Kruber, S., Schutkowski, M., and Fischer, G. (1998). Side-chain effects on peptidyl-prolyl *cis/trans* isomerization. J Mol Biol 279, 449-460.

Sood, A. and Panchagnula, R. (2001). Peroral route: an opportunity for protein and peptide drug delivery. Chem Rev 101, 3275-3303.

Stein, R. L. (1993). Mechanism of enzymatic and nonenzymatic prolyl *cis-trans* isomerization. Adv Protein Chem 44, 1-24.

Stewart, D. E., Sarkar, A., and Wampler, J. E. (1990). Occurance and role of *cis* peptide bonds in protein structures. J Mol Biol 214, 253-260.

Vanhoof, G., Gossens, F., De Meester, I., Hendriks, D., and Scharpe, S. (1995). Proline motifs in peptides and their biological processing. FASEB J. 9, 736-44.

Walker, J. R., Altman, R., Warren, J., Altman, E. (2002). Stabilization of peptides using protein-based motifs. Submitted.

Walker, J. R., Roth, J. R., and E. Altman. 2001 An *in vivo* study of novel bioactive petides that inhibit the growth of *Escherichia coli*. J Peptide Res 58, 380-388.

Warren, J. W., Walker, J. R., Roth, J. R. and E. Altman. 2000. Construction and characterization of a highly regulable expression vector, pLAC22 and pLAC33. Plasmid 44, 138-151.

Weiner, S. J., Kollman, P. A., Nguyen, D. T., Case, D. A. (1986) All atom force field for simulations of proteins and nucleic acids. J Comp Chem 7, 230-252.

Williamson, M. P. (1994). The structure and function of proline-rich regions in proteins. Biochem J 287 249-260.

Yao, J., Feher, V. A., Espejo, F., Reymond, M. T., Wright, P. E., and Dyson, H. J. (1994). Stabilization of a type VI turn in a family of linear peptides in water solution. J Mol Biol 243, 736-753.

Yaron, A. and Naider, F. (1993). Proline-dependent structural and biological properties of peptides and proteins. Crit Rev Biochem Mol Biol 28, 31-81.

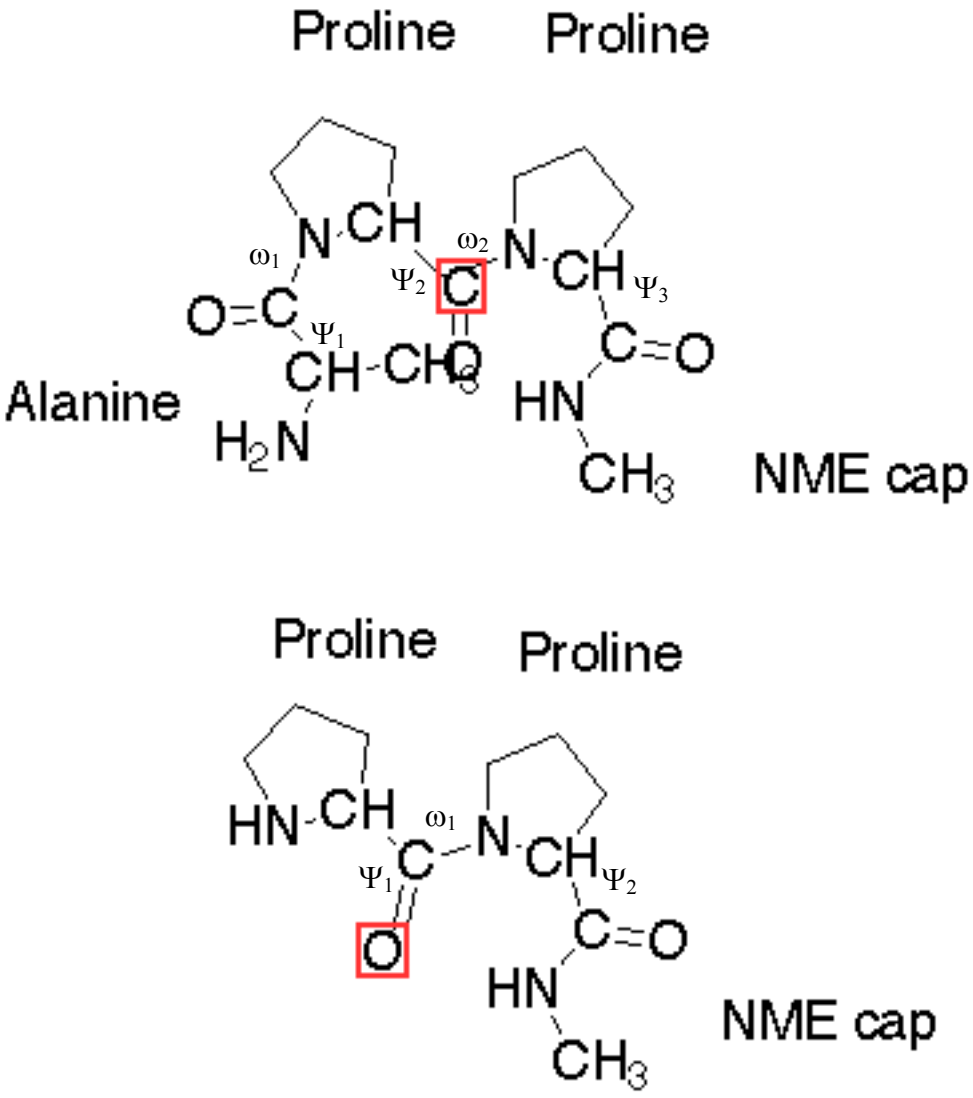


Figure 4.1 The APP (top) and PP structure used for the systematic search by Sybyl with the peptide bond angles (Ψ and ω) chosen for incremental rotations.

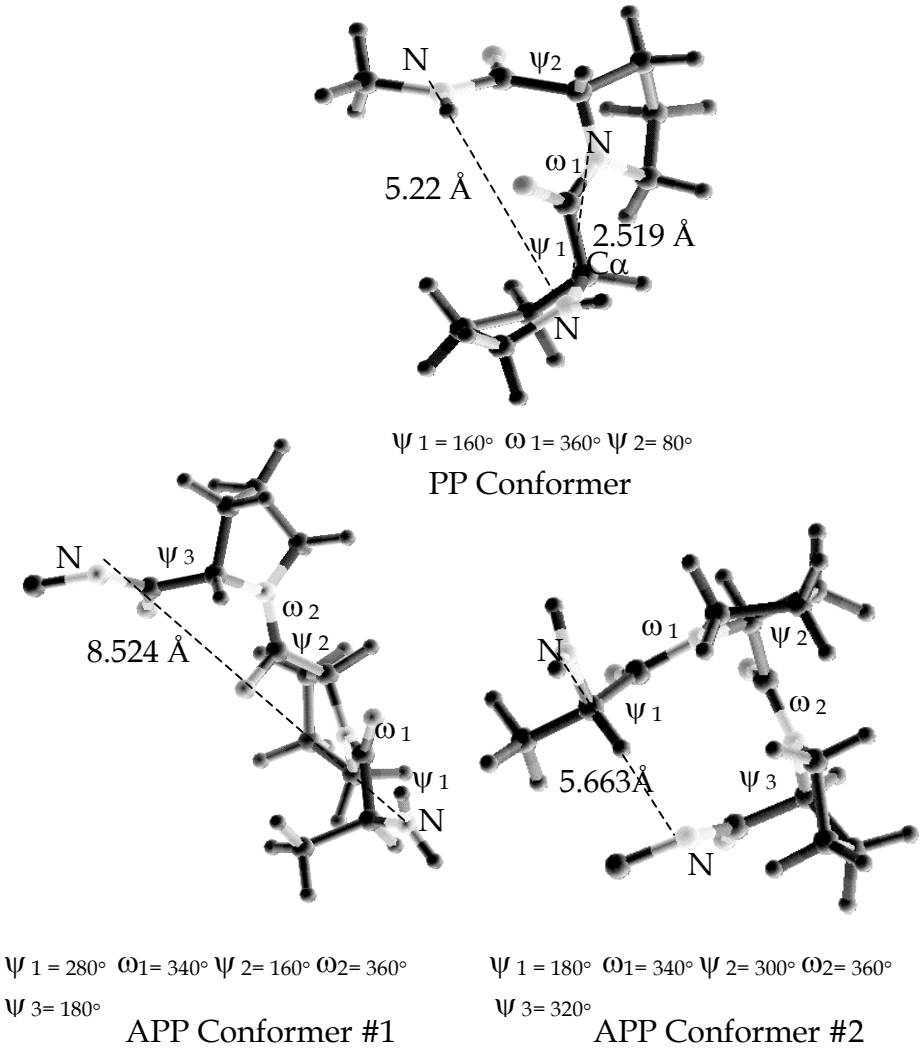
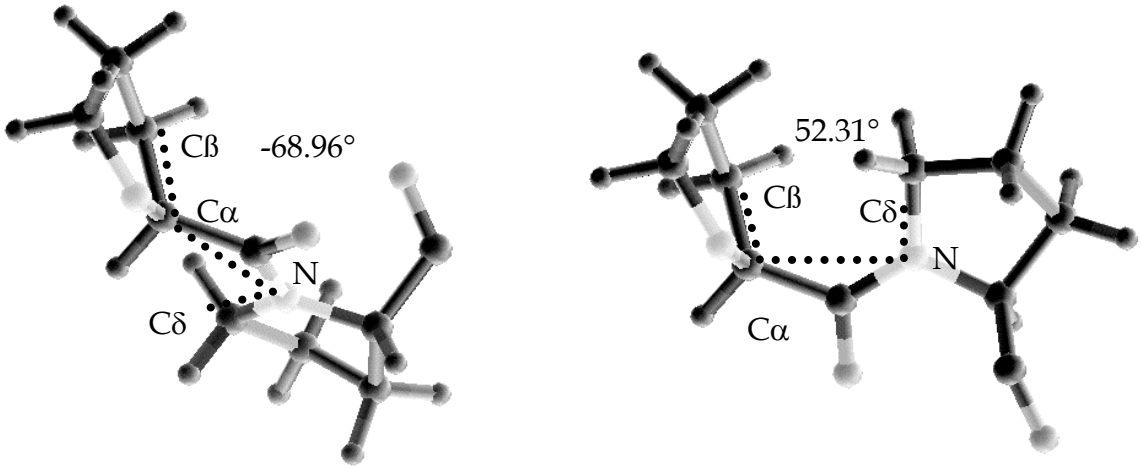


Figure 4.2 Lowest energy conformers calculated of PP (PP) and APP (APP). Torsional angles (Ψ and ω) and distances (Å) measured as indicated in figure.



PP dihedral from APP
Confirmation #1

PP dihedral from APP
Conformation #2

Figure 4.3 The different lowest energy conformers with dihedrals calculated between the two prolines of APP.

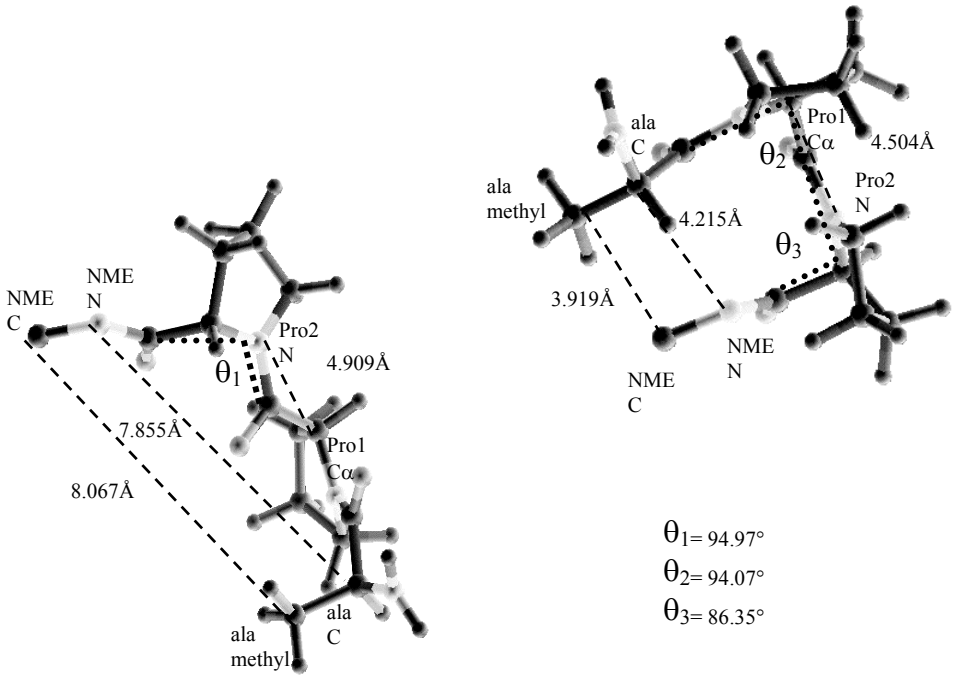


Figure 4.4 The different low energy conformers of APP with measured distances as indicated as well as angles observed within each conformer.

CHAPTER 5

STABILIZING PEPTIDES WITH IMPROVED PROLINE PROTECTIVE MOTIFS

Introduction

Synthetic peptide drugs with high efficacy often exhibit short half-lives when administered in whole animal systems. For example, glucagon-like peptide 1 (GLP-1), a peptide used for the treatment of diabetes, and the novel anti-HIV peptide, T140, have half-lives on the order of a few minutes (Gallwitz, *et al.,* 2000; Tamamura, *et al.,* 2001). Degradation of peptide drugs is due to the activity of peptidases - proteins that hydrolyze peptide bonds. Peptidases can attack internal peptide bonds, splitting the peptide into fragments, or they can cleave off one or two amino acids from the terminal ends (Bai, *et al.,* 1992; Brownlees and Williams, 1993; Miller, 1996; Rawlings and Barrett, 1993). One solution to avoiding terminal degradation is the addition of protective end groups such as amide or acetyl groups (Wang, 1999). Unfortunately, these motifs are not protective in all situations nor are they without effects on all peptide drugs.

Novel protective motifs based on the amino acid proline were identified in previous studies (Walker, *et al.,* 2001). In particular, a motif of three amino acids, alanine-proline-proline (APP), increased the half-life of synthetic randomized peptides over three-fold in rat serum. Proline bonds are known for their steric

strain on peptide bonds and are able to resist cleavage by many peptidases (Mentlein, 1988; Yaron and Naider, 1993). Additionally, proline bonds found in *cis* conformation are known to resist cleavage by proline specific peptidases (Cunnigham and O'Connor, 1997; Walter, *et al.,* 1980). Interestingly, it was predicted that 10-30% of proline peptide bonds that occur in nature would prefer the *cis* conformation (Williamson, 1994; MacArthur and Thornton, 1991). Later, 43% of proteins listed in Brookhaven protein database were found to contain one or more *cis* prolyl bonds (Reimer, *et al.,* 1998).

Formation of the prolyl *cis* bond is affected by the preceding amino acid to proline (MacArthur and Thornton, 1991; Williamson, 1994). In general, aromatic residues, which tend to possess bulkier side chains, increased the occurrence of a *cis* isomer by 20% (Stewart, *et al.,* 1990; Frömmel and Preissner, 1990; Grathohl and Wüthrich, 1976; Yao, *et al.,* 1995). More specifically, tryptophan, tyrosine, and phenylalaine, can cause high ratios of proline *cis* bonds. Another amino acid that is commonly found to flank proline in *cis* conformation is glycine. Analysis of peptides in the protein structure database (PDB) indicates that a significant number of *cis* prolyl bonds also occurred between two prolines (Reimer, *et al.,* 1998).

Given the frequency of prolyl *cis* bonds in native proteins and proline's propensity to resist peptidase cleavage, the APP motif was modified by substituting alanine with amino acids having greater tendency to form a *cis* bond within the motif. The protein modeling program, Sybyl, was used to calculate

lowest energy conformations of modified proline motifs most likely to form at least one *cis* bond. Four modified motifs, all having one calculated *cis* bond, were chosen and the new motifs were incorporated into synthetic randomized 13-amino acid peptides. Based on degradation rates in rat blood serum, half-lives were calculated and compared to the original motif, APP.

Material and Methods

Modeling of modified motifs

All protein conformation studies were performed with the Tripos program, Sybyl, and amino acid sequences were created with the protein building feature of Sybyl. An N- methyl group (NME) was added to the carboxyl end of the second proline to simulate the effect of a peptide chain attached to the protective motif. Alanine was replaced with the amino acids tryptophan, tyrosine, histidine, phenylalanine, proline and glycine. Two other amino acids, serine and lysine, were also used as control motifs containing only *trans* bonds. Kollman-all charges were loaded on each structure and then minimizations were performed with the Tripos force field (Clark, *et al.,* 1989) with settings of 1000 iterations, a gradient of 0.5 kcal/mol and a dielectric constant of 4.0. Figure 5.1 details the amide bonds chosen for rotation and rotations were performed in increments of 20°. Lowest energy conformers were computed only for a span of 10 kcal/mol since an energy minimum was desired.

Peptide synthesis

Biotinylated randomized peptides were synthesized by Sigma-Genosys. Peptide sequences with their respective designation were as follows: XXXXXXKXXXXXA (XXX), APPXXXKXXXXXX (APP), GPPXXXKXXXXXX (GPP), HPPXXXKXXXXXX (HPP), SPPXXXKXXXXXX (SPP), FPPXXXKXXXXX (FPP), and PPPXXXKXXXXXX (PPP), where A, P, G, H, and F, are standard single letter amino acid designation, and X designated an equal molar mixture of all 20 natural L-amino acids. Lysine (K) was required at the middle of each peptide for biotinylation, which in turn was used for quantification.

Rat serum

A Sprague-Dawley rat was euthanized with CO_2, whole blood was extracted via heart puncture and serum was prepared as described in Chapter 3.

Results

The lowest energy conformer for each substituted motif was examined for the presence of predicted *cis* bonds. Figure 5.2 shows the structures of all five motifs along with the APP motif. The motifs containing tryptophan, tyrosine, phenylalanine, proline, glycine, and histidine all exhibited a *cis* bond between proline 2 and proline 3, while motifs with alanine, lysine, and serine all exhibited *trans* bonds. Figure 5.3 shows the special difference between *trans* and *cis* bonds between two prolines. FPP, GPP and HPP all exhibited very similar *cis* arrangements with regard to the Cα and carboxyl groups of proline 3. Proline 3 of PPP however, was slightly different in its configuration. The doubly bonded

oxygen of the carboxyl group, as well as the NME group, was rotated 180° in comparison to FPP, GPP and HPP.

The half-lives for the substituted motifs were determined using rat serum as described previously (Walker, *et al.,* submitted), and results are shown in Table 5.1. Rat serum was chosen as the extract of choice for degradation studies because the APP motif had shown significant ability to improve peptide half-life, and rat serum is an accepted prognosticator for pharmaceutically relevant models.

Substituting histidine and serine in the proline motif provided enough protection to give the randomized peptides half-lives similar to those for the APP motif. Phenylalanine and glycine substituted motifs showed little increase in peptide half-life above the APP standard, while the PPP motif yielded an almost three-fold increase over APP, and over a five-fold increase over unprotected peptides.

Discussion

Prolyl residues can assume either of two distinct conformations, *trans* or *cis*, when incorporated into peptides. The ratio of *trans* to *cis* prolyl conformers can vary in different peptides. The *trans* conformer is usually preferred due to its lower energy, and is generally found in greater abundance (MacArthur and Thornton, 1991; Reimer, *et al.,* 1998). Although less common, *cis* conformers are required for certain biological functions, and are less likely to be degraded by peptidases (Brauer, *et al.,* 2002; Merker, *et al.,* 1996). This study suggests that *cis*

isomers may be used to increase resistance to peptidase activity. The PPP motif, which extended peptides' half-life, displayed a unique *cis* conformation between the two prolyl residues in comparison to the *cis* conformers of other motifs. Although the FPP, HPP and GPP motifs were also predicted to possess *cis* bonds between their prolyl residues, the ratio of *trans*:*cis* bonds may be higher in these motifs. Degradation studies suggest that GPP and FPP had a greater ratio of *cis* to *trans* isomers than HPP, while the ratio was highest in PPP.

Another study that investigated the effect of the ratio of *trans* to *cis* conformers in relation to susceptibility to degradation examined the angiotensin-converting enzyme (ACE) substrate, benzoyl-phenylalanine-glycyl-proline (BPGP). This tripeptide exists as *trans* and *cis* isomers at a ratio of 56:44. After a single passage through rabbit lung, nuclear magnetic resonance spectroscopy showed that only the *cis* isomer remained and the *trans* isomer had been hydrolyzed by ACE. This provided strong evidence that the *cis* bond protected the peptide from cleavage by ACE (Merker, *et al.,* 1993; Merker, *et al.,* 1996). In another study, dipeptidyl peptidase IV was shown cleave only peptides containing the *trans* conformer of proline and not the *cis* conformer (Fisher, *et al.,* 1983). The results support the contention that the PPP motif in *cis* configuration provided better protection for the randomized peptide by limiting peptidase degradation.

Currently, terminus improvement of peptide drugs is limited to the use of acetylation and amidation. Previous studies have shown that the APP motif

performs better than either acetylation or amidation in head-to-head comparative studies (Walker, *et al.,* submitted). These results could provide researchers with a protein-based stabilization motif that is superior to those currently available.

References

Bai, J. P. F. and Amidon, G. L. (1992). Structural specificity of mucosal cell transport and metabolism of amino acids and peptide drugs: implication for oral peptide delivery. Pharm Res 9, 969-978.

Brauer, A. B. E., Domingo, G. J., Cooke, R. M., Matthews, S. J., and Leatherbarrow, R. J. (2002). A conserved *cis* peptide bond is necessary for the acitivity of Bowman-Birk inhibitor protein. Biochemistry 41, 10608-10615.

Brownlees, J. and Williams, C.. H. (1993). Peptidases, peptides at the mammalian blood-brain barrier. J Neurochem 60, 793-803.

Clark, M., Cramer, III, R. D., Van Opdenbosch, N. (1989). Validation of the general purpose Tripos 5.2 force field. J Comp Chem 10, 982-1012.

Cunningham, D. F. and O'Connor, B. (1997). Proline specific peptidases. Biochim Biophys Acta 1343, 160-186.

Fisher, G., Heins, J., and Barth, A. (1983). The conformation around the peptide bond between the P_1- and P_1-position is important for catalytic activity of some proline-specific proteases. Biochem Biophys Acta 742, 452-462.

Frömmel, C. and Preissner, R. (1990). Prediction of prolyl residues in *cis*-conformation in protein structures on the basis of the amino acid sequence. FEBS Lett 277, 159-163.

Gallwitz, B., Ropeter, T., Morys-Wortmann, C., Mentlein, R., Siegel, E. G., and Schmidt, W. E. (2000). GLP-1 analogues resistant to degradation by dipeptidyl-peptidase IV in vitro. Regul Pept 86, 103-111.

Grathwohl, C. and Wüthrich, K. (1976). The X-Pro peptide bond as an NMR probe for conformational studies of flexible linear peptides. Biopolymers 15, 2025-2041.

MacArthur, M. W. and Thorton, J. M. (1991). Influence of proline residues on protein conformation. J Mol Biol 218, 397-412.

Mentlein, R. (1988). Proline residues in the maturation and degradation of peptide hormones and neuropeptides. FEBS Lett 234, 251-256.

Merker, M. P., Armitage, I. M., Audi, S. H., Kakalis, L. T., Linehan, J. H., Maehl, J. R., Roerig, D. L., and Dawson, C. A. (1996). Impact of angiotensin-converting

enzyme substrate conformation on fractional hydrolysis in lung. Am J Physiol 270, L251-L259.

Merker, M. P., Dawson, C. A., Bongard, R., Roerig, D., Haworth, S. and Linehan, J. (1993). Angiotensin converting enzyme preferentially hydrolyzes the *trans* isomer of a proline containing substrate. J Appl Physiol 62, 1519-1524.

Miller, C. G. (1996). Protein degradation and proteolytic modification. In *Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology, 2nd edition, Ed. Neidhardt, F.C. (ASM Press: Washington, D.C..), pp. 938-954.

Rawlings, N. D., and Barrett, A. J. (1993). Evolutionary family of peptidases. Biochem J 290, 205-218.

Reimer, U., Scherer, G., Drewello, M., Kruber, S., Schutkowski, M., and Fischer, G. (1998). Side-chain effects on peptidyl-prolyl *cis/trans* isomerization. J Mol Biol 279, 449-460.

Stewart, D. E., Sarkar, A., and Wampler, J. E. (1990). Occurrence and role of cis peptide bonds in protein structures. J Mol Biol 214, 253-60.

Tamamura, H., Omagari, A., Hiramatsu, K,. Gotoh, K., Kanamoto, T., Xu, Y., Kodama, E., Matsuoka, M., Hattori, T., Yamamoto, N., Nakashima, H., Otaka, A., and Fujii. N. (2001). Development of specific CXCR4 inhibitors possessing high selectivity indexes as well as complete stability in serum based on anti-HIV peptide Ti40. Bioorg Med Chem Lett 11, 1897-1902.

Wang, W., Jiang, J., Ballard, C. E., and Wang, B. (1999). Prodrug approaches to the improved delivery of peptide drugs. Curr Pharm Des 5, 265-287.

Walker, J. R., Roth, J. R. and Altman, E. (2001). An *in vivo* study of novel bioactive peptides that inhibit the growth of *Escherichia coli*. J Peptide Res 58, 380-388.

Walker, J. R., Altman, R., Warren, J., and Altman, E. (2002). Stabilization of peptides using protein based motifs. Submitted.

Walter, R., Simmons, W. H., and Yoshimoto, T. (1980). Proline specific endo- and exopeptidases. Mol Cell Biochem 30, 111-127.

Williamson, M. P. (1994). The structure and function of proline-rich regions in proteins. Biochem J 287 249-260.

Yao, Z-J., Kao, M. C.. C., and Chung, M. C.. M. (1995). Epitope identification by polyclonal antibody from phage-display random peptide library. J Protein Chem 14, 161-166.

Yaron, A. and Naider, F. (1993). Proline-dependent structural and biological properties of peptides and proteins. Crit Rev Biochem Mol Biol 28, 31-81.

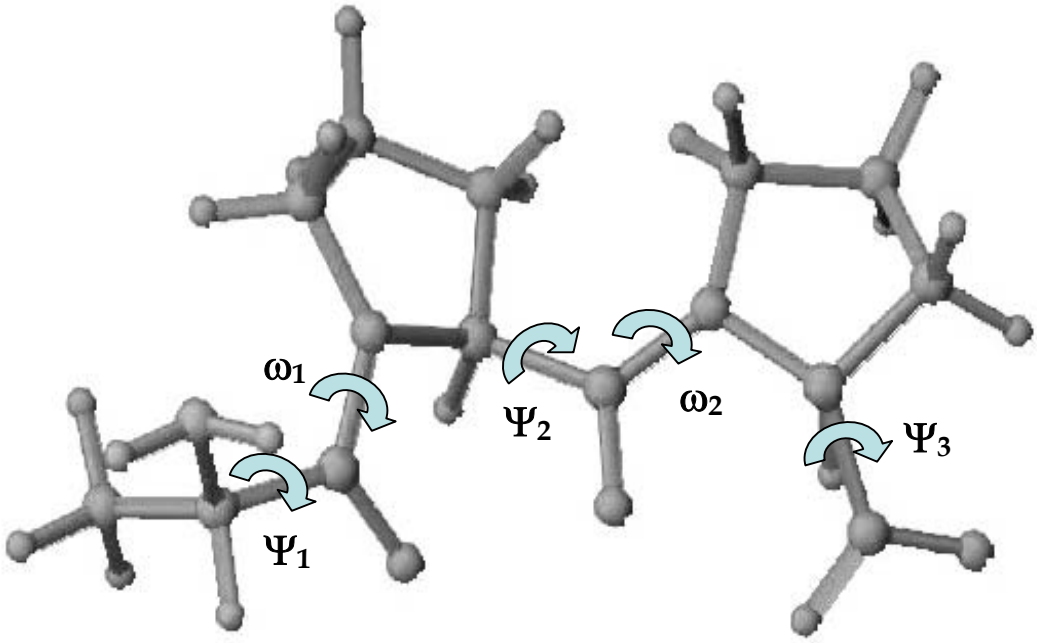


Figure 5.1 Ψ and ω indicate the peptide bonds that were selected for rotation in determining lowest energy conformers. APP is shown here. Rotations occurred in 20° increments using the systematic search feature of the Tripos molecular modeling program Sybyl.

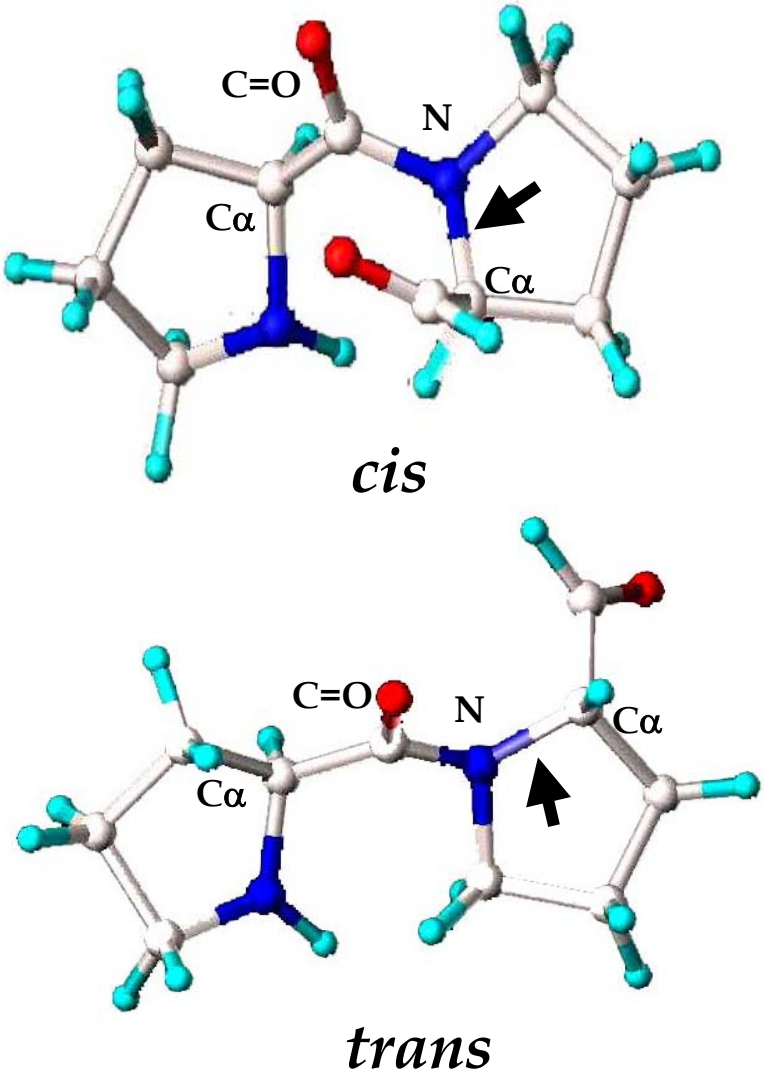


Figure 5.2 *Cis* and *trans* arrangements of the planar proline-proline peptide. The arrow indicates the change in arrangement of the second proline's N-Cα bond in relation to the first proline's C=O group.

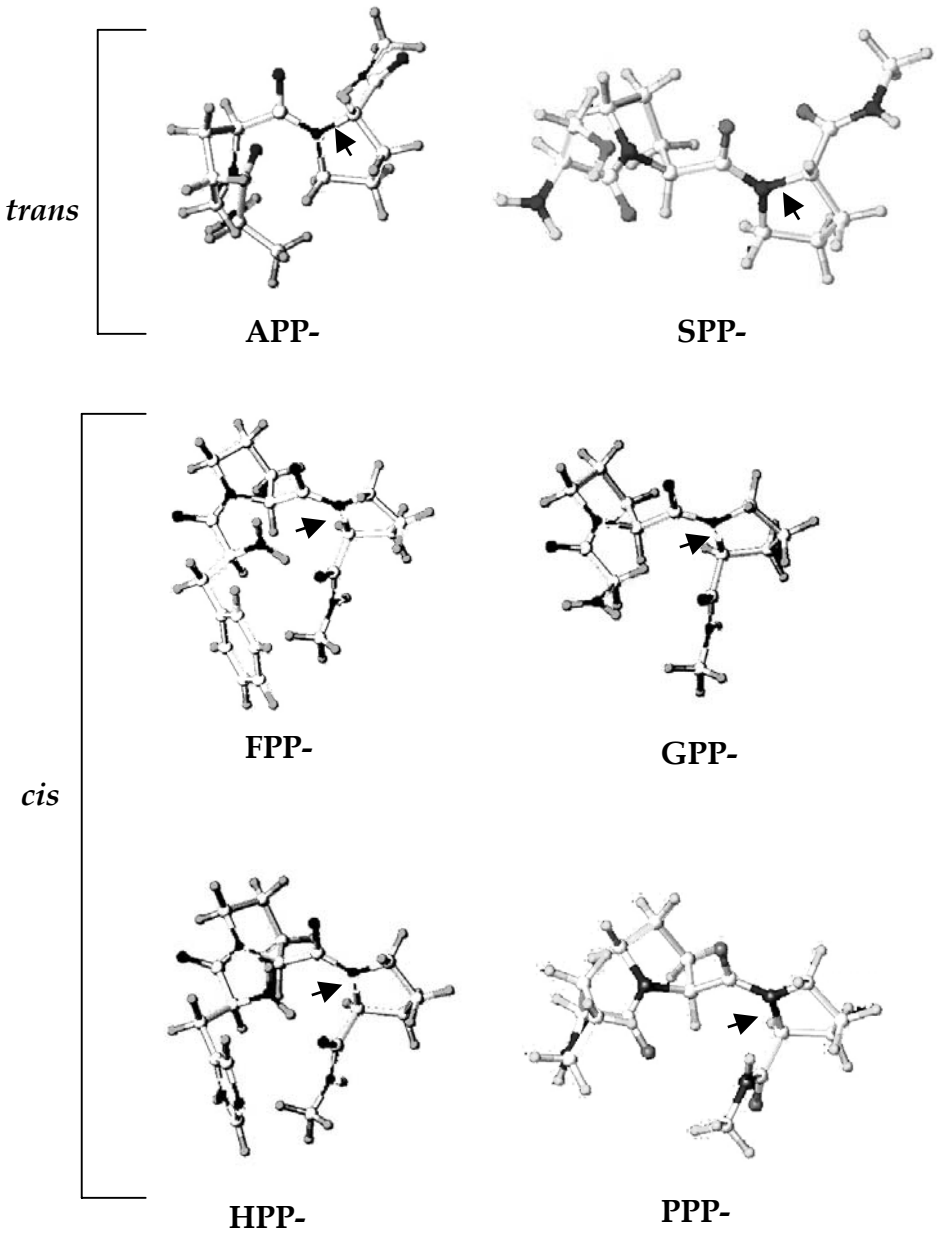


Figure 5.3 Lowest energy conformers of modified motifs arranged with arrows to show the *cis* or *trans* peptide bond between proline 2 and proline 3.

Table 5.1 Half-life increases of peptides containing modified proline motifs in rat serum

Sample	Half-life in minutes with 10mg/ml rat serum	Fold increase
XXX	34.3	-
APP-	65.1	1.9
HPP-	62.0	1.8
FPP-	104.4	3.0
SPP-	67.8	2.0
PPP-	188.0	5.5
GPP-	109.3	3.2

CHAPTER 6

BIOTINYLATED PEPTIDE UPTAKE BY *ESCHERICHIA COLI* AND OTHER GRAM-NEGATIVE BACTERIA[1]

[1] Walker, J. R. and E. Altman. To be submitted to *Science*.

Abstract

Biotinylated peptides of up to 30 amino acids can be taken up by *Escherichia coli.* Uptake is dependent on the biotin transporter encoded by *birB/bioP* and can be inhibited competitively by free biotin or avidin. Biotinylated peptides can also be taken up by other Gram-negative bacteria, such as *Salmonella typhimurium* and *Pseudomonas aeruginosa*. This finding may make it possible to create new peptide antibiotics for use against Gram-negative pathogens.

Introduction

The outer membrane of Gram-negative bacteria functions as a molecular sieve and allows few molecules to passively diffuse into the periplasm. Porins in the outer membrane allow the transport of larger molecules, and may be specific or non-specific in their molecular recognition. Non-specific porins, such as OmpF, OmpC and PhoE, allow rapid passage of small hydrophilic molecules (Nikaido, 1996; Nikaido and Vaara, 1985). The Opp permease has a specificity for oligopeptides and their uptake is dependent upon size, hydrophobicity and charge (Payne and Gilvarg, 1968; Barak and Gilvarg, 1975; Naider and Becker, 1975).

It is well documented that *Escherichia coli* cannot take up peptides larger than 650 daltons, about the size of a penta- or hexapeptide. The size exclusion limit for peptide uptake in other Gram-negative organisms, such as *Salmonella typhimurium,* has been found to be similar to that of *E. coli* (Payne, 1976, 1980; Payne and Smith, 1994). In contrast, Gram-positive bacteria can take up much

larger peptides. For example, *Lactococcus lactis* can take up peptides over 18 residues in length (2140 daltons) (Detmers, *et al*., 1998), while *Bacillus megaterium* can import molecules of up to 10,000 daltons (Scherrer and Gerhardt, 1971).

While conducting an *in vivo* screen for peptides that could inhibit the growth of *Staphylococcus aureus*, an eleven amino acid biotinylated peptide was added extracellularly to cultures of *S. aureus* and *E. coli,* which should not have been able to take up the 1,300 dalton peptide. The peptides had been biotinylated so they could be quantitated on Western blots using a chemiluminescent reagent. Surprisingly, the peptide was taken up by both *S. aureus* and *E. coli* within 5 minutes of addition. This observation contradicted the known size exclusion limit of *E. coli* and suggested that biotinylation of peptides may provide for peptide uptake via the biotin transport system.

Material and Methods

Bacterial strains

E. coli MG1655 (wild-type F-λ-), *E. coli* S1036 (Δ*bio*61 *bioP*98 (up promoter) *recA*1 *thi rpsL* λ *b*515 *b*519 *galq*6 *red*270 *cI*857), *E. coli* S1039 (*birB*ts13 Δ*bio*61 *bioP*98 (up promoter) *recA*1 *thi rpsL* λ *b*515 *b*519 *galq*6 *red*270 *cI*857), *Pseudomonas aeruginosa* ATCC9721, *S. typhimurium* LT2, and *S. aureus* ATCC25923 were the bacterial strains used in this study. *E. coli* S1036 and S1039 were derived from SK121, which is a derivative of SK98 (Ketner and Campbell, 1975) that contains a mutation in the λ prophage enabling SK121 to grow at 43^0C (A. Campbell, personal communication).

Media

Rich LB and minimal M9 media, as described by Miller, 1972, were used for *E. coli* MG1655 and *S. typhimurium* cultures. Rich LB and minimal media, as described by Gilleland, *et al.*, 1974, were used for *P. aeruginosa*. Tryptic soy broth and minimal media as described by Mah, *et al.*, 1967, were used for *S. aureus*. Rich LB and minimal media as described by Campbell, 1961, was used for *E. coli* S1036 and S1039.

Peptides and reagents

The randomized biotinylated peptides AXXXX[KBtn]XXXXA (11mer) and AXXXXXXXXXXXXX[KBtn]XXXXXXXXXXXXXXX (30mer) were synthesized by Sigma Genosys, The Woodlands, Texas, United States, where A denotes L-alanine, X denotes an equimolar mixture of all 20 natural L-amino acids, and KBtn denotes the L-lysine to which biotin has been attached. Biotin, thiamine, avidin, and bovine serum albumin were purchased from Sigma. NeutrAvidin Horseradish Peroxidase Conjugate and SuperSignal West Dura Extended Duration Chemiluminescent Substrate were purchased from Pierce, Rockford, Illinois.

Uptake assays

Cell cultures were started from overnight cultures in LB medium, washed once using minimal medium, diluted into minimal medium, and then incubated at 37°C overnight. Cultures were diluted again in minimal medium and incubated until they reached an OD_{550} of 0.5. The 30mer and 11mer biotinylated

peptides were added to medium at a concentration of 1 ug per mL of culture. After addition of the peptide to the cultures, 1 mL aliquots were extracted at time intervals up to an hour, cells were washed of extracellular peptide using fresh minimal media, and then boiled with SDS-PAGE gradient sample buffer. Samples were run on 10-16% tricine gradient gels (Schagger and von Jagow, 1987) and transferred to nitrocellulose membranes. The resulting Western blots were treated with NeutrAvidin Horseradish Peroxidase Conjugate and SuperSignal West Dura Extended Duration Chemiluminescent Substrate. The membranes were incubated for 5-10 minutes, then exposed to autoradiography film for 1 minute. Bands on the film were quantified using a gel analysis system, the AlphaEase 5.5 Densitometry Program from Alpha Innotech, San Leandro, California, United States.

To test for competitive inhibition, the 30-amino acid biotinylated peptide and an either equal molar amount or 10-fold molar excess of biotin, thiamine, avidin, or BSA was added to midlog cell cultures. After 10 minutes of incubation, 1 ml samples were removed and analyzed as described above for the general uptake assay.

Results

The ability of *E. coli* and *S. aureus* to import an 11-amino acid biotinylated peptide was tested using randomized peptides, instead of peptides with a specific sequence, in order to avoid nonspecific uptake that might be caused by certain amino acid sequences. Peptides were added to mid-log cultures of

bacteria, which were allowed to incubate for time intervals of up to 60 minutes. Cells were removed at specific times, pelleted, washed to remove peptide that had been not taken up by the cells, and then analyzed as described in Materials and Methods. As shown in Figure 6.1, both *E. coli* and *S. aureus* readily imported the 11-amino acid biotinylated peptide. Up to 75% of the peptide was imported within the first 5 minutes of incubation as determined using densitometry. To determine whether the import due to biotinylation was limited in *E. coli* to smaller peptides, a 30-amino acid biotinylated peptide was also tested for import into *E. coli* and *S. aureus*. As with the 11-amino acid biotinylated peptide, the 30-amino acid biotinylated peptide was also taken up by both *E. coli* and *S. aureus* (data not shown).

Competitive inhibition of import by biotin or avidin

To test whether large peptide uptake was specifically mediated by biotin, a competition experiment was conducted in both *E. coli* and *S. aureus* using biotin. Since large peptides can be readily taken up by Gram-positive bacteria, such as *S. aureus*, biotin should have no competitive effect. However, in *E. coli*, if the import was due to biotin, then free biotin should competitively block uptake. Figure 6.2 shows that the uptake of biotinylated peptides was blocked in *E. coli* by the addition of biotin, whereas biotin had no effect on the uptake of biotinylated peptides by *S. aureus*. Additionally, the competitive inhibition in *E. coli* was specific to biotin as another similarly sized vitamin, thiamine, had no effect.

Because avidin is known to bind biotin (Green, 1963), avidin was also tested for the ability to competitively inhibit the uptake of biotinylated peptides in *E. coli*. Figure 6.3 shows that avidin competitively inhibited the uptake of biotinylated peptides in *E. coli*, but another similarly sized protein, bovine serum albumin, had no effect.

Import of biotinylated peptides is dependent on the biotin transport system

The biotin transport system in *E. coli* has been well characterized and mutants of the biotin transporter, *birB*/*bioP*, are available (Eisenberg, *et al.*, 1975; Campbell, *et al.*, 1980). If the import of biotinylated peptides in *E. coli* was occurring via the biotin transport system, then *birB* mutants should not be able to import biotinylated peptides. Figure 6.4 shows that a wild-type $birB^{+}$ isogenic strain was able to take up biotinylated peptide, while a $birB^{-}$ mutant strain was not.

Biotinylated peptide uptake by other Gram-negative bacteria

In a test of other Gram-negative bacteria it was found that both the 11- and 30-amino acid biotinylated peptides were readily imported by both *S. typhimurium* and *P. aeruginosa*. Figure 6.5 shows uptake of the 30-amino acid biotinylated peptide by *S. typhimurium* and *P. aeruginosa*.

Discussion

It has been well established that Gram-negative bacteria, such as *E. coli,* can only import small peptides. In this study, biotinylated peptides up to 30-amino acids in length were shown to be taken up by *E. coli,* and that uptake was

dependent on the biotin transporter encoded by *birB*/*bioP*. This uptake can be competitively inhibited by free biotin or avidin. What is known about biotin function in *E. coli* is consistent with the finding that biotin can be used to facilitate the uptake of peptides via the biotin transporter in *E. coli*.

Biotin can be synthesized, as well as imported, by *E. coli,* and the genes involved in biotin biosynthesis and transport are repressible by biotin (Guha, *et al.*, 1971). The biotin transport system is regulated independently of the biosynthetic pathway (Pai, 1973). *E. coli* readily imports the vitamin when it is available, and concomitantly, represses biotin synthesis. Biotin uptake is specific and energy-dependent, thus the vitamin can accumulate against a concentration gradient (Prakash and Eisenberg, 1974; Piffeteau, *et al.*, 1982; Piffeteau and Gaudry, 1985). The maximal rate of uptake is observed during exponential growth phase and glucose has been shown to increase biotin uptake slightly (Piffeteau, *et al.*, 1982). The rate of biotin uptake has also been shown to increase proportionally to the amount of extracellular biotin available (Prakash and Eisenberg, 1974).

The first biotin transporter mutant was discovered by Campbell, *et al.*, 1972. The mutant was named *bir* for biotin retention, and the mutation abolished the ability of *E. coli* to take up biotin. Eisenberg, *et al.*, 1975 independently isolated a mutant that abolished biotin uptake, which they termed *bioP*. Campbell, *et al.*, 1980, renamed their original *bir* mutant to *birB* and showed via genetic mapping experiments that *birB* and *bioP* mutants were identical.

In some respects it seems surprising that the biotin transport system can be used to facilitate the uptake of large peptides. Biotin has a molecular weight of 244, making it relatively small in comparison to an 11-amino acid peptide having an average molecular weight of 1269 or a 30-amino acid peptide with an average molecular weight of 3361. Clearly the biotin uptake system is flexible enough to accommodate large molecules.

There is contradictory evidence with regards to how biotin's structure affects its ability to be taken up by *E. coli*. Prakash and Eisenberg, 1974, stated that while the ureido ring of biotin must be intact for uptake, modification of the side chain has little effect. However, Piffeteau, *et al.*, 1982, suggested that modifications to the side chain of biotin could drastically affect biotin's ability to be taken up, and that the carboxyl group on the side chain was essential for biotin uptake. As shown in Figure 6.6, for the biotinylated peptides used in this study, the biotin carboxyl group is joined to the side chain of lysine via an amide bond, making the carboxyl group of biotin unavailable for recognition. This supports the argument of Prakash and Eisenberg that the side chain of biotin is not critical for uptake. Thus, with respect to biotinylated peptides, it appears that the ureido ring is more likely responsible for biotin recognition and uptake.

Interestingly, biotinylated molecules are currently being investigated for drug delivery in mammalian cells. Avidin-based drugs that bind to biotinylated vectors are being used to promote delivery across the blood brain barrier (Bonfils, *et al.*, 1992; Pardridge, 2002; Song, *et al.*, 2002), while

antitumor toxins and imaging agents coupled to streptavidin are being delivered using biotinylated antibodies (Press, *et al.*, 2001; Hussey and Peterson, 2002). Biotinylation has been shown to promote the delivery of polyethylene glycol camptothecin conjugates into human ovarian carcinoma cells (Minko, *et al.*, 2002), and it can increase the cellular uptake of polyethylene glycol TAT nonapeptide conjugates into mammalian cell lines (Ramanathan, *et al.*, 2001).

The finding that biotinylated peptides can be taken up by Gram-negative bacteria such as *E. coli*, *S. typhimurium* and *P. aeruginosa*, presents an intriguing possibility for the development of antibacterial peptides. Given the abundance of naturally occurring antibacterial peptides and the increased interest in designing new synthetic peptide drugs, researchers have so far been trying to develop novel peptide antibiotics that can inhibit the function of key intracellular targets identified through genomics. Researchers have been focusing on Gram-positive bacteria where the uptake of large peptides is not problematic. The use of biotinylated peptides may make it possible to use this same approach to develop antibacterial peptides that can target Gram-negative bacteria.

References

Barak, Z. and Gilvarg, C. (1975). Specialized peptide transport system in *Escherichia coli*. J Bact 122, 1200-1207.

Bonfils, E., Mendes, C., Roche, A.-C., Monsigny, M., and Midoux, P. (1992). Uptake by macrophages of a biotinylated oligo-α-deoxythymidylate by using mannosylated streptavidin. Bioconj Chem 3, 277-284.

Campbell, A. (1961). Sensitive mutants of bacteriophage λ. Virology 14, 22-32.

Campbell, A., del Campilllo-Campbell, A., and Chang, R. (1972). A mutant of *Escherichia coli* that requires high concentrations of biotin. Proc Nat Acad Sci USA 69, 676-680.

Campbell, A., Chang, R., Barker, D., and Ketner, G. (1980). Biotin regulatory (*bir*) mutations of *Escherichia coli*. J Bact 142, 1025-1028.

Detmers, F. J. M., Kunji, E. R. S., Lanfermeijer, F. C., Poolman, B., and Konings, W. N. (1998). Kinetics and specificity of peptide uptake by the oligopeptide transport system of *Lactococcus lactis.* Biochem 37, 16671-16679.

Eisenberg, M. A., Mee, B., Prakash, O. M., and Eisenberg, M. R. (1975). Properties of α-dehydrobiotin-resistant mutants of *Escherichia coli* K-12. J Bact 122, 66-72.

Gilleland, Jr., H. E., Stinnet, J. D., and Eagon, R. G. (1974). Ultrastructural and chemical alteration of the cell envelope of *Pseudomonas aeruginosa*, associated with resistance to ethylenediaminetetraacetate resulting from growth in a Mg^{2+}-deficient medium. J Bact 117, 302-311.

Green, N. M. (1963). Avidin. The nature of the biotin-binding site. Biochem J 89, 599-609.

Guha, A. (1971). Divergent orientation of transcription from the biotin locus of *Escherichia coli*. J Mol Biol 56, 53-62.

Hussey, S. L. and Peterson, B. R. (2002). Efficient delivery of streptavidin to mammalian cells: clathrin-mediated endocytosis regulated by a synthetic ligand. J Am Chem Soc 124, 6265-6273.

Ketner, G. and Campbell, A. (1975). A deletion mutation placing the galactokinase gene of *Escherichia coli* under control of the biotin promoter. Proc Natl Acad Sci USA 7, 2698-2702.

Mah, R. A., Fung, D. Y. C., and Morse, S. A. (1967). Nutritional requirements of *Staphylococcus aureus* S-6. Appl Microb 15, 866-870.

Miller, J. H. (1972). Experiments in Molecular Genetics (Cold Springs Harbor Laboratory Press: Cold Springs Harbor, N. Y.)

Minko, T., Paranjpe, P. V., Qiu, B., Lalloo, A., Won, R., Stein, S., and Sinko, P. J. (2002). Enhancing the anticancer efficacy of camptothecin using biotinylated poly(ethylene glycol) conjugates in sensitive and multidrug-resistant human ovarian carcinoma cells. Cancer Chemother Pharmacol 50, 143-50.

Naider, F. and Becker, J. M. (1975). Multiplicity of oligopeptide transport systems in *Escherichia coli*. J Bact 122, 1208-1215.

Nikaido, H. (1996). Outer membrane In *Escherichia coli* and *Salmonella* cellular and molecular biology. 2nd ed. Ed. Neidhardt F. C. (ASM Press: Washington D. C.), pp. 29-47.

Nikaido, H. and Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. Microbiol Rev 49, 1-32.

Pai, C. H. (1973). Biotin uptake in biotin regulatory mutant of *Escherichia coli*. J Bact 116, 494-496

Pardridge, W. M. (2002). Targeting neurotherapeutic agents through blood-brain barrier. Arch Nuerol 59, 35-40.

Payne, J. W. and Gilvarg, C. (1968). Size restriction on peptide utilization in *Escherichia coli*. J Bio Chem 243, 6219-6299.

Payne, J. W. and Smith, M. W. (1994). Peptide transport by micro-organisms. Adv Micro Physiol 36, 2-80.

Payne, J. W. (1976). Peptides and micro-organisms. Adv Microb Physiol 13, 55-113.

Payne, J. W. (1980). Transport and utilization of peptides by bacteria. In Microorganisms and Nitrogen Sources, Ed. Payne, J. W. (John Wiley & Sons: New York), pp. 212-256.

Piffeteau, A., Zamboni, M., and Gaudry, M. (1982). Biotin transport by a biotin-deficient strain of *Escherichia coli*. Biochim Biophys Acta 688, 29-36.

Piffeteau, A. and Gaudry, M. (1985). Biotin uptake: influx, efflux, and countertransport in *Escherichia coli*. Biochim Biophys Acta 816, 77-82.

Prakash, O. M. and Eisenberg, M. A. (1974). Active transport of biotin in *Escherichia coli* K-12. J Bact 120, 785-791.

Press, O. W., Cocoran, M., Subbiah, K., Hamlin, D. K., Wilbur, D. S., Johnson, T., Theodore, L., Yau, E., Mallett, R., Meyer, D. L., and Axworthy, D. (2001). A comparative evaluation of conventional and pretargeted radioimmunotherapy of CD20-expressing lymphoma xenografts. Blood 98, 2535-2543.

Ramanathan, S., Qiu, B., Pooyan, S., Zhang, G., Stein, S., Leibowitz, M. J., and Sinko. P. J. (2001). Targeted PEG-based bioconjugates enhance the cellular uptake and transport of a HIV-1 TAT nonapeptide. J Control Release 77, 199-212.

Scherrer, R. and Gerhardt, P. (1971). Molecular sieving by the *Bacillus megaterium* cell wall and protoplast. J Bact 107, 718-735.

Schägger, H. and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1-100 kDa. Anal Biochem 166, 368-379.

Song, B.-W., Vinters, H. V., Wu, D., and Pardridge, W. M. (2002). Enhanced neuroprotective effects of basic fribroblast growth factor in regional brain ischemia after conjugation to a blood-brain delivery vector. J Pharm Exper Therap 301, 605-610.

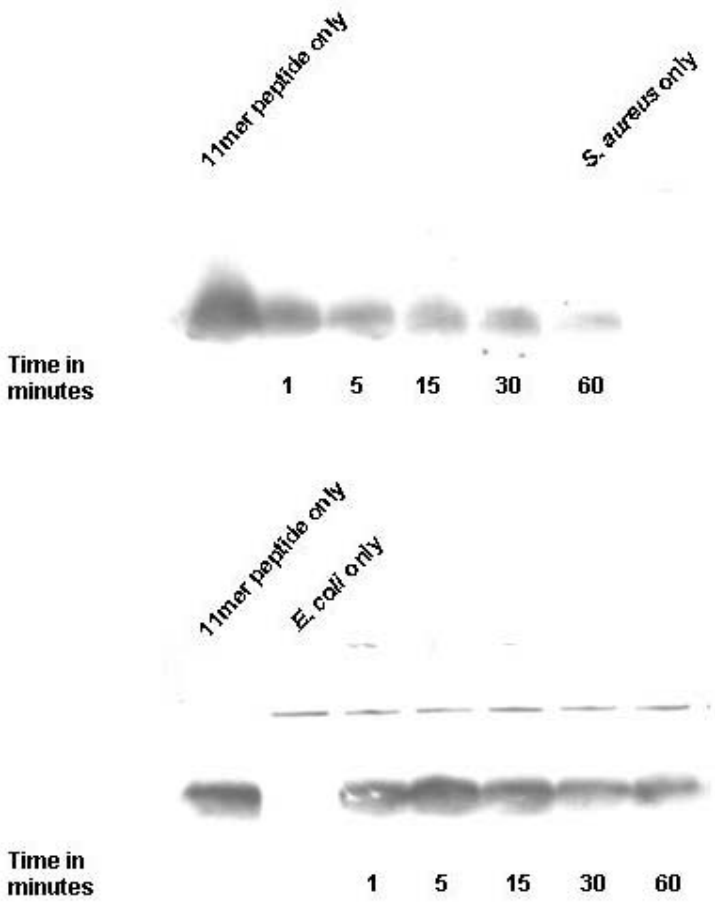


Figure 6.1 Uptake of an 11-amino acid biotinylated peptide by *S. aureus* and *E.* coli. The biotinylated peptide was added to mid-log cultures, aliquots were taken at different time intervals and analyzed as described in Material and Methods. Peptide only and cell only samples were included as controls.

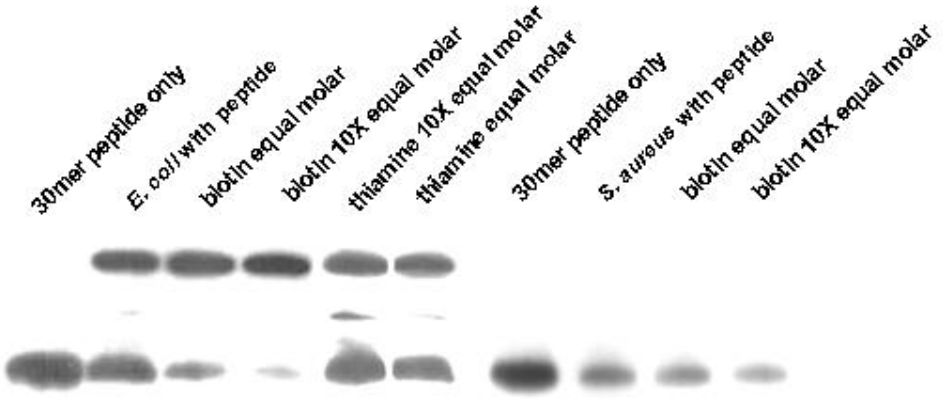


Figure 6.2 Effect of biotin on the uptake of a 30-amino acid biotinylated peptide in *E. coli* and *S. aureus*. Equal molar and 10X molar excess amounts of biotin or thiamine, and the biotinylated peptide were added to mid-log cultures. Aliquots were taken after 10 minutes of incubation and analyzed as described in Materials and Methods.

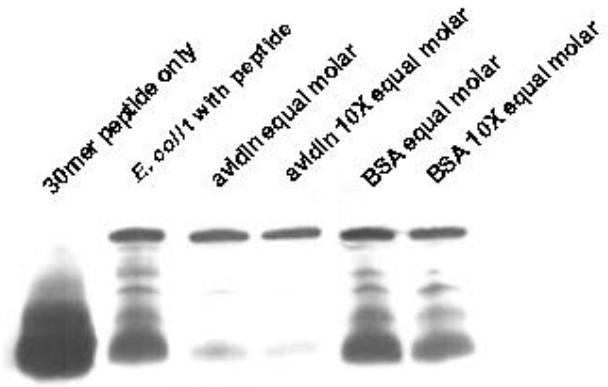


Figure 6.3 Effect of avidin on the uptake of a 30-amino acid biotinylated peptide in *E. coli*. Equal molar and 10X molar excess amounts of avidin or bovine serum albumin, and the biotinylated peptide were added to mid-log cultures. Aliquots were taken after 10 minutes of incubation and analyzed as described in Materials and Methods.

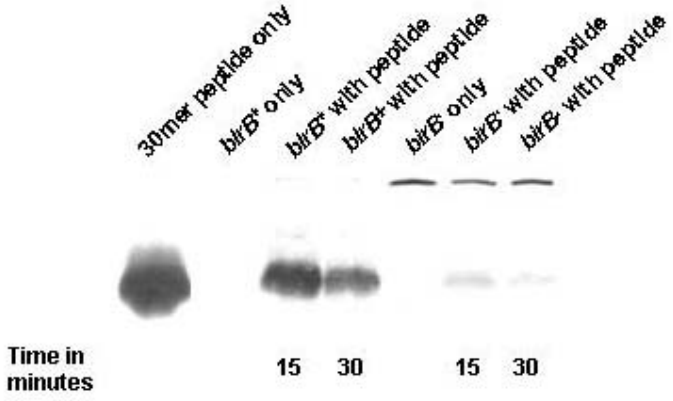


Figure 6.4 Effect of a *birB*$^{-}$ mutation on the uptake of a 30-amino acid biotinylated peptide in *E. coli*. The biotinylated peptide was added to mid-log cultures of *birB*$^{+}$ and *birB*$^{-}$ cells. Aliquots were taken after 10 minutes of incubation and analyzed as described in Materials and Methods. Peptide only and cell only samples were included as controls.

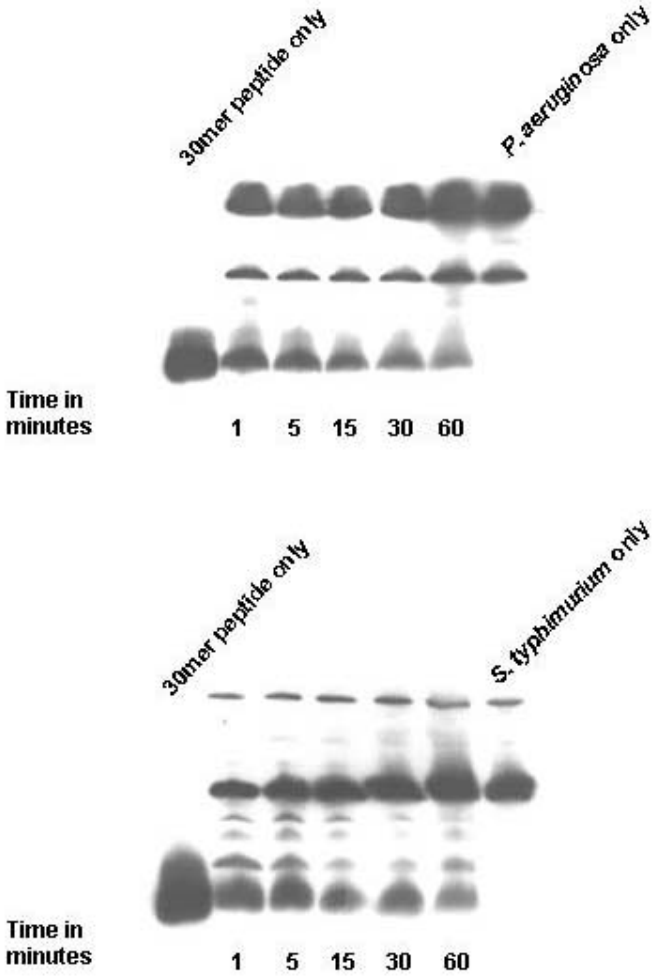


Figure 6.5 Uptake of a 30-amino acid biotinylated peptide by *S. typhimurium* and *P. aeruginosa*. The biotinylated peptide was added to mid-log cultures and aliquots taken at different time intervals were analyzed as described in Materials and Methods. Peptide only and cell only samples were included as controls.

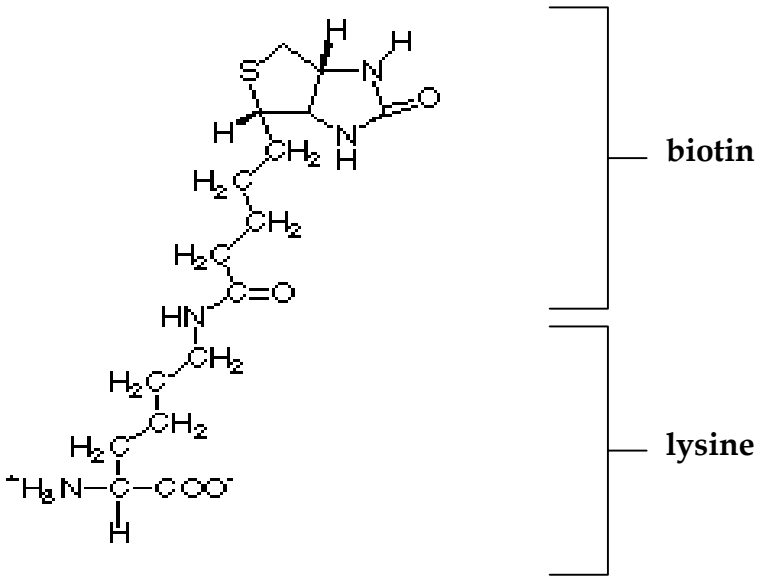


Figure 6.6 Attachment of biotin to lysine. The biotin and lysine moieties are indicated.

CHAPTER 7

CONCLUSIONS

Stability is an important aspect in the development of peptide drugs. This study investigated protective peptide motifs, the improvement of these protective motifs and, in addition, identified an uptake system in Gram-negative bacteria for biotinylated peptides. Novel protective motifs were identified by screening *E. coli* cells transformed with randomized peptide libraries that encoded 20-amino acid peptides. Two stabilizing motifs discovered in the study were a fusion to the protein, Rop, and prolines at peptide termini. Randomized peptide libraries modified by fusion of Rop to the amino or carboxyl end of the peptide produced potent inhibitory peptides at an averaged frequency of 1 in 527 transformed colonies. This was a 38-fold increase in the frequency of inhibitors identified from randomized peptide libraries. Additionally, libraries encoding proline at both termini of peptides yielded inhibitory peptides at a frequency of 1 in 625 – a 32-fold increase in frequency over unprotected libraries. These results indicated that both Rop and proline may provide peptide terminal protection from peptidases.

The proline motif was further evaluated using an *in vitro* assay to assess whether improvement of peptide half-life was due to an effect on peptidase susceptibility. Various motifs were tested, including one and two prolines and

the addition of a third amino acid, alanine, to the two proline motif. All motifs were added to either or both ends of the randomized peptides. The results indicated that APP protected peptides had longer half-lives compared to those having P or PP motifs.

The terminal end to which the motif was attached was also important for peptide half-life. The APP motif attached to the amino terminus of randomized peptides provided the longest half-lives compared to APP at both termini, as well as amidation or acetylation at the amino or carboxy termini, respectively. Molecular modeling of the APP and the PP motifs using Sybyl indicated that APP may form two distinct conformers while the PP motif forms only one. One of APP's conformers is a structure featuring two right angles along the peptide backbone that creates a highly "kinked" structure. In comparison to the extended structures of the other conformer of APP and the single conformer of PP, this "kinked" arrangement of APP's second conformer may confer to the peptide increased resistance to peptidase attack. The more protective nature of APP at the amino end of peptides suggested that aminopeptidases may be more of a threat in the intercellular environment than peptidases targeting carboxyl termini.

Improvement of the APP motif was accomplished by using molecular modeling to identify four derivative motifs with a preference for *cis* prolyl bonds. The modeled motifs were added to randomized peptides and tested with *in vitro* degradation assays. PPP, the best motif, provided a greater than five-fold

increase in peptide half-life over the unprotected peptide and almost a three-fold increase over the APP motif. PPP may prefer the *cis* conformers over the *trans* conformers, which may provide greater resistance to aminopeptidases.

A novel peptide uptake system in Gram-negative bacteria was also characterized during this study. Biotin is bound and transported across Gram-negative bacterial membranes by a biotin-specific uptake porin. Biotinylated peptides as long as 30 amino acids were transported across the cell wall via the biotin uptake system. These results suggest the biotin uptake system to be highly flexible with regard to molecular size of transported molecules. This process of utilizing a specific uptake system to transport other molecules may be considered a type of illicit transport. The use of biotin as an illicit peptide transporter may be applicable to the development of novel peptide delivery systems for Gram-negative organisms, especially for the delivery of antibiotic peptides targeting intracellular sites, which otherwise might not pass through the outer membrane.