THE BACTERIAL PRODUCTION OF ANTIMICROBIAL, CATIONIC PEPTIDES AND THEIR EFFECTS ON THE OUTER MEMBRANES OF GRAM-NEGATIVE BACTERIA

by

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ABSTRACT

Natural polycationic antibiotic peptides have been found in many different species of animals and insects and shown to have broad antimicrobial activity. To permit further studies on these peptides, bacterial expression systems were developed. Attempts to express these peptides with an N-terminal signal sequence were unsuccessful due to the lability of the basic peptides. Therefore, different fusion protein systems were tested, including fusions to glutathione-*S*-transferase (GST) and *Staphylococcus aureus* protein A. For GST, fusions to the defensin, human neutrophil peptide 1 (HNP-1), or a synthetic cecropin/melittin hybrid (CEME) were generally unstable if found in the soluble fraction of lysed cells, but were stable if found as insoluble inclusion bodies. In the course of these studies, we developed a novel method of purifying inclusion bodies, using the detergent octyl-polyoxyethylene, as well as establishing methods for preventing fusion protein proteolytic breakdown. Cationic peptides could be successfully released from the GST carrier protein with high efficiency by chemical means (cyanogen bromide digestion) and with low efficiency by enzymatic cleavage (using factor X_a). Fusions of protein A to cationic peptides were expressed in the culture supernatant of *S. aureus* clones and after affinity purification, CNBr digestion and column chromatography, pure cationic peptide was obtained. CEME produced by this procedure had the same amino acid content, amino acid sequence, gel electrophoretic mobility and antibacterial activity as CEME produced by protein chemical procedures.

Three cationic peptides, CEME, CEMA and melittin, were all found to have a broad range of antibacterial activity at concentrations that were comparable to conventional antibiotics. All three were found to permeabilize the outer membrane of *Pseudomonas aeruginosa* and *Enterobacter cloacae* to lysozyme and the hydrophobic probe 1-*N*-phenylnaphthylamine. CEMA

permeabilized membranes at concentrations 2- to 5-fold lower than CEME and 20-fold lower than melittin. In some cases, it disrupted membranes better than polymyxin B, a known potent permeabilizer. CEMA also had the highest binding affinity for purified *P. aeruginosa* LPS and whole cells, although CEME and melittin also bound strongly.

These data are discussed with special reference to the mechanism by which these peptides cross the outer membrane of Gram-negative bacteria. It is proposed that they utilize the self-promoted uptake pathway which has been suggested previously for other cationic antibiotics. As well, the potential for the use of cationic peptides as therapeutic antibiotics is discussed.

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LIST OF ABBREVIATIONS

Ap	ampicillin
AU	acid urea
bp	base pair
CEMA	CEME analogue
CEME	cecropin/melittin hybrid peptide (CA1-8M1-18)
CFU	colony forming unit
Cm	chloramphenicol
CNBr	cyanogen bromide
DEAE	diethylaminoethyl
EDTA	ethylenediamine tetraacetic acid
FPLC	fast protein liquid chromatography
GST	glutathione-S-transferase
HEPES	(N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) sodium salt
HNP	human neutrophil peptide
IGF-I	human insulin-like growth factor I
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactoside
Kan	kanamycin
kb	kilobase pair
kDA	kilodalton
KCN	potassium cyanide
LB-S	Luria-Bertani, no salt broth
LBNS	Luria-Bertani, normal salt broth
LPS	lipopolysaccharide
MCP	macrophage cationic peptide
MIC	minimum inhibitory concentration

MTPBS	mouse tonicity phosphate-buffered saline
NMR	nuclear magnetic resonance
NPN	1-*N*-phenylnaphthylamine
OD	optical density
O-POE	octyl-polyoxyethylene
PA	truncated *S. aureus* protein A
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMBN	polymyxin B nonapeptide
PMSF	phenylmethylsulfonyl fluoride
RP	reverse phase
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
Tet	tetracycline
TFA	trifluoroacetic acid
TLCK	Nα-p-tosyl-L-lysine chloromethyl ketone
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
TSS	transformation and storage solution
TST	50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.05% Tween 20
UV	ultra violet
WCLB	whole cell lysing buffer
Z	synthetic IgG binding domain

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DEDICATION

Throughout the duration of these studies, my wife, Gloria, has been the one person to share every up and down. Whether I needed a shoulder for sympathy, an ear for my frustrations or a smile of encouragement, she always had them just at the right time. This thesis, therefore, is not only a reflection of my work, it is a testimony to the love, patience and dedication that she has shown to me over these last years. Gloria, it is to you that I dedicate this thesis.

INTRODUCTION

A. *Pseudomonas aeruginosa.*

1. Clinical Considerations.

Pseudomonas aeruginosa is a Gram-negative, rod-shaped organism that can grow with limited minerals on a large number of carbon and energy sources. These minimal growth requirements enable *P. aeruginosa* to exist in very diverse environments, including soil, standing water and sediments. It also has other properties such as slime layers and adhesins that make it difficult to remove from colonized sites by mechanical means. *P. aeruginosa* is not usually a pathogen for healthy human beings, since it is unable to penetrate the host's first defense barrier, namely the skin and mucosa. However if that physical barrier is compromised and host defenses are weak, *P. aeruginosa* becomes prevalent in human infections of burns, eye injuries, and sites of major surgery and medical interventions involving instruments such as catheters or shunts. Another occurrence of *P. aeruginosa* infections is in patients whose non specific defense or immune systems are under duress, such as premature infants, patients with cystic fibrosis or neoplasia, and patients who are undergoing immunosuppressive drug treatment or whole body irradiation treatment. *P. aeruginosa* can therefore be categorized as a nosocomial opportunistic human pathogen.

Many different toxins which are produced by *P. aeruginosa* have been described (Döring et al., 1987) and it has been suggested that these may all contribute variably in different *P. aeruginosa* infections (Nicas and Iglewski, 1985). Given this multifactorial virulence, it is not suprising that *P. aeruginosa* is able to cause a wide variety of different medical problems such as

bacteremia, urinary tract infections, endocarditis and gastrointestinal infections (Pollack, 1990).

The clinical significance of this pathogen has been increasing steadily. During the period between 1975 and 1984, the relative frequency of *P. aeruginosa* in nosocomial infections (expressed as a percentage of all isolates) increased from 6.3% to 11.4%, making it second only to *E. coli* as the most frequently acquired hospital pathogen. Not only is the increase in *P. aeruginosa* infections a cause for concern, but also the fact that these infections are associated with a high mortality rate (Young, 1984; Hancock and Bell, 1989).

The increase in the incidence of *P. aeruginosa* infections is largely a testimony of its resistance to many forms of antimicrobial therapy (Bryan, 1979). Antibiotics that have been used to treat *P. aeruginosa* in the past, such as streptomycin, carbenicillin, tetracycline, chloramphenicol and trimethoprim, have become ineffective due to the emergence of resistant strains (Bryan, 1979). One of the characteristics of *P. aeruginosa* that confers such intrinsic resistance is the low permeability of its outer membrane (Angus et al., 1982). Although the outer membrane of *P. aeruginosa* is not remarkably different to those of other Gram-negative organisms, its permeability has been estimated to be twelve-fold (Nicas and Hancock, 1983b) to 100-fold (Yoshimura and Nikaido, 1982) lower than that of the *E. coli* outer membrane.

2. Outer Membrane Structure.

The envelope of *P. aeruginosa* is made up of three distinct components: the cytoplasmic or inner membrane, the periplasmic space and the outer membrane. The cytoplasmic membrane is a phospholipid bilayer membrane that is studded with as many as one hundred different proteins (Cronan et al.,

1987). The periplasm can be dissected into inner and outer periplasmic spaces. The former is relatively un-crosslinked and therefore appears as a gelatinus structure (Hobot et al., 1984). The latter, conversely, is highly crosslinked and plays a large role in the structural integrity of the cell (Oliver, 1987). It contains peptidoglycan (murein) which is a macromolecular combination of polysaccharides and peptide (Park, 1987). In addition to these components, the periplasm is filled with an array of proteins that carry out binding, scavenging and detoxifying functions (Oliver, 1987).

The outer membrane of *P. aeruginosa*, unlike the uniform cytoplasmic membrane, is an asymmetric lipid bilayer (Figure 1). The inner leaflet consists of the chloroform-methanol extractable phospholipids that include phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol (cardiolipin), and some other unknown phospholipids (Nikaido and Hancock, 1986). In all cases, these phospholipids possess two fatty acid chains that extend from the polar head group. Interspersed among the phospholipids are lipoproteins such as OprL and OprI. The latter, which is the *Pseudomonas* equivalent of the *E. coli* Braun Lipoprotein (Mizuno, 1979), is covalently attached to the peptidoglycan (Mizuno and Kageyama, 1979) thus providing an "anchor" for the outer membrane. In addition to these lipoproteins, there are many other different outer membrane proteins in *P. aeruginosa* (for reviews, see Nikaido and Hancock, 1986; Hancock et al., 1990).

The component of the Gram-negative outer membrane that distinguishes it from the cytoplasmic membrane is the lipopolysaccharide (LPS), which is found only in the outer leaflet (Smit et al., 1975). It is an amphipathic molecule consisting of three domains: a) the hydrophilic O-antigen side chain that extends outward from the cell, b) a core polysaccharide region consisting of various conserved components, and c) the hydrophobic lipid A tail which

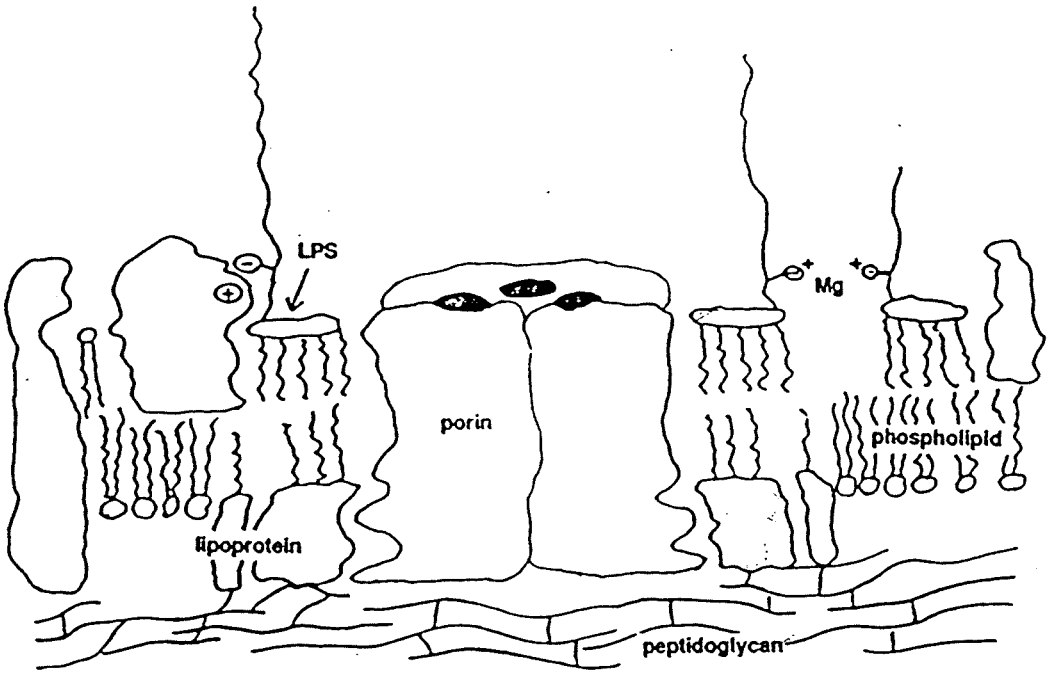


Figure 1: Schematic representation of the outer membrane and peptidoglycan of *P. aeruginosa* .

anchors the molecule into the membrane. Lipid A consists of a 4-phosphoglucosaminyl-(1-6)-glucosamine-1-phosphate backbone (Wilkinson, 1983) to which four to six saturated fatty acids are either ester or amide linked (Karunaratne et al., 1992). These linkages can be either directly on the sugar backbone, or through the hydroxyl groups of other fatty acid chains (Kropinski et al., 1985). The fatty acid composition of the lipid A has been found to include 3-hydroxydodecanoic acid, 2-hydroxydodecanoic acid, 3-hydroxydecanoic acid, and decanoic acid (Kropinski et al., 1985) as well as minor amounts of others (Pier et al., 1981). The core region of LPS consists of D-glucose, D-galactosamine, L-rhamnose, L-glycero-D-*manno*heptose and 2-keto-3-deoxyoctulosonic acid (KDO) (Rowe and Meadow, 1983; Wilkinson, 1983; Kropinski et al., 1985). Besides these saccharide components, the core also contains L-alanine, ethanolamine and phosphate (Rowe and Meadow, 1983; Wilkinson, 1983). Although the core constituents remain the same in different *P. aeruginosa* strains, variations in sugar ratios, sugar arrangement and phosphate composition have been found (Kropinski et al., 1985; Rivera et al., 1988a). The O-antigen side chain, conversely, varies greatly between strains. This highly immunogenic component of LPS is composed of unbranched tri- or tetra-saccharide repeat units (Kropinski et al., 1985) which are rich in N-acetylated sugars and whose repeat number varies from strain to strain. The O-antigen side chain structures of all *P. aeruginosa* serotypes have now been elucidated (Yu et al., 1988).

Another key feature of the outer membrane is the interaction between LPS molecules. The presence of negatively charged groups on or near the disaccharide backbone of LPS, combined with the juxtaposition of the LPS molecules in the outer membrane, suggest that a strong electrostatic repulsion would exist thus causing a destabilization of the membrane (Nikaido and Vaara,

1985). It has been found that neighboring LPS molecules are stabilized by divalent cation bridges (Schindler and Osborn, 1979). The importance of these cations, such as Mg^{2+} and Ca^{2+}, is shown by treating intact cells with the divalent cation chelator ethylenediaminetetraacetate (EDTA) which disrupts the cells and results in the loss of LPS molecules (Leive, 1965). The combination of LPS and divalent cations results in a stable hydrophilic barrier that is virtually impermeable to many hydrophobic molecules (see below). In addition to divalent cations there are protein-LPS interactions that are also crucial to maintaining outer membrane integrity. An example is *P. aeruginosa* protein H1 which is induced in low Mg^{2+} conditions and results in increased resistance to polymyxin B, gentamicin, and EDTA (Nicas and Hancock, 1980). It has been hypothesized that since OprH has a putative pI of 8.6 and therefore is positively charged at physiological pH, it will interact with the negatively charged LPS in limiting divalent cation conditions (Bell et al., 1991), resulting in a stable outer membrane.

3. Outer Membrane Permeability and Antibiotic Uptake.

The outer membrane of *P. aeruginosa* has been shown to contribute significantly to the intrinsic resistance of this organism to antibiotics by forming an effective permeability barrier. In addition to antibiotics, this barrier restricts the access of many hydrophobic and large hydrophilic compounds (for reviews, see Nikaido and Vaara, 1985; Nikaido and Hancock, 1986). There is a group of compounds able to disrupt the organization of the outer membrane and allow the uptake of normally impenetrable molecules. This group is collectively known as outer membrane permeabilizers.

The antibiotic polymyxin B is one of the most well studied permeabilizers. Its structure consists of a polycationic decapeptide "head" region (in which seven of the amino acids form a cyclic structure) and a fatty acyl tail. The fatty acyl tail has been implicated in the association with, and subsequent lysis of, the cytoplasmic membrane (Teuber and Bader, 1976), since polymyxin B nonapeptide (PMBN), a polymyxin B derivative that lacks this fatty acyl chain, shows very weak killing activity (Vaara and Vaara, 1983). Both polymyxin B and PMBN exhibit potent membrane permeabilizing activity on the outer membranes of enteric bacteria as evidenced by their enhancement of hydrophobic antibiotic uptake (Vaara and Vaara, 1983). Polymyxin B was also shown to permeabilize the outer membrane of *P. aeruginosa* to the protein lysozyme, the hydrophobic fluorescent probe 1-*N*-phenylnaphthylamine (NPN) and the chromogenic β-lactam nitrocefin (Hancock and Wong, 1984). This permeabilization could be antagonized by the addition of Mg^{2+}, indicating that polymyxin B must initially associate with the negatively charged sites on the LPS molecules that are normally occupied by Mg^{2+}. Evidence supporting this association came from the observation that dansylated polymyxin B had a strong (0.4 μM) affinity for purified LPS and that Mg^{2+} could competitively displace the dansyl polymyxin from the LPS (Moore et al., 1986).

Studies with bacteria that are resistant or sensitive to polymyxin B or other polycations seem to corroborate the theory that polycationic compounds initially interact with the negatively charged sites on LPS. A *P. aeruginosa tolA* mutant that was susceptible to aminoglycosides was shown to have altered LPS that resulted in an increased affinity for polymyxin B (Rivera et al., 1988b). Conversely, polymyxin resistant (*pmr*) mutants of *S. typhimurium* (Mäkelä et al., 1978) and *E. coli* (Meyers et al., 1974) have been isolated and shown to have increased esterification of their LPS molecules (Vaara et al., 1981; Peterson et

al., 1987). The esterification results in a less acidic LPS and consequently in a decreased binding affinity for polymyxin B and other cationic compounds.

Antibiotics are taken up through a number of different pathways. Many β-lactams are believed to cross the outer membrane by diffusing through the small water filled channels of porin proteins (Zimmermann and Rosselet, 1977). Much of the evidence supporting this hypothesis comes from the fact that some β-lactam MIC values for porin-deficient mutants are much higher than their isogenic wild type strains (Hancock and Bell, 1989). Some of these mutants, however, still demonstrate residual uptake of antibiotics such as chloramphenicol and tetracycline, suggesting that there may be other alternative pathways (Hancock and Bell, 1989). In some cases, porins involved in substrate-specific uptake may become an uptake pathway for an antibiotic that mimics the structure of the substrate. For example, the β-lactam imipenem is taken up through the basic amino acid-specific channel OprD in *P. aeruginosa* (Trias and Nikaido, 1990a; Trias and Nikaido, 1990b).

A second pathway, the partitioning of hydrophobic antibiotics into the outer membrane, is virtually non existent in wild type *P. aeruginosa*, as indicated by high MIC values for these hydrophobic antibiotics (Nikaido and Hancock, 1986) and the inability of NPN to penetrate the outer membrane (Loh et al., 1984). The uptake of these compounds can occur in deep rough mutants (Hancock and Bell, 1989) or be enhanced by membrane-disrupting compounds such as EDTA (Loh et al., 1984), suggesting that the stabilized LPS interactions on the surface of the outer membrane, serve as a barrier to this group of antibiotics.

4. Self-Promoted Uptake of Antibiotics.

Mutant *P. aeruginosa* cells that overproduce the H1 protein (OprH) and cells grown in limited Mg^{2+} conditions (which induce OprH production) were found to be resistant to polymyxin B and gentamicin (Nicas and Hancock, 1980) but had no change in the susceptibility of to other antibiotics such as β-lactams and tetracyclines (Nicas and Hancock, 1983a). From these data it was hypothesized that OprH blocked an uptake pathway that was utilized by cationic compounds. Given the membrane permeabilizing activities (Hancock and Wong, 1984) and LPS binding capabilities (Moore et al., 1986) of these cationic compounds, a third antibiotic uptake pathway, called self-promoted uptake, was proposed (Figure 2; Hancock et al., 1981). This pathway involves three steps: (1) the initial displacement of the Mg^{2+} ions from the LPS cross-bridges by the cationic antibiotic (Figure 2B); (2) the subsequent disruption of the outer membrane structure which can permeabilize it to other, normally excluded compounds (Figure 2C); and (3) the eventual uptake of the compound itself into the periplasmic space (Figure 2D). The nature of the final uptake process is not well understood, but its efficacy is undoubtedly related to both the structure of the disrupted membrane and the conformation of the compound once it has bound to the LPS. Recently, Sawyer et al. (1988) were able to show that the interactions of antimicrobial rabbit macrophage defensins (see below) with the outer membrane of *P. aeruginosa* were consistent with the self-promoted uptake hypothesis (discussed below).

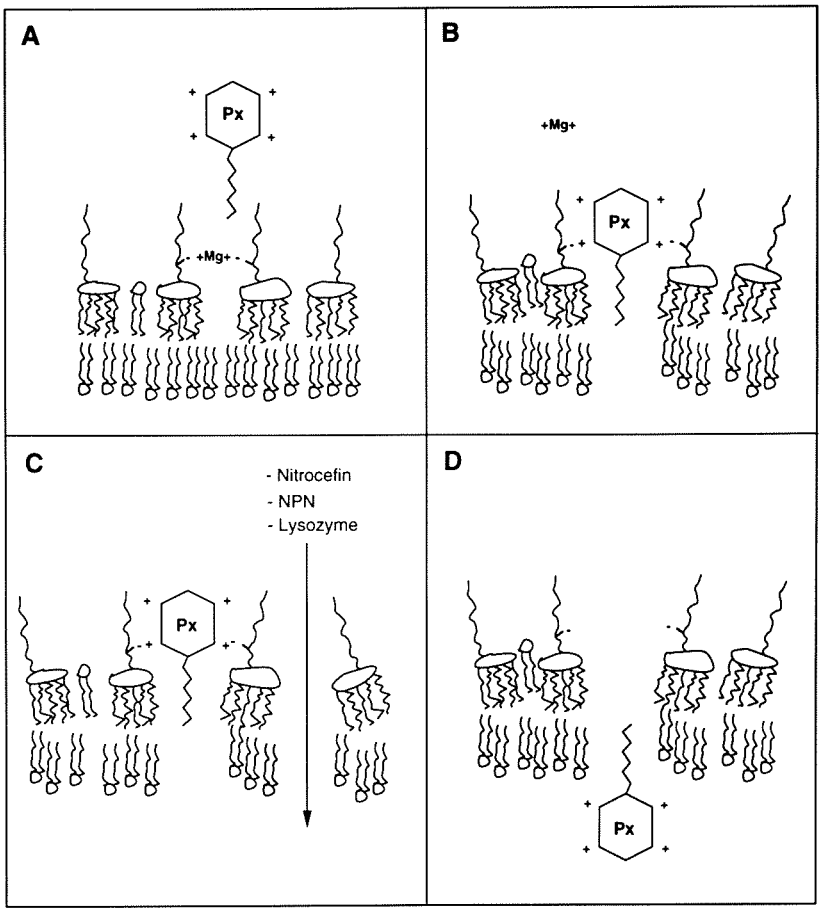


Figure 2: Schematic diagram of the self-promoted uptake model.

A, Typical Gram-negative bacterial outer membrane with a Mg^{2+} crossbridge (outer membrane proteins are omitted for simplicity); **B**, Displacement of Mg^{2+} by a cationic antibiotic (polymyxin B, Px, is used as an example) and the initial perturbations of the lipid bilayer; **C**, Further disruption of the outer membrane resulting in the uptake of normally excluded compounds; **D**, Uptake of the antibiotic across the outer membrane.

B. Cationic Peptides.

1. Introduction.

Antibiotics produced by microorganisms have long been used to fight many infectious diseases. Within the past several decades, however, a new breed of antimicrobial agents, termed "peptide antibiotics", has been discovered. Zeya and Spitznagel (1966) were the first to describe such peptides in mammalian polymorphonuclear leukocytes and noted that they tended to be cationic in nature. Since then, further investigation has led to the discovery of many different peptides from a wide range of organisms (examples in Table I). Considering the location of some of these peptides in the organisms, it is not certain that their primary role is host defense. Nonetheless, they all show a broad range of antimicrobial activity and therefore are potentially useful in antibiotic therapy.

2. Defensins.

The term "defensins" was originally used to describe a family of antimicrobial and cytotoxic peptides from mammalian neutrophils that ranged from 29-35 amino acids in length (for reviews, see Ganz et al., 1990; Lehrer et al., 1990; Lehrer et al., 1993). They are invariably cationic, containing between four and ten arginine residues, and include six conserved cysteine residues that form three disulfide bonds (Figure 3). The first, N-terminal cysteine forms a disulfide bond with the last, C-terminal cysteine, resulting in an effectively cyclic peptide (Selsted and Harwig, 1989). Defensins constitute 5-7% of the total cellular protein in neutrophils and 30-50% of the total protein in the neutrophil's primary granules (Rice et al., 1987). They are actually synthesized

Table I: Cationic Peptides.

Peptide	Origin	Size[a]	Structure[b]	Reference
Indolicidin	Bovine neutrophils	13	Undetermined	Selsted, et al., 1992
Apidaecins	*Apis mellifera* gut	18	Undetermined	Casteels, et al., 1989
Magainins	*Xenopus laevis* skin	23	α-helix	Zasloff, 1987
Melittin	*Apis mellifera* venom	26	α-helix	Habermann and Jentsch, 1967
Defensins	Human	29-30	β-sheet	Ganz, et al., 1985
	Rabbit	33-34	β-sheet	Selsted, et al., 1984
	Guinea Pig	31	β-sheet	Selsted and Harwig, 1987
	Rat	29-32	β-sheet	Eisenhauer, et al., 1989
	Equine	46	β-sheet	Couto, et al., 1992
Cecropin P1	Pig small intestine	31	α-helix	Lee, et al., 1989
Cryptdin	Mouse Paneth cells	35	β-sheet	Eisenhauer, et al., 1992
Cecropins	*Hyalophora cecropia*	35-37	α-helix	Steiner, et al., 1981

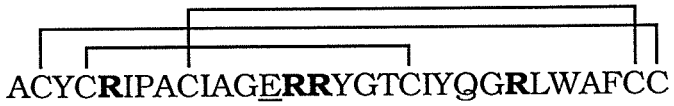
con't...

Table I: Cationic Peptides (con't).

Peptide	Origin	Size[a]	Structure[b]	Reference
Charybdotoxin	Scorpion venom	37	β-sheet	Miller, et al., 1985
β-defensins	Bovine neutrophils	38-42	Undetermined	Selsted, et al., 1993
Sarcotoxins	*Sarcophaga peregrina*	39	α-helix	Okada and Natori, 1983
Phormicins	*Phormia terranovae*	40	Mixed	Lambert, et al., 1989
Sapecin	*Sarcophaga peregrina*	40	Mixed	Matsuyama and Natori, 1988
Seminalplasmin	Bovine semen	48	α-helix	Theil and Scheit, 1983
Bactenecins	Bovine neutrophils	42	Undetermined	Frank, et al., 1990
Diptericin	*Phormia terranovae*	82	Undetermined	Dimarcq, et al., 1988
Hymenoptaecin	*Apis mellifera* gut	93	Undetermined	Casteels, et al., 1993
Attacins	*Hyalophora cecropia*	187-188	Undetermined	Hultmark, et al., 1983

a, number of amino acid residues
b, predominant secondary structure found in the peptide

HNP-1



Cecropin A

KW**K**LF**KK**IE**K**VGQNI**R**DGII**K**AGPAVAVVGQATQIA**K**

Melittin

GIGAVL**K**VLTTGLPALISWI**KRKR**QQ

CEME

KW**K**LF**KK**IGIGAVL**K**VLTTGLPALIS

CEMA

KW**K**LF**KK**IGIGAVL**K**VLTTGLPAL**K**LT**K**

defensin pre pro region

pre: M**R**TLAILAAILLVALQAQA

pro: EPLQA**R**ADEVAAAPEQIAADIPEVVVSLAWDESLAP**K**HPGS**RK**N

Figure 3: Amino acid sequences of selected cationic peptides.

Designations include: HNP-1, human neutrophil peptide 1; CEME, cecropin/melittin hybrid (CA(1-8)M(1-18)); CEMA, a frameshift analogue of CEME. Positively charged amino acids are in bold and negatively charged amino acids are underlined. The disulfide bond array for mammalian defensins is demonstrated for HNP-1.

as 94-95 amino acid prepropeptides. The negatively charged residues in the pro region (Figure 3) virtually balance the positively charged residues in the defensin sequence, which led to speculation that the pro region was necessary for host protection prior to processing and packaging of the mature defensin into the granules (Michailson et al., 1992). From the granules, the peptides are released into the microbe-containing phagosome through degranulation where they exert their microbiocidal activity. The importance of defensins to the host defense system is evidenced by the high infection rate that occurs in patients whose granular components are defective or missing (Ganz et al., 1988).

Defensins show a broad range of antimicrobial activity. At concentrations of 10-100 μg/mL, they are able to kill Gram-negative and Gram-positive bacteria, although the are generally more active against the latter (Lehrer et al., 1993). For Gram-negative bacteria, rough strains are more sensitive to defensin activity than their isogenic smooth strains (Ganz et al., 1990). In addition to bacteria, various defensins have been shown to kill *Candida albicans* (Selsted et al., 1985), *Treponema pallidum* (Borenstein et al., 1991), and some enveloped viruses such as herpes simplex virus (Lehrer et al., 1985).

The first information concerning the 3-dimensional structure of defensins came with the elucidation of the solution structure of rabbit neutrophil defensin NP-5 (Pardi et al., 1988). The basic structural backbone of the defensin molecule was a triple-stranded antiparallel β-sheet (Figure 4A), with the charged amino acids at one end of the molecule and the hydrophobic ones at the other end. This basic structure was confirmed by the crystal structure of HNP-3 (Hill et al., 1991) which showed the human defensin as an elongated ellipsoid. The HNP-3 crystallized as a basket-shaped dimer (Figure 4B) which, due to the monomer's amphipathicity, had an apolar base and a

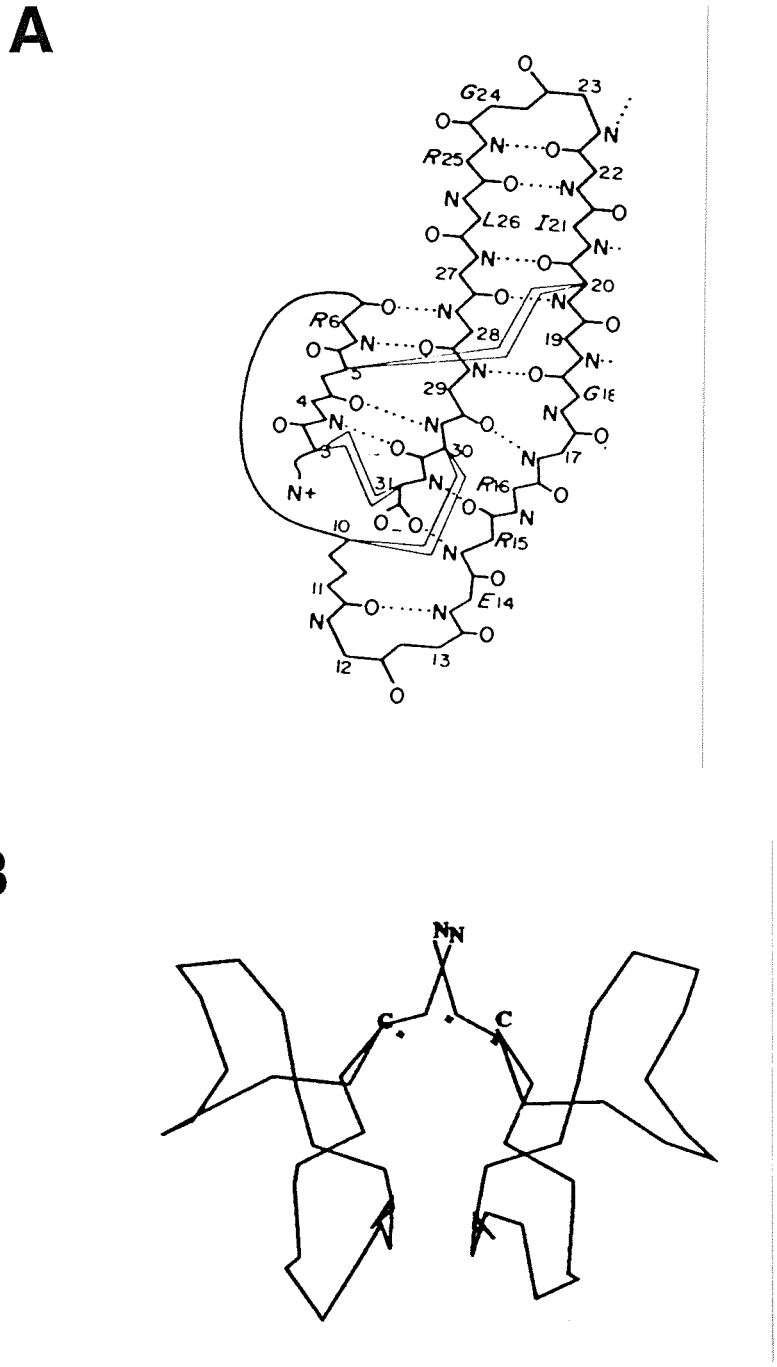


Figure 4: Model of the mammalian defensin structure.

A, The triple-stranded anti-parallel β-sheet structure of an HNP-3 monomer. The disulfide bonds are represented by lightening bolts. **B**, An HNP-3 dimer with a polar top and an apolar bottom. Both figures were reproduced by copyright permission from Hill, et al. (1991) © American Association for the Advancement of Science.

polar top. The invariant Gly^{18} in the defensin amino acid sequence was found at the dimer interface, suggesting that dimerization is required for activity. This is further supported by the fact that upon dimerization, a water-filled channel is formed. These observations led to the speculation that HNP-3 monomers wedge their hydrophobic base into lipid membranes, and eventually form dimer-pores or multimer-pores (Hill et al., 1991). This is consistent with the data from Kagan et al. (1990) who showed that rabbit defensins can form voltage dependent ion-permeable channels in planar lipid bilayers.

These structural data, however, do not provide any information on how these peptides cross the asymmetric outer membrane in Gram-negative bacteria. Sawyer et al. (1988) showed that rabbit MCP-1 and MCP-2 (equivalent to NP-1 and NP-2) were able to bind to and permeabilize the outer membrane of *P. aeruginosa* and that these defensins could bind purified LPS. These data resulted in the proposal that defensins are taken up across the outer membrane by the self-promoted uptake pathway. Human defensins were also shown, albeit only at bactericidal or bacteriostatic concentrations, to permeabilize the outer membrane of various Gram-negative bacteria to rifampicin and Triton X-100 (Viljanen et al., 1988). A detailed examination of HNP-1-mediated bactericidal activity against *E. coli* revealed that the defensin caused sequential permeabilization of the outer and inner membranes, and that it was likely that the latter event that resulted in cell death (Lehrer et al., 1989). This study also showed that HNP-1 was a relatively poor permeabilizer of the outer membrane since it required up to 50 μg/mL for 20 min before outer membrane permeabilization was detected.

Another group of defensins was isolated from a number of different insects such as *Phormia terranovae* (Lambert et al., 1989), *Sarcophaga peregrina* (Matsuyama and Natori, 1988), and others (for a review, see

Hoffmann and Hetru, 1990)(Table I). These peptides were initially called insect defensins since they were small, cationic and contained six cysteine residues. Upon further investigation, however, the positions of these cysteine residues in the amino acid sequence, and even the disulfide array were different from those of mammalian defensins (Selsted and Harwig, 1989; Lepage et al., 1991). The insect defensins are also slightly longer (38-43 residues) and less cationic (pI 8.0-8.5)(Hoffmann and Hetru, 1990) than their mammalian counterparts. The biggest differences between these two groups of peptides are in their 3-dimensional structures. While the mammalian defensins are dominated by β-sheet structures (Hill et al., 1991), the insect defensins have a loop, α-helix, and β-sheet structures (Hoffmann and Hetru, 1990). Therefore, while these two groups share similarities in name, size, charge and antibacterial activity, they cannot be considered structural homologues.

3. Cecropins.

Along with defensins, insects produce another family of cationic antimicrobial peptides in response to a bacterial challenge. This group is collectively called cecropins, after the moth *Hyalophora cecropia* in which they were first discovered (Hultmark et al., 1980). Cecropin-like peptides have now been found in many different insects (for reviews, see Boman and Hultmark, 1987; Boman et al., 1991; Hultmark, 1993), as well as in mammalian cells (Lee et al., 1989)(Table I). The peptides are 35-39 amino acids in length and have net positive charges that range from +3 to +8. Unlike the defensins, they do not contain cysteine residues (Figure 3) and therefore adopt a completely different tertiary structure (see below). A number of residues are conserved throughout the cecropin family, including Trp^{2}, Phe^{5}, Lys^{6}, Glu^{9}, Gly^{12}, Arg^{16}, Ala^{22}, Gly^{23},

Pro^{24}, Ala^{25}, and Ala^{32}. The role of these residues remains unclear; however, they may be involved in inter-protein contacts in oligomeric structures (Durell et al., 1992). The necessity of Trp^{2} was demonstrated since upon its deletion, the lytic activity of the peptide decreased dramatically (Steiner et al., 1988).

In response to an infection, *H. cecropia* produces three major cecropins, A and B (Hultmark et al., 1980) and D (Hultmark et al., 1982). The genes for these peptides have been cloned and sequenced (Xanthopoulos et al., 1988; Gudmundsson et al., 1991) revealing that, like defensins, they were produced as prepropeptides. The amino acid sequence was analyzed for potential secondary structures which led Steiner (1982) to propose that the peptide consisted of two α-helices joined by a hinge region (Figure 5A). This was later confirmed by the two-dimensional NMR structure determination of cecropin A (Holak et al., 1988). The strongly basic N-terminal α-helix is almost perfectly amphipathic, while the more hydrophobic C-terminal α-helix is less so. These two regions are joined by the flexible hinge region encoded by Ala-Gly-Pro-Ala, which is conserved in most cecropins. The design of cecropin-like model peptides and cecropin analogs demonstrated that all three structural features were required for full antibacterial activity (Steiner et al., 1988; Fink et al., 1989).

Cecropins have a broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria as determined by zone inhibition assays (Boman et al., 1991). Cecropins A and B have a much broader range of activity than cecropin D which may reflect the high positive charge densities of their N-terminal α-helices as compared to cecropin D. None of the cecropins are able to lyse eukaryotic cells (Steiner et al., 1981; Wade et al., 1990) in contrast to defensins, which possess potent cytotoxic activity (Lehrer et al., 1993). Like the defensins, however, cecropins were able to form voltage dependent ion channels

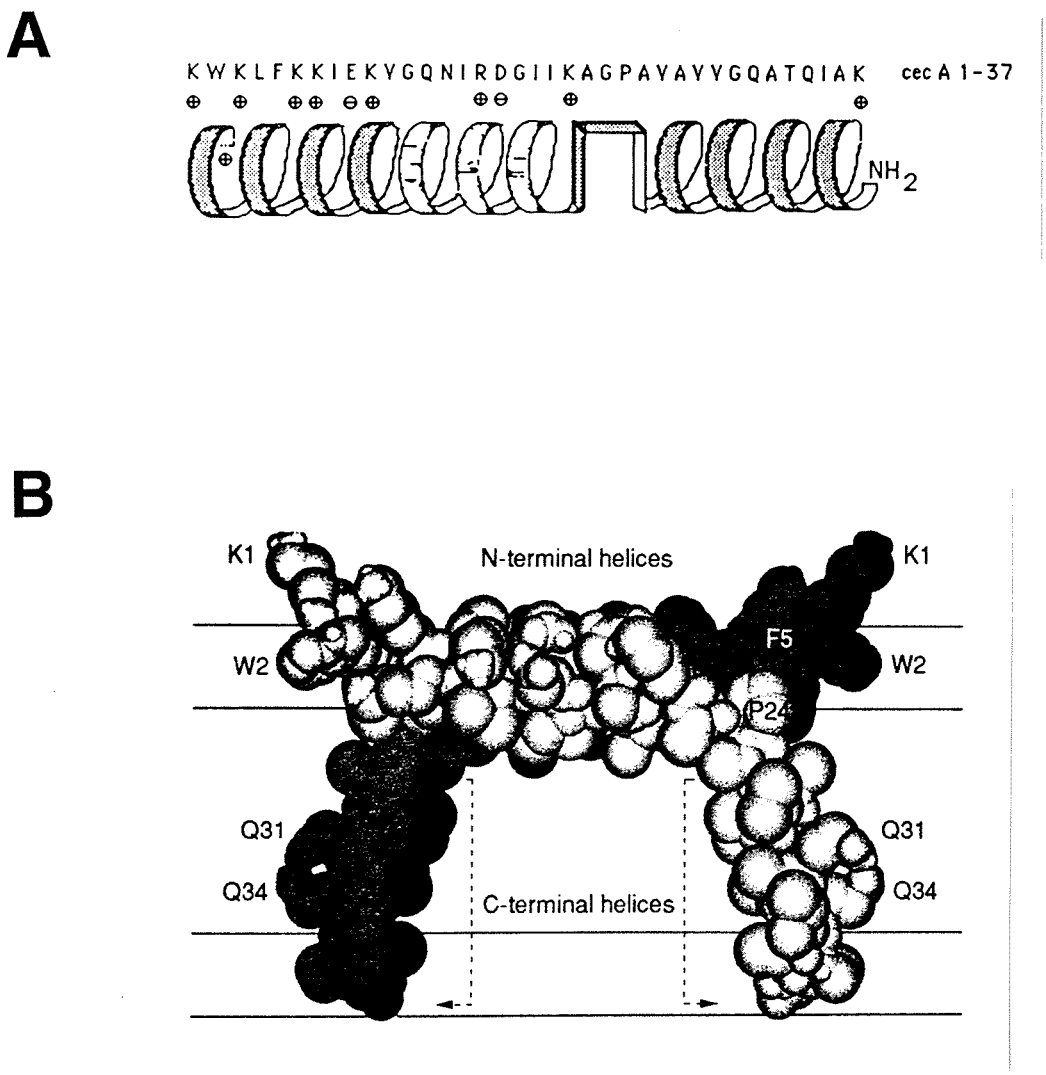


Figure 5: Model of the structure of cecropin A.

A, Cecropin A primary and secondary structures showing its helix-hinge-helix nature. The charged residues are shown to indicate the amphipathic nature of the N-terminal α-helix. Reproduced by copyright permission from Steiner, et al. (1988). **B**, Antiparallel dimer of cecropin A in a membrane bilayer. The N-terminal α-helices are embedded into the lipid head-group layer and the C-terminal α-helices traverse the hydrophobic phase of the membrane. Selected residues are highlighted. Reproduced by copyright permission from Durell, et al. (1992) © Biophysical Society.

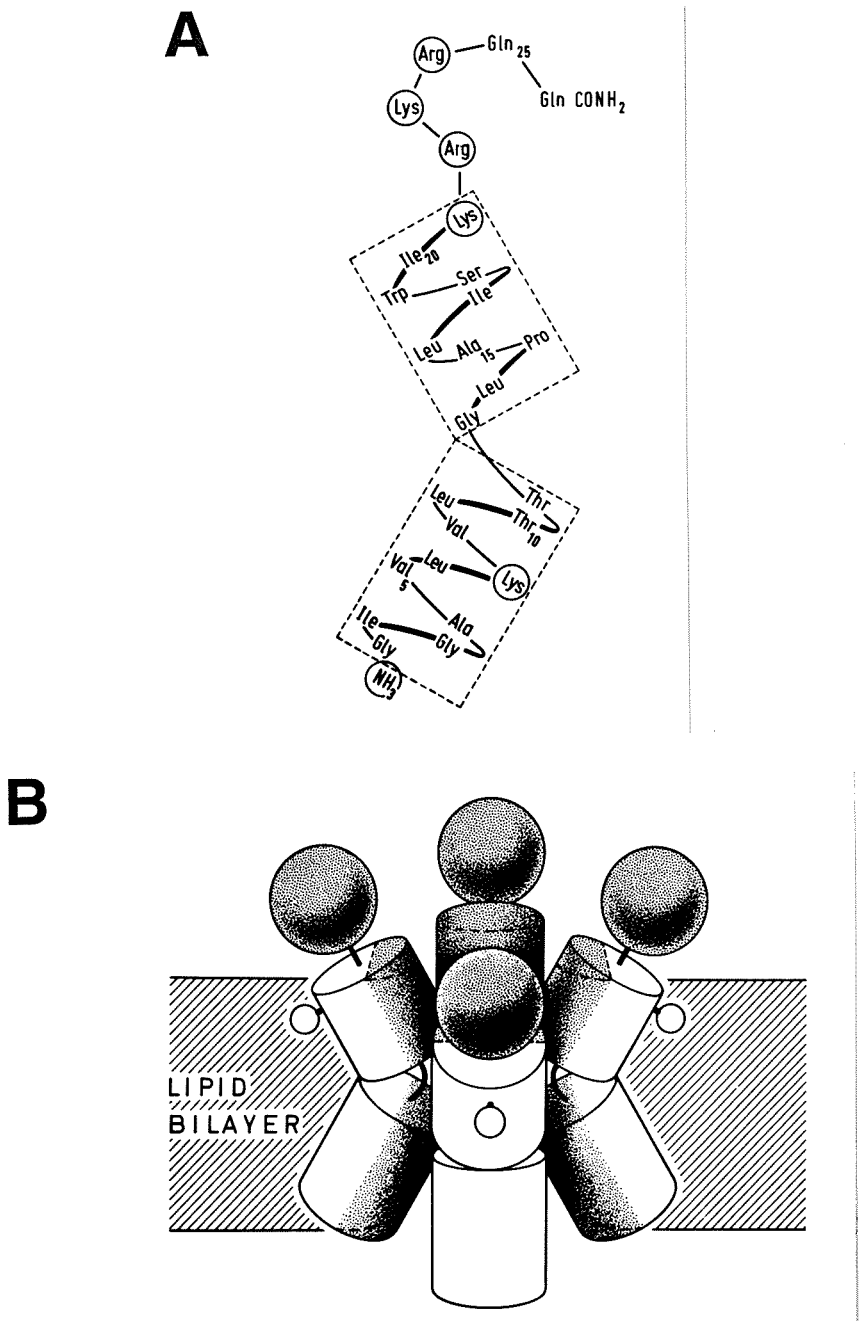


Figure 6: Model of the structure of melittin.

A, Monomeric, membrane-bound melittin showing the helix-hinge-helix structure and the C-terminal random coil motif. Circles denote positively charged amino acids. **B**, Tetrameric melittin associated with a lipid membrane. The shaded spheres represent the non-helical C-terminal residues, and the cylinders represent the α-helices with the hydrophobic sides (white) facing the membrane and the hydrophilic sides (shaded) facing each other, thus forming a channel. Both figures were reproduced by copyright permission from Vogel and Jähnig (1986) © Biophysical Society.

in planar lipid membranes (Christensen et al., 1988). By studying a number of synthetic analogs, these researchers were able to determine that the flexible hinge region separating the two amphipathic α-helices was required for pore-forming activity. The observed correlation between the peptides' bactericidal and pore-forming activities led to the conclusion that the lethal effect of cecropins on bacterial cells was the formation of large pores in the cytoplasmic membrane which led to cell lysis (Christensen et al., 1988). Although ion channel formation has not been demonstrated for the porcine cecropin P1, it was shown to kill *E. coli* bacteria by cell lysis (Boman et al., 1993) which suggests that all cecropins have a similar mechanism of activity. Lipid bilayers which had either a positive surface charge or included cholesterol were highly resistant to the pore-forming action of cecropins. The latter result was consistent with the insensitivity of eukaryotic cells to lysis by cecropins, and the former suggested that the positively charged cecropins required a negatively charged membrane to initiate bactericidal activity. Although required, this binding was not sufficient for killing, since bacteria that were resistant to cecropin activity, were still found to bind large amounts of the peptide (Steiner et al., 1988).

The observations on cecropin binding and lysis led to the formulation of a three step mechanism of cecropin activity on lipid membranes (Christensen et al., 1988). The first step involved the electrostatic adsorption of cecropin oligomers to the negatively charged surface of the membrane. In the second step, the hydrophobic C-terminal α-helix would insert into the membrane while the amphipathic N-terminal α-helix would remain at its interfacial position. This step could only occur with peptides that possessed the flexible hinge between the two helices. Finally, an applied voltage (positive to negative going into the membrane) would drive the N-terminal α-helix into the membrane, with

its hydrophilic residues forming a water-filled channel. This model was expanded by Durell et al. (1992) who, using atomic-scale computer models, proposed that cecropins form antiparallel dimers whose C-terminal α-helices can span the membrane (Figure 5B). These dimers would then aggregate into a hexagonal geometry (based on pore dimensions from Christensen et al., 1988), resulting in a small ion channel formed by six adjacent C-terminal α-helices. This "type I" channel would further undergo a conformational change in which the hydrophilic residues of the N-terminal α-helices would move from the membrane surface to the pore lining, creating a larger ("type II") channel. Although these proposals may accurately describe the mechanism of cecropin action on the cytoplasmic membrane, they cannot be applied to the more complex, asymmetric outer membrane of Gram-negative bacteria. It is interesting to note that despite their high potency against Gram-negative bacteria, the effects of cecropins on outer membranes have never been studied directly.

4. Melittin.

The venom of the honey bee *Apis mellifera* contains, as its major protein component, a 26 amino acid peptide that is highly cationic (Figure 3; Habermann and Jentsch, 1967) and possesses strong antibacterial and potent hemolytic activities (for a review, see Dempsey, 1990). Many investigators have studied the structure/function relationship of melittin, particularly with respect to its interaction with membranes. This has resulted in some conflicting ideas on how this peptide antibiotic exerts its mechanism of activity.

Melittin adopts different structures depending on its environment. In water, or in other low ionic strength environments, it exists as a monomer that

has no detectable secondary structure. In higher ionic strength buffers, or other environments that promote self association, the peptide forms a tetramer consisting of monomeric subunits that are predominantly in a helical conformation (Dempsey, 1990). Residues 1-21 of melittin form a hydrophobic α-helix that is bent in the middle at a glycine residue (Figure 6A). The C-terminal portion of the peptide is very hydrophilic and is not believed to form an α-helix. This structure differs from the α-helical peptide cecropin A in a few ways. First the polarity of the peptides is reversed, with the N-terminus of cecropin A and the C-terminus of melittin being hydrophilic, while the C-terminus of cecropin A and N-terminus of melittin are hydrophobic. Secondly, the bend in the peptides is found in a different position. In melittin, the bend is in the middle of the N-terminal hydrophobic α-helix, while in the cecropins, it separates the hydrophilic and hydrophobic α-helical segments. These differences have ramifications for hybrid peptides created from melittin and cecropin A (see below).

The amphipathicity of the N-terminal α-helix of melittin suggests that the monomers must aggregate when interacting with lipid membranes. Figure 6B shows a schematic model of tetrameric melittin in a membrane. The hydrophilic faces of the four α-helices face the inside of the tetramer and probably form a pore (see below). Although the antibacterial properties of melittin have been documented (Boman et al., 1989a), it is the hemolytic activity that continues to be the focus of intense research. Melittin has been shown to cause cell lysis at concentrations of greater than 1 μg/mL (DeGrado et al., 1982; Hider et al., 1983). It is also able to form voltage-dependent ion-permeable channels in lipid bilayers (Tosteson and Tosteson, 1981). The conductance of these channels was shown to change with the fourth power of melittin concentration indicating that tetrameric association of the monomers

forms the channel (Tosteson and Tosteson, 1981). This conclusion seemed to be inconsistent with the observation that melittin could form multi-state pores, indicating that there was heterogeneity in the pore structures (Hanke et al., 1983). This is in contrast to the cecropins which showed discreet channel sizes (Christensen et al., 1988).

The structure of melittin has been examined to determine what features are responsible for its channel-forming and hemolytic activities. Blondelle and Houghten (1991) created a series of 24 individual omission analogues of melittin to determine which residues were necessary for hemolytic activity. Their results showed that the deletion of any residue in the hydrophobic α-helix resulted in a marked decrease in the hemolytic activity, while the omission of any C-terminal residues had no effect. This was in contrast to Werkmeister et al. (1993) who found that either the incubation of melittin with a C-terminal specific monoclonal antibody or the deletion of one of the two Lys-Arg motifs in the C-terminus significantly reduced the hemolytic activity of the peptide. This suggested that the C-terminus was necessary for hemolysis, and that the deletion of one residue from that sequence (Blondelle and Houghten, 1991) was not sufficient to reduce that activity. This was supported by the fact that a melittin analogue that was missing the last four amino acids (and therefore one Lys-Arg motif) had a 30% weaker affinity for phospholipid membranes and a much lower lytic activity compared to native melittin (Otoda et al., 1992). Interestingly, this shortened melittin analogue could still form voltage-dependent ion channels in lipid bilayers (Otoda et al., 1992). This indicated that membrane binding and cell lysis were not directly related to ion channel formation. Therefore, one can only conclude that the actions of melittin on lipid membranes are varied and complex. At low concentrations, melittin will form heterogeneously sized ion channels, probably consisting of melittin aggregates

that do not significantly perturb the membrane, while at higher concentrations, it will cause cell lysis.

Other activities of melittin have been studied and recently reviewed (Dempsey, 1990; Fletcher and Jiang, 1993). Again, since the focus of melittin research has been its interaction with phospholipid membranes, virtually no research on the peptide's interaction with the outer membrane has been done. One study, however, showed that melittin bound strongly to purified *Salmonella typhimurium* LPS (David et al., 1992), suggesting that melittin may be taken up by the self-promoted uptake pathway since the first step in this pathway is the binding of the compound to LPS.

5. Synthetic and Hybrid Peptides.

The discovery of cationic peptides and their potent antimicrobial activities has led to attempts to improve this activity. Most of this research has focused on the α-helical peptides since their structural features are relatively simple (compared to those of defensins) and their basic functional domains have been identified (see above).

Early studies in the field of synthetic and hybrid peptides focused on the creation of peptides that were predicted to have amphipathic α-helix structure. Lee et al. (1989) synthesized a number of peptides that contained varying numbers of repeated tri-, tetra-, or penta-peptide fragments that would give rise to an α-helical structure. They tested these peptides for activity against six organisms and found a direct correlation between the peptide's ability to form a stable, amphipathic α-helix and its antibacterial activity against Gram-positive bacteria. (It is interesting to note that these synthetic peptides were inactive against Gram-negative bacteria, suggesting that cationicity and α-helicity are

not sufficient to cross the outer membrane). The peptide that possessed the highest bactericidal activity (Ac-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$) also had the highest α-helix content in a lipid environment (Lee et al., 1989). This peptide was also the best facilitator at forming ion channels in lipid membranes at bactericidal concentrations (Anzai et al., 1991). The conductance of these channels ranged from 2-750 pS which indicated a heterogeneity in the conformation or assembly of the channels, as was demonstrated for melittin (Hanke et al., 1983). The requirement for amphipathic and α-helical structure was supported by Blondelle and Houghten (1992) who showed that both these features were necessary for bacterial cell lysis.

In addition to creating new peptides, analogues of some naturally occurring peptides have been synthesized in an attempt to augment their activity. One example is the magainins, a small group of peptides from the skin of the African frog *Xenopus laevis* (Table I), which are 23 amino acids in length and possess broad spectrum antimicrobial activity (Zasloff, 1987). Synthetic magainin analogues with enhanced α-helical structure were shown to have a 50-fold increase in their antimicrobial activity (Chen et al., 1988). The addition of groups of ten or more arginine or lysine residues to the N- or C-terminal ends of magainin-2 resulted in MIC reductions of 5- to 10-fold (Bessalle et al., 1992). The peptide with positive chain extension at the C-terminal end had no increase in hemolytic activity. This was in contrast to melittin whose positively charged C-terminus was shown to be necessary for hemolytic activity (Otoda et al., 1992; Werkmeister et al., 1993).

As described above, two other naturally occurring peptides, cecropin A and melittin, have been altered to enhance their activity. In addition to changes in the parent peptides, much work has gone into hybrid peptides that contain sequences from both cecropin A and melittin. Boman et al. (1989a) described

the first hybrid peptides which were the same size as melittin, and contained different combinations of the hydrophobic and hydrophilic regions of cecropin A and melittin. In this process, they synthesized a peptide consisting of the first 13 amino acids of cecropin A followed by the first 13 amino acids of melittin (CA(1-13)M(1-13)). This peptide was 100-fold more active against *S. aureus* than cecropin A and had antimalarial activity that was 10-fold better than cecropin B (Boman et al., 1989a). Unlike melittin, however, this cecropin-like peptide possessed no hemolytic activity. The two-dimensional structure of CA(1-13)M(1-13) revealed that it still consisted of a helix-hinge-helix structure, but that the hydrophobic C-terminal α-helix came from the N-terminal sequence of melittin (Sipos et al., 1991). The conclusion from this work was that hemolytic activity was dependent on a specific design and location of the basic "head" region, while antibacterial activity was not sequence specific (Boman et al., 1989a).

Wade et al. (1990) investigated whether or not these peptides interacted with chiral membrane components to initiate antibacterial activity. They synthesized D-enantiomers of melittin, cecropin A, magainin-2 (all normally found in the L-form) and a few cecropin-melittin hybrids, and found that these peptides showed the same antibacterial and ion channel forming activities as their corresponding L-forms. They concluded that these peptides do not require specific interaction with chiral receptors or enzymes for antibacterial activity but rather an appropriate hydrophobic environment (Wade et al., 1990). During these studies, a new hybrid peptide was constructed [CA(1-8)M(1-18), hereafter referred to as CEME; Figure 3], which had even better antimicrobial activity and lower hemolytic activity than CA(1-13)M(1-13). Although the two-dimensional structure for CEME has not been elucidated, its amino acid sequence indicates that it may have two hinge regions, one from the G-I-G-A

domain of melittin (which is the domain responsible for the hinge in CA(1-13)M(1-13)), and another from the G-L-P domain of the melittin sequence which provides the only bend in native melittin (Figure 6A). The presence of two flexible hinge regions would predict an overall reduction of the α-helical content of CEME, which was demonstrated by circular dichroism analysis (Wade et al., 1990). Confirmation of this double-hinge structure and its ramifications for antibacterial activity requires further investigation.

Using CEME as a parent peptide, a number of truncated peptides were synthesized to determine how short the peptide could be made without compromising its bactericidal activity (Andreu et al., 1992). C-terminal deletions brought the peptide size down to 18 amino acids with no apparent loss of activity as compared to CEME. A series of 15-mers was created by combining the first 7 amino acids of cecropin A with different 8 amino acid segments from the N-terminal sequence of melittin. Although most of these peptides showed good activity, they all had slightly higher lethal concentrations than CEME, and in some cases were only weakly active against specific bacteria (Andreu et al., 1992). The most active of these 15-mers were peptides that had a disrupted G-I-G-A domain and therefore no bend in the α-helix. This suggested that shorter peptides required a full length α-helix to disrupt membranes, but the mechanism of action of these shorter hybrid peptides was not explored further.

A recent, ambitious study used 30 chimeric peptides to define further the structural requirements for antibacterial activity (Wade et al., 1992). The results confirmed that the general structural requirements were an amphipathic N-terminal α-helix, a flexible hinge region, and a hydrophobic C-terminal α-helix, but no new requirements were discovered. None of the

peptides tested in this study improved on the potent, broad host range activity of CEME.

C. Fusion Protein Technology.

1. Introduction.

Recent advances in the area of gene cloning and expression have placed new demands on the field of protein purification. It is now commonplace to take a cloned gene, whether it is prokaryotic or eukaryotic, and express it in a prokaryotic host such as *E. coli.* This process has resulted in the purification of many different proteins in quantities that would have been unattainable if purifying them from original sources. However, while providing larger amounts of starting material, this procedure did not simplify the tedious process of purifying the desired protein from a complex array of cellular components.

The development of affinity tag fusion protein technology (reviewed in Sassenfeld, 1990; Uhlén and Moks, 1990) was a way to simplify the purification procedure. In one example of this technology, the gene encoding the protein of interest is fused to a gene encoding a protein that has a strong binding affinity to a specific ligand. The heterologous protein produced from such a gene fusion can be purified by affinity chromatography on a matrix to which the ligand is bound. In other examples, the affinity tag is a short, poly-amino acid "tail" that is added on to the protein, increasing its charge density or hydrophobicity and enabling its purification by ion exchange or hydrophobic chromatography. Examples of these two types of affinity tags are found in Table II.

This technology provides several advantages for producing foreign proteins in a host such as *E. coli.* Many heterologous proteins produced by direct expression of a cloned gene are proteolytically degraded or insoluble.

Table II: Commonly Used Affinity Tags.

Affinity Tag	Binding Matrix	Elution Conditions	Reference
β-galactosidase	TPEG-Sepharose	0.1 M borate	Ullman, 1984
Cellulose-binding domain	Cellulose	water	Ong, et al., 1989
Flag™	Anti-Flag™ antibody	0.1 M glycine pH 3.0 or 2-5 mM EDTA	Hopp, et al., 1988
Glutathione-S-transferase	Glutathione agarose	5 mM reduced glutathione	Smith and Johnson, 1988
Maltose binding protein	Crosslinked amylose	10 mM maltose	Maina, et al., 1988
Polyarginine	Cationic exchange	NaCl gradient	Sassenfeld, 1984
Polyhistidine	Nitrolotriacetic acid-Sepharose (Ni^{2+})	low pH or ≤ 250 mM imidazole	Hochuli, et al., 1988
Protein A	IgG-Sepharose	0.5 M acetic acid pH 3.4	Uhlén, et al., 1983
Synthetic IgG-binding domain	IgG-Sepharose	0.5 M acetic acid pH 3.4	Moks, et al., 1987a

There are several examples of proteins that, when produced as fusion proteins, had decreased susceptibilities to proteolysis and increased solubility, compared to when they were produced directly (Marston, 1986). Another consideration of fusion protein production is the localization of the gene product. Use of a secretory system can have many advantages, such as the proper formation of disulfide bonds in the oxidative environment outside the cell, increased stability by the avoidance of intracellular proteases, increased solubility, the enhancement of purification procedures and, in some cases, the avoidance of host toxicity from the heterologous protein. Unfortunately, not all proteins are compatible with the secretory requirement of membrane translocation.

The major obstacle of fusion protein technology is how to obtain the target protein free of the fusion partner with no extra amino acids on the N- or C-terminal ends. One advantage of the technology is the ability to cleave specifically the protein *in vitro*, as opposed to relying on the *in vivo* removal of the formylmethionine residue or the signal sequence which can result in heterogeneous N-terminal ends. In order to utilize this advantage, site-specific cleavage sites must be engineered in the fusion protein to allow the removal of all extraneous amino acids from the target protein. There are many different cleavage methods which are divided into chemical and enzymatic methods (Table III). Chemical methods tend to be quite efficient but are not feasible with larger target proteins which usually contain the low specificity recognition sequences internally. The cleavage conditions for these methods are also quite harsh and could be detrimental to the protein. In contrast, most enzymatic methods, especially the endopeptidases, are very specific but can be expensive to scale up and are sometimes quite inefficient due to the inaccessibility of the recognition site to the enzyme. Therefore, the method best used to cleave the

Table III: Methods for the Site-Specific Cleavage of Fusion Proteins.

Method	Recognition Sequence/Cleavage Site	Reference
Chemical		
Acid	-X-D-↓-P-X-	Szoka, et al., 1986
CNBr	-X-M-↓-X-	Itakura, et al., 1977
BNPS-skatole	-X-W-↓-X-	Knott, et al., 1988
Hydroxylamine	-X-N-↓-G-X-	Moks, et al., 1987b
Enzymatic		
Carboxypeptidase A	-X-↓-X_1 $X_1 \neq$ R or K	Hochuli, et al., 1988
Carboxypeptidase B	-X-↓-R or -X-↓-K	Sassenfeld, 1984
Chymotrypsin	-X-W-↓-X-, -X-F-↓-X-, -X-Y-↓-X-	Dahlman, et al., 1989
Collegenase	-X-P-X-↓-G-P-X-	Germino and Bastia, 1984
Enterokinase	-X-D-D-D-K-↓-X-	Maroux, et al., 1971
Factor X_a	-X-I-E-G-R-↓-X-	Nagai and Thøgersen, 1987
Thrombin	-X-L-V-P-R-↓-G-S-X-	Smith and Johnson, 1988
Trypsin	-X-R-↓-X-, or -X-K-↓-X-	Shine, et al., 1980

fusion protein will vary on an individual basis and depend on a number of factors including protein size and lability.

2. Glutathione-S-transferase.

One commonly used affinity tag is glutathione-S-transferase (GST), which was originally identified in *Schistosoma japonicum* (Smith et al., 1986) and can be expressed as an active, soluble protein in *E. coli* (Smith et al., 1988). This protein was the basis for a number of fusion protein vectors which were initially used to purify over 30 eukaryotic polypeptides (Smith and Johnson, 1988). Since then, this system has been used in the purification of many different proteins such as the murine leukemia inhibitory factor (Gearing et al., 1989), the *Plasmodium falciparum* antigen Pf155/RESA (Ståhl et al., 1990), epidermal growth factor receptor sequences (Koland et al., 1990), rat interleukin-6 (Frorath et al., 1992), *Poa p*IX grass pollen allergens (Olsen and Mohapatra, 1992), the transactivation domain of Vmw65 from herpes simplex virus type 1 (Donaldson and Capone, 1992), mouse mammary tumor virus protease (Menéndez-Ariau et al., 1992), and human cellular retinoic acid binding protein II (Redfern and Wilson, 1993). The affinity of GST for reduced glutathione allows the purification of soluble GST fusion proteins by adsorption to glutathione agarose beads and subsequent desorption using free reduced glutathione (Smith and Johnson, 1988). The GST moiety of the purified fusion proteins can be released by specific proteolytic cleavage using thrombin or factor X_a (Smith and Johnson, 1988). In several instances, the GST fusion proteins were found to be partially degraded (Koland et al., 1990; Olsen and Mohapatra, 1992), although there does not appear to be a correlation between the amino acid sequence of the target protein and the lability of the fusion

protein. Another potential problem with this system occurs when the fusion proteins form insoluble inclusion bodies (Smith and Johnson, 1988; discussed in detail below) which require harsh purification techniques that are incompatible with that of affinity chromatography. This problem was recently addressed by Hartman et al. (1992) who described conditions which enabled the conversion of insoluble fusion protein aggregates to a soluble form amenable to glutathione agarose affinity purification.

3. Protein A.

Another affinity tag that has been used extensively is the IgG-binding domain of S. *aureus* protein A (Nilsson and Abrahmsén, 1990). The gene was originally isolated by Löfdahl et al. (1983) and its complete sequence confirmed earlier suggestions that it consisted of two functional domains (Uhlén et al., 1984). The N-terminal region has a 58 amino acid unit which is repeated 5 times and is believed to be responsible for IgG binding. The C-terminal region has an 8 amino acid unit which is repeated 12 times and is thought to be involved in the binding of the protein to the cell wall. The former region was used as the basis for a number of fusion protein vectors that had a truncated protein A gene (Uhlén et al., 1983). These vectors were first used to create protein A/β-galactosidase fusion proteins that could be purified by adsorption to IgG-Sepharose and desorption using either a glycine buffer pH 3.0 (Löfdahl et al., 1983) or excess pure protein A (Uhlén et al., 1983) depending on the pH stability of the target protein. The original vectors were improved to give ones that were temperature inducible (pRIT2) or allowed for efficient secretion of the fusion protein to the *E. coli* periplasm or the S. *aureus* extracellular medium (pRIT5)(Nilsson et al., 1985a). These vectors have since been used to produce

fusion proteins to alkaline phosphatase (Nilsson et al., 1985a), human insulin-like growth factor I (Moks et al., 1987a), human apoA-1 protein (Monaco et al., 1987), a transactivation domain of the herpes simplex virion protein VP16 (Stringer et al., 1990), yeast calmodulin (Stirling et al., 1992), and a tyrosine kinase domain of the c-*src* protein (Saya et al., 1993). A new series of fusion protein vectors was constructed using different multiplicities of a synthetic DNA fragment that encoded a small IgG-binding domain (designated "Z"). This Z region lacked asparagine-glycine dipeptide sequences as well as methionine residues which rendered it resistant to hydroxylamine and CNBr treatments respectively. This allowed the specific release of a target protein that also lacked these residues, by engineering one of these chemical cleavage sites at the junction of the fusion protein. This was already demonstrated with the production of IGF-I (Moks et al., 1987a). The ZZ-IGF-I protein was purified by IgG-Sepharose affinity chromatography, cleaved with hydroxylamine to release IGF-I, and passed over the affinity matrix a second time to obtain pure IGF-I in the flow through. This process was scaled up to a 1000 L which led to the production of large quantities of IGF-I (Moks et al., 1987b).

4. Inclusion Body Formation and Protein Degradation.

The use of affinity tags for the production and purification of foreign proteins in *E. coli* is not without problems. Many foreign proteins produced either directly or as fusion proteins in *E. coli* are found to form insoluble, electron dense aggregates (reviewed in Marston, 1986; Kane and Hartley, 1988; Fischer et al., 1993). It has been, and continues to be, a mystery as to why some heterologous proteins form inclusion bodies while others do not. Early hypotheses suggested that solubility limitations, the size of the protein, the type

of promoter, the extent of expression and the formation of incorrect intra- and inter-chain disulfide formation played key roles in inclusion body formation. However, upon comparing different insoluble and soluble fusion proteins with respect to these parameters, no correlation could be determined (Kane and Hartley, 1988; Schein, 1989). It should be noted that with a complex process such as inclusion body formation, the lack of any such correlations does not necessarily mean that these factors are not important for any specific protein. For example although not all cysteine-containing proteins form inclusion bodies, improper disulfide bond formation may contribute to the stability of the inclusion bodies in ones that do (Mitraki and King, 1989).

One parameter that is consistently observed to affect inclusion body formation is growth temperature, with lower temperatures (30°C or lower) favoring soluble protein production (Schein and Noteborn, 1988). Mitraki and King (1989) suggested that inclusion bodies were formed by the aggregation of specific, partially folded intermediates and that this was dependent on temperature and pH. This would explain why some heterologous proteins are insoluble and others are not since each has its own specific folding pathways. This hypothesis was supported by a study in which the soluble production of a protein A/β-galactosidase fusion protein was dependent on specific temperature and pH conditions (Strandberg and Enfors, 1991). In addition, they showed that changing the amino acid sequence around the junction between the two proteins abolished inclusion body formation, perhaps as a result of alterations in the ability of the fusion protein to fold properly.

The formation of inclusion bodies results in advantages and disadvantages with respect to fusion protein purification. Substantial purification of the protein by the removal of cytoplasmic contaminants can be obtained by differential solubilization of the inclusion bodies and has resulted

in protein that was used for crystallography (Nagai et al., 1988). As well, some proteins have been shown to be protected from proteolysis when produced as inclusion bodies (Cheng et al., 1981). One major disadvantage of inclusion bodies is that one must denature them with high concentrations of a chaotropic agent, such as urea or guanidine-HCl, to solubilize them (Marston, 1986). In the case of disulfide-containing proteins, a sulfhydryl reducing agent is also required (for a review, see Fischer et al., 1993). This denaturation necessitates the refolding of the heterologous protein to recover its biological activity. This process comes with its own set of problems and complications which have to be solved for each individual protein, although recent advances in protein refolding have improved the efficacy of this procedure (reviewed in Schein, 1990).

Another problem associated with fusion protein production is that of proteolytic degradation. There are believed to be 8 proteases in *E. coli*, 5 of which are found in the cytoplasm (Swamy and Goldberg, 1981), where most heterologous proteins accumulate. There are a few different ways to prevent this potential problem. One is to use a cocktail of protease inhibitors such as PMSF, EDTA, pepstatin A and leupeptin which inhibit serine, metallo, acid and thiol proteases respectively (Deutscher, 1990). Another alternative is to use *E. coli* hosts that are defective in one or more protease genes such as *lon* and *htpR* (Goff et al., 1984) or *ompT* (Elish et al., 1988). One conclusion that can be drawn from all this work is that each heterologous protein is different, thus making it difficult to predict their solubility and stability characteristics in a specific recombinant production system.

5. Expression of Cationic Peptides.

The study of cationic peptides such as cecropins and defensins has been somewhat limited by the paucity of the material and the complexity of their purification schemes. Although chemical synthesis has been used to make small peptides like NP-1 (Rao et al., 1992) and cecropin A (Boman et al., 1989b), for larger peptides and those with complex disulfide arrays this is not economically feasible. Therefore, there have been several attempts to establish expression systems in which to produce these peptides.

The first successful attempts at producing cecropin A from *H. cecropia* were accomplished in a baculovirus system. Hellers et al. (1991) cloned a cDNA fragment encoding the preprocecropin A gene into a baculovirus which was used to infect insect hosts. The cecropin A peptide was properly processed to a mature, active form and secreted into the hemolymph of the insects, although only 70% of the peptide was properly amidated at the C-terminus (Hellers et al., 1991). This recombinant construct, however, was not able to produce any peptide in cultured insect cells. When the cecropin A gene was fused to a synthetic IgG-binding domain of *S. aureus* protein A (ZZ; Nilsson et al., 1987) and placed in a baculovirus system, pure fusion protein was obtained from both cultured insect cells and larvae hosts (Andersons et al., 1991). Active cecropin A was obtained by CNBr cleavage of the fusion protein. Although the baculovirus system could be used to produce biologically active cecropin A, it is not a system that lends itself well to scale up.

Saccharomyces cerevisiae has also been used as a host for the production of cationic peptides. The insect defensin A gene from *P. terranovae* was fused to the gene encoding a leader sequence from the yeast pheromone mating factor α (MFα1) and expressed in yeast (Reichhart et al., 1992). The protein expressed from this gene was processed and secreted, resulting in active defensin A in the culture supernatant.

A scorpion insectotoxin, I_5A, was expressed in yeast, *E. coli*, and tobacco plants (Pang et al., 1992). The peptide, which is 35 amino acids in length and contains 4 disulfide bonds, was secreted from both bacterial and yeast cells, and produced in the cytoplasm of the tobacco plant cells. Although the peptide could be purified from each host, no biological activity was detected. The peptides produced in yeast and bacteria were both found to contain extra amino acid residues at their N-termini, which may have had a detrimental effect on their activity. This work confirmed that producing cationic peptides by direct expression in bacteria was not very feasible. In fact, producing these peptides by fusion protein technology has been equally unsuccessful. Fusions of mammalian defensins to β-galactosidase (T. Ganz, personal communication) and cecropin A to the ZZ domain (Andersons et al., 1991) were found to be highly susceptible to proteolysis when produced in bacterial expression systems. There has been only one known report of successful production of a cationic peptide in bacteria, that being the disulfide-containing peptide charybdotoxin (Park et al., 1991). This peptide was produced in *E. coli* as a fusion protein to the gene 9 protein of the T7 phage. The peptide was successfully released from the gene 9 protein by factor X_a and refolded to give a peptide that was shown to have biological activity. It is unknown if this system has been attempted for the production of other cationic peptides.

D. Aims of This Study.

The goals of this thesis were (1) to establish a bacterial expression system for cationic peptides in order to purify large quantities for further study, (2) to study the outer membrane permeabilizing activity and the bactericidal activity of selected peptides, and (3) to search for evidence that would support

the hypothesis that these peptides cross the outer membrane via the self-promoted uptake pathway. HNP-1 and CEME were the peptides chosen for this study due to their documented, strong activity against *P. aeruginosa.* In addition, CEMA, a C-terminal variant of CEME that contains two extra positive charges, and melittin were also studied.

MATERIALS AND METHODS

A. Strains, Plasmids and Growth Conditions.

All strains used in this study are listed in Table IV, and all plasmids used are found in Table V. Most strains were grown on Luria Broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract) supplemented with 0.5% (w/v) NaCl (LBNS) and 1.5% (w/v) agar. Plasmids were maintained using antibiotics such as ampicillin (75 μg/mL for *E. coli*), kanamycin (25 μg/mL for *E. coli*) and chloramphenicol (10 μg/mL for *S. aureus*). For membrane permeabilization studies such as lysozyme lysis assays and NPN uptake assays the bacteria were grown in LB without any salt supplement (LB-S). Bacteria used in dansyl polymyxin displacement assays and minimum inhibitory concentration (MIC) assays were also grown in LB-S. All medium components were obtained from Difco Laboratories, Detroit, Michigan.

B. Genetic Manipulations.

1. General DNA Techniques.

All general DNA techniques such as DNA isolation, agarose gel electrophoresis, radioactive labeling of oligonucleotides, colony blotting and Southern and Northern blotting were performed as described in Ausubel et al. (1987) and Sambrook et al. (1989). Other methods used included RNA isolation (von Gabain et al., 1983), and slot lysis gel electrophoresis (Sekar, 1987). DNA restriction and modifying enzymes (Bethesda Research Laboratories (BRL), Burlington, Canada; Boehringer Mannheim, Mannheim, Germany; Pharmacia,

Table IV: Strains.

Strain	Description	Reference/Source
E. coli		
DH5α	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Hanahan, 1983
BL21	F^- *hsd*^{s} *gal* non suppressing host (r_B^- m_B^-)	Studier and Moffatt, 1986
HMS174	F^- *hsd*^{s} *recA rif*^{R} non suppressing host (r_K^- m_K^+)	Studier and Moffatt, 1986
K38	HfrC (λ)	Russel and Model, 1984
UT5600	*ompT* protease mutant	Elish, et al., 1988
C474 (SC122)	*lac*(am) *trp*(am) *pho*(am) *mal*(am) *supC*^{ts} *rpsL*	Goff, et al., 1984
C475	SC122 *lon*	Goff, et al., 1984
C476	SC122 *htpR*165-Tn*10*	Goff, et al., 1984
UB1005	Parent for DC2	Richmond, et al., 1976
DC2	Antibiotic supersusceptible mutant	Richmond, et al., 1976
SC9251	Parent for SC9252	Meyers, et al., 1974
SC9252	Polymyxin B resistant	Meyers, et al., 1974

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Table IV: Strains (con't).

Strain	Description	Reference/Source
S. aureus		
RN4220	Transformation recipient and expression host, methicillin sensitive	Kreiswirth, et al., 1983
SAP0017	Methicillin resistant, clinical isolate	T. Chow, UBC
P. aeruginosa		
H103	PAO1 prototroph	Hancock and Carey, 1979
H309	H103 containing plasmid RP1	Hancock and Wong, 1984
K799 (or H187)	Wildtype isolate; parent for Z61	Angus, et al., 1982
Z61 (or H188)	Antibiotic supersusceptible mutant	Angus, et al., 1982
S. typhimurium		
C587 (14028s)	Parent of C590	Fields, et al., 1989
C590 (MS7953s)	*phoP/phoQ* mutant; defensin sensitive	Fields, et al., 1989
E. cloacae		
218S	Clinical isolate and parent of 218R1	Marchou, et al., 1987
218R1	β-lactamase overproducing strain	Marchou, et al., 1987

Table V: Plasmids.

Plasmid	Description	Reference/Source
pTZ19R	general cloning vector, Ap^R	Pharmacia
pKP196	pTZ19R containing a 196 bp *Sma*I fragment encoding a ribosome binding site, the *E. coli* alkaline phosphatase signal sequence and the HNP-1 gene	This study
pT7-5	T7 RNA polymerase expression vector, Ap^R	Tabor and Richardson, 1985
pKP190	pT7-5 containing a 190 bp *Sst*I/*Hin*dIII fragment from pKP196	This study
pT7-7	T7 RNA polymerase expression vector that contains the ribosome binding site from T7 gene 10, Ap^R	Tabor and Richardson, 1985
pKP160	pT7-7 containing a 160 bp *Nde*I/*Hin*dIII fragment from pKP196 which lacks the ribosome binding site	This study
pGP1-2	A P15A based plasmid that encodes the T7 RNA polymerase under control of the P_L promotor. It also contains the λ repressor cI-857, Kan^R	Tabor and Richardson, 1985
pGEX-3X	glutathione-S-transferase fusion protein expression vector, Ap^R	Pharmacia
pPCR	pTZ19R containing the 500 bp *Bcl*I/*Eco*RI fragment that had been changed by PCR	This study
pGEX-KP	pGEX-3X derivative with an *Sph*I/*Hin*dIII/*Eco*RI multiple cloning site	This study

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Table V: Plasmids (con't).

Plasmid	Description	Reference/Source
pGEX-HNP-1	pGEX-KP with a 97 bp *Sph*I/*Hin*dIII fragment encoding the HNP-1 gene	This study
pGEX-proHNP-1	pGEX-HNP-1 with a 207 bp *Sph*I fragment encoding the HNP-1 pre pro region	This study
pGEX-CEMA	pGEX-KP with a 110 bp *Sph*I/*Hin*dIII fragment encoding the CEMA gene	This study
pGEX-CEME	pGEX-CEMA with a 25 bp *Nae*I/*Eco*RI fragment replaced with oligonucleotides K and L correcting the 2 bp deletion	This study
pGEX-proCEME	pGEX-CEME with a 207 bp *Sph*I fragment encoding the HNP-1 pre pro region	This study
pGEX-CEME-S	pGEX-CEME with adaptors at the 5' and 3' ends that create a CEME *Sal*I fragment	This study
pGEX-CEMA-S	pGEX-CEMA with adaptors at the 5' and 3' ends that create a CEMA *Sal*I fragment	This study
pGEX-HNP1-B	pGEX-HNP1 with adaptors at the 5' and 3' ends that create an HNP-1 *Bam*HI fragment	This study
pGEX-proHNP1-B	pGEX-proHNP1 with adaptors at the 5' and 3' ends that create a proHNP-1 *Bam*HI fragment	This study

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Table V: Plasmids (con't).

Plasmid	Description	Reference/Source
pRIT5	protein A based fusion protein expression vector for use in *E. coli* or S. *aureus*, Amp^R, Cm^R	Pharmacia
pPA-CEME	pRIT5 with a 136 bp *Sal*I fragment encoding the CEME gene	This study
pPA-CEMA	pRIT5 with a 142 bp *Sal*I fragment encoding the CEMA gene	This study
pPA-HNP-1	pRIT5 with a 112 bp *Bam*HI fragment encoding the HNP-1 gene	This study
pPA-proHNP-1	pRIT5 with a 295 bp *Bam*HI fragment from pGEX-proHNP-1 encoding the proHNP-1 gene	This study
RP1	broad host range plasmid encoding the TEM-2 β-lactamase; Neo^R Kan^R Tet^R Amp^R	Nicas and Hancock, 1983b

Uppsala, Sweden) and DNA ligase (BRL) were used according to the manufacturer's method.

2. DNA Fragment Isolation.

Specific DNA fragments were isolated by the band intercept technique (Winberg and Hammarskjörd, 1980) using DEAE paper and manufacturer's method (Schleicher and Schuell Inc., Keene, N.H.). Alternatively, a method of isolating DNA fragments directly from agarose plugs was used. Briefly, a piece of agarose containing the fragment of interest was cut from a gel and placed in a 1.5 mL eppendorf tube containing siliconized glass wool. A small hole was poked in the bottom of this tube which was then placed in another 1.5 mL eppendorf tube. Upon spinning these tubes at 5000 g for 10 min in a microfuge, the liquid and DNA contained in the agarose plug was collected in the bottom tube. The DNA was extracted once with water-saturated N-butanol and ethanol precipitated.

3. DNA Sequencing.

Plasmid DNA for sequencing was isolated using Qiagen columns (Qiagen Inc., Chatsworth, California) following the manufacturer's protocol. Sequencing reactions were set up according to the manufacturer's method, containing 1 μg of template DNA, 3.2 pmol of primer and components from an Applied Biosystems Inc. (ABI; Foster City, California) Taq DyeDeoxy Terminator Cycle Sequencing Kit. Sequencing reactions were carried out using an Ericomp thermocycler (98°C for 1 sec, 50°C for 15 sec, 60°C for 4 min; 25 cycles), run on

an ABI 370A automated DNA sequencer, and analyzed using ABI 373A Data Collection and Analysis programs for the Macintosh computer.

4. Polymerase Chain Reaction.

Reactions were set up to include 1 ng of template DNA, 20 pmoles of each oligonucleotide primer, 0.8 mM dNTPs, and 1 unit of DNA Taq polymerase (BRL) in 10 mM Tris-HCl pH 8.2, 50 mM KCl, 0.8 mM $MgCl_2$ and 0.01% gelatin. Reactions were covered with mineral oil and placed in an Ericomp thermocycler for 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, with a final step of 72°C for 10 min to complete all chain extensions. The entire contents of the reaction tube were placed on a piece of parafilm and rolled around to remove the mineral oil. Samples were then diluted and analyzed by agarose gel electrophoresis.

5. Transformation and Electroporation.

Although some transformations of *E. coli* cells with plasmid DNA were done using 0.1 M $CaCl_2$ (Sambrook et al., 1989), most were done using the method of Chung et al. (1989). Briefly, cells to be transformed were grown to an OD_{600} of 0.3-0.4 in LBNS. For each transformation, 1 mL of these cells were pelleted in a 1.5 mL microcentrifuge tube for 1 min and resuspended in 100 µL of TSS solution (LBNS broth containing 10% (w/v) PEG 8000, 5% (v/v) dimethyl sulfoxide, 25 mM $MgCl_2$, pH 6.5). DNA (≈ 1 ng) was added to the cells and the mixture was placed on ice. After 30 min, 0.9 mL of LBNS was added to the cells which were then incubated at 37°C for 1 hr to allow the expression of the

plasmid's antibiotic resistance gene. Aliquots of the cells were plated on selective medium and grown overnight at 37°C.

The method for electroporation of S. *aureus* cells was similar to that of Compagnone-Post et al. (1991). Cells were grown in 200 mL of LBNS to an OD_{600} of 0.4-0.6 before being chilled and harvested. Cells were washed twice with 80 mLs of cold electroporation buffer (7 mM HEPES pH 7.2, 272 mM sucrose) and finally resuspended in 2.4 mL of the same buffer. Between 10 and 100 ng of plasmid DNA was added to 160 µL of cells and stored on ice for a few minutes. Electroporation was carried out in 0.1 cm electroporation cuvettes using a Bio-Rad (Mississauga, Canada) Gene Pulser set to 200 ohms, 25 µF and 1.8 kV. Immediately following electroporation, 1 mL of SMMP medium (2.8% (w/v) Bacto Pennassay Broth with 275 mM sucrose, 11 mM maleic acid, 11 mM $MgCl_2$ and 0.25% (w/v) BSA) was added. The cells were incubated for 90 min at 37°C before plating for transformants on LBNS plates containing 10 µg/mL of chloramphenicol.

6. Oligonucleotide Purification.

Oligonucleotides were obtained from T. Atkinson (Department of Biochemistry, University of British Columbia). Oligonucleotides longer than 50 bases were gel purified according to the method of Atkinson and Smith (1984) using 8% polyacrylamide gels. The oligonucleotides were visualized by UV shadowing and cut out of the gel with a sterile scalpel. To elute the oligonucleotides, the polyacrylamide gel slices were placed in 1.5 mL Eppendorf tubes containing 1 mL of 0.5 M ammonium acetate and incubated overnight at 37°C. The eluted oligonucleotides were further purified on C_{18} SEP-PAK cartridges (Waters, Milford, Massachusetts) as described by Atkinson and Smith

(1984). The 0.5 M ammonium acetate solution containing the oligonucleotides was loaded onto a prepared C_{18} SEP-PAK column, washed with water and eluted with 40% acetonitrile. The oligonucleotides were either lyophilized or ethanol precipitated before quantification by A_{260} absorbance. In the case of oligonucleotides of < 50 bases, these were dissolved in 0.5 M ammonium acetate and purified directly on C_{18} SEP-PAK columns using 20% acetonitrile as the eluting solution.

C. Vector Construction.

1. Direct Expression Vectors.

Six overlapping, complementary oligonucleotides (Table VI, A-F) encoding the gene for HNP-1 (with *E. coli* biased nucleotides at the wobble position of each codon), preceded by the alkaline phosphatase signal sequence and a ribosome binding site, were annealed together (heated for 3 min at 90°C and cooled slowly to room temperature) and ligated into pTZ19R that was previously cleaved with *Sma*I to form pKP196 (Figure 7). From this plasmid, a 190 bp *Sst*I/*Hin*dIII fragment containing the HNP-1 gene was cloned into pT7-5 to create pKP190 (Figure 8). This plasmid was transformed into K38 (pGP1-2) which possessed a plasmid-encoded T7 RNA polymerase gene under the control of the temperature inducible λ P_L promoter and would allow HNP-1 expression from the T7 RNA polymerase promoter. A 160 bp *Nde*I/*Hin*dIII fragment from pKP196 that no longer contained the ribosome binding site was inserted into pT7-7 to make pKP160 (Figure 8). This was also transformed into K38 (pGP1-2) for HNP-1 expression.

Table VI: Oligonucleotides.

Name	Sequence 5'- 3'	Description
A	GGGAGCTCCTAACTAACTAAGGAGGAGACATATGAAAC AAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTT ACCCC	81 mer used in construction of HNP-1 gene
B	CCAGTGCAATAGTGCTTTGTTTCATATGTCTCCTCCT TAGTTAGTTAGGAGCTCC	56 mer used in construction of HNP-1 gene
C	TGTGACAAAAGCCGCATGCTACTGCCGTATACCGGCCT GCATCGCGGGCGAACGTCGTTACGGTA	65 mer used in construction of HNP-1 gene
D	CAGGCCGGTATACGGCAGTAGCATGCGGCTTTTGTCAC AGGGGTAAACAGTAACGGTAAGAGTG	64 mer used in construction of HNP-1 gene
E	CCTGCATCTACCAGGGCCGTCTGTGGGCGTTCTGCTG CTAAAAGCTTCGC	50 mer used in construction of HNP-1 gene
F	GCGAAGCTTTTAGCAGCAGAACGCCCACAGACGGCCCT GGTAGATGCAGGTACCGTAACGACGTTCGCCCGCGATG	76 mer used in construction of HNP-1 gene
G	CCGATGGCCATCATACGTTATATAGCTGAC	30 mer used in converting pGEX-3X to pGEX-KP
H	GCGGGAATTCAAGCTTGCATGCACGACCTTCGATCAGA TCCG	42 mer used in converting pGEX-3X to pGEX-KP
I	CGGGGATCCGCATATGAAATGGAAACTGTTCAAGAAGA TCGGCATCGGCGCCGTGCTGAAAGTGCTGACCACCGGT CTGCCGGCGCTGAAGCTAACTAAGTA	102 mer encoding CEMA

con't...

Table VI: Oligonucleotides (con't).

Name	Sequence 5'- 3'	Description
J	AGCTTACTTAGTTAGCTTCAGCGCCGGCAGACCGGTGG TCAGCACTTTCAGCACGGCGCCGATGCCGATCTTCTTG AACAGTTTCCATTTCATATGCGGATCCCCGCATG	110 mer encoding CEMA
K	GGCGCTGAAGCTAACTAAGTAAGCTTG	27 mer used in converting CEMA to CEME
L	AATTCAAGCTTACTTAGTTAGCTTCAGCGCC	31 mer used in converting CEMA to CEME
M	CCATATGAGGACCCTCGCCATCCTTGCTGCCATTCTCC TGGTGGCCCTGCAGGCCCAGGCTGAGCCACTCCAGGCA AGAGCTGATGAGGTTGCAGCAGCCCCGGAGCAGA	110 mer used in construction of pre pro cartridge
N	TTGCAGCTGACATCCCAGAAGTGGTTGTTTCCCTTGCA TGGGACGAAACGTTGGCTCCAAAGCATCCAGGCTCAAG GAAAAACATGGCATG	91 mer used in construction of pre pro cartridge
O	CCATGTTTTTCCTTGAGCCTGGATGCTTTGGAGCCAA GCTTTCGTCCCATGCAAGGGAAACAACCACTTCTGGGA TGTCAGCTGCAATCTGCTCCGGGGCTGCTGCAAC	109 mer used in construction of pre pro cartridge
P	CTCATCAGCTCTTGCCTGGAGTGGCTCAGCCTGGGCCT GCAGGGCCAGCAGGAGAATGGCAGCAAGGATGGCGAGG GTCCTCATATGGCATG	92 mer used in construction of pre pro cartridge
Q	AGCTTGTCGACA	12 mer encoding a *Hin*dIII to *Sal*I adaptor
R	CGTCGACATCGAAGGTCGTGCATG	24 mer encoding factor Xa recognition site and an *Sph*I to *Sal*I adaptor
S	CACGACCTTCGATGTCGACGCATG	24 mer encoding factor Xa recognition site and an *Sph*I to *Sal*I adaptor

con't...

Table VI: Oligonucleotides (con't).

Name	Sequence 5'- 3'	Description
T	CGGATCCATGGCATG	15 mer encoding a methionine residue and an *Sph*I to *Bam*H1 adaptor
U	CCATGGATCCGCATG	15 mer encoding a methionine residue and an *Sph*I to *Bam*H1 adaptor
V	AATTCGGATCCG	12 mer encoding an *Eco*R1 to *Bam*H 1 adaptor
W	TATGGGATCCCA	12 mer encoding an *Nde*I to *Bam*H1 adaptor
X	CCAAAATCGGATCTGATCGAAGG	23 mer used as sequencing primer
Y	CAGATCGTCAGTCAGTCACG	20 mer used as sequencing primer

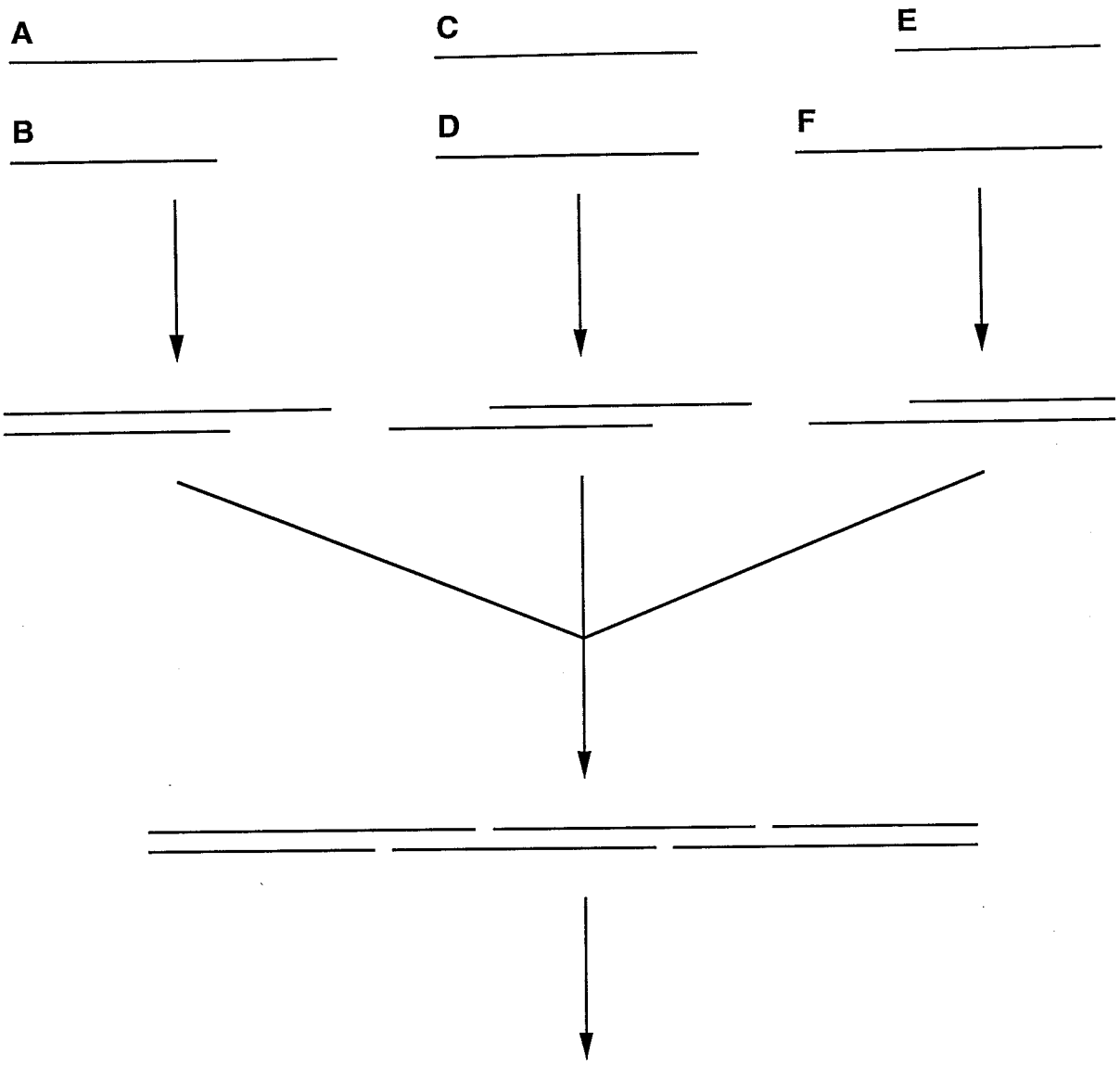

Figure 7: Schematic representation of the strategy used to anneal the six oligonucleotides that encoded the HNP-1 gene.

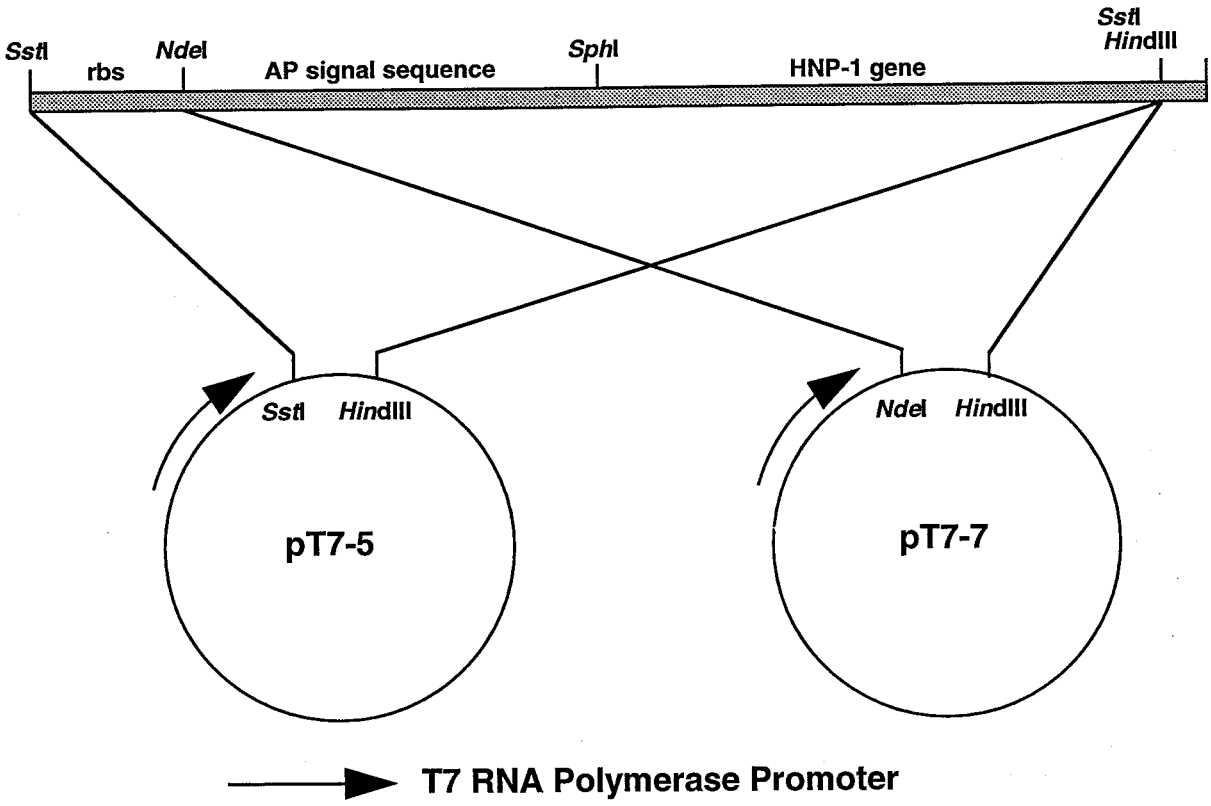

Figure 8: Construction of pKP190 and pKP160.

The HNP-1 gene was inserted into pT7-5 and pT7-7 to form pKP190 and pKP160 respectively as described in Materials and Methods, section C.1. Only relevant restriction sites are shown. The large arrow indicates the position and direction of the T7 RNA polymerase promotor. AP, alkaline phosphatase; rbs, ribosome binding site.

2. GST Expression Vectors.

The multiple cloning site of the fusion protein expression vector pGEX-3X had to be changed before the HNP-1 gene could be cloned into it. Therefore, a 500 bp *Bam*HI/*Bal*I fragment was isolated from pGEX-3X and subjected to PCR using oligonucleotides G and H, the latter which had a 21 base overhang encoding *Sph*I, *Hin*dIII and *Eco*RI restriction enzyme sites. The PCR reaction (Materials and Methods, section B.3.) was performed in an Ericomp thermocycler. The reaction products were blunt-ended using Klenow fragment and ligated into pTZ19R cleaved with *Sma*I to produce pPCR. A 200 bp *Bcl*I/*Eco*RI fragment from pPCR was used to replace the *Bcl*I/*Eco*RI fragment from pGEX-3X to produce pGEX-KP, which now had a multiple cloning site consisting of *Sph*I, *Hin*dIII and *Eco*RI instead of *Bam*HI, *Sma*I and *Eco*RI. A 90 bp *Sph*I/*Hin*dIII fragment from pKP196 containing just the HNP-1 gene was cloned into pGEX-KP to create pGEX-HNP-1, which was sequenced using oligonucleotides X and Y as primers to ensure it was the correct construct.

The plasmid pGEX-CEME was created by a two step process. Two complementary oligonucleotides encoding *Sph*I and *Hin*dIII sticky ends and the CEME gene preceded by a methionine codon were cloned into pGEX-KP. Upon sequencing, however, a 2 bp deletion was discovered, which resulted in a gene encoding a peptide (CEMA) with an additional 2 amino acids and a net charge change of +2 as compared with CEME (oligonucleotides I and J). This plasmid was termed pGEX-CEMA. In order to fix the frameshift mutation, a *Nae*I/*Eco*RI fragment containing the deletion was removed from pGEX-CEMA and replaced with oligonucleotides K and L to form pGEX-CEME. This correction was confirmed by DNA sequencing.

The plasmids pGEX-proHNP-1 and pGEX-proCEME were created using oligonucleotides M-P which encode the pre pro region of the HNP-1 gene (Daher et al., 1988). These oligonucleotides, which have *Sph*I sticky ends when annealed, were inserted into *Sph*I-cleaved pGEX-HNP-1 and pGEX-CEME. The insertion orientation of the prepro fragment was confirmed by restriction enzyme analysis.

3. Protein A Expression Vectors.

The limited restriction sites available for cloning into pRIT5 (Nilsson et al., 1985a) and the requirement for obtaining the correct reading frame, necessitated changing the 5' and 3' ends of the CEME, CEMA and HNP-1 genes. For CEME and CEMA, the 3' ends were altered by inserting the self-annealing oligonucleotide Q encoding a *Hin*dIII to *Sal*I adapter into the *Hin*dIII sites of pGEX-CEME and pGEX-CEMA. These were further altered at the 5' end by cloning, into the *Sph*I sites, the annealed oligonucleotides R and S which encode an *Sph*I to *Sal*I adapter including a factor X_a recognition site. The orientation of this asymmetric insertion was confirmed by DNA sequencing. The *Sal*I fragments from the resulting two vectors (pGEX-CEME-S and pGEX-CEMA-S) were cloned into pRIT5 to produce pPA-CEME and pPA-CEMA. The HNP-1 gene was modified in a similar manner. An *Sph*I to *Bam*HI adapter containing a methionine codon (oligonucleotides T and U) was inserted into the *Sph*I site on the 5' end of the HNP-1 gene (the orientation was confirmed by DNA sequencing), while an *Eco*RI to *Bam*HI adapter (oligonucleotide V) was used at the 3' *Eco*RI site. The resulting *Bam*HI fragment from pGEX-HNP-1-B was cloned into pRIT5 to form pPA-HNP-1. The plasmid pGEX-proHNP-1 was changed to pGEX-proHNP-1-B using oligonucleotide V at the 3' end and

oligonucleotide W, an *Nde*I to *Bam*HI adapter, at the 5' end. The cloning of this *Bam*HI fragment into pRIT5 resulted in the production of pPA-proHNP-1. All pPA plasmids were isolated and electroporated into *S. aureus* RN4220

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D. Immunological Techniques.

1. Production and Purification of Antibodies.

Antibodies were prepared as described by Harlow and Lane (1988). A preparation of GST/CEMA was solubilized in SDS-PAGE loading buffer, heated at 100°C for 10 min, and loaded onto a preparative SDS polyacrylamide gel. GST/CEMA contained in gel slices was passively eluted into water and lyophilized. The sample was resuspended and mixed with an equal volume of Freund's complete adjuvant before injecting 1 mg of protein subcutaneously into a rabbit. Booster shots of 1 mg of protein mixed with Freund's incomplete adjuvant were given at 3 and 7 weeks. After 10 weeks, the rabbits were bled and the serum was isolated.

2. Western Blotting.

Western immunoblotting (Mutharia and Hancock, 1983) and immunodetection (Mutharia and Hancock, 1983; Harlow and Lane, 1988) were done as previously described.

E. Electrophoresis.

1. SDS-PAGE.

SDS-PAGE was performed as previously described (Hancock and Carey, 1979).

2. AU-PAGE.

AU-PAGE was done according to the method of Panyim and Chalkey (1969). Briefly, 15% polyacrylamide gels containing 5 M urea (w/v) and 5% acetic acid were pre-electrophoresed at 100 V for two hours (for mini gels) or 150 V overnight (for large gels) in 5% acetic acid. The buffer was replaced with new 5% acetic acid before the samples were loaded. Samples were solubilized in 5% acetic acid and diluted 2:1 with sample buffer (9 M urea in 5% acetic acid containing methyl green as a tracking dye). Gels were run in the reverse polarity (towards the cathode) at either 100 V or 250 V until the methyl green migrated off the bottom of the gel.

F. Protein Expression.

1. Direct Expression.

Attempts to express HNP-1 from pKP190 and pKP160 using the T7 RNA polymerase system were performed as previously described (Ausubel et al., 1987). Briefly, K38 (pGP1-2) cells containing pKP190 or pKP160 were grown in LBNS with kanamycin and ampicillin at 37°C to an OD_{600} of 0.5. A sample of cells was removed (as an uninduced control) before the culture was shifted to 42°C for 30 min to induce the T7 RNA polymerase. Again, a sample of cells was

removed before the culture was shifted back to 37°C and 200 μg/mL of rifampicin simultaneously added to prevent *E. coli* RNA polymerase activity. The cells were harvested and the basic proteins extracted as previously described (Lindahl and Zengel, 1979). Proteins extracted from all strains were analyzed by SDS-PAGE and AU-PAGE.

2. GST Fusions.

Cells of *E. coli* strain DH5α containing pGEX-KP, pGEX-CEME, pGEX-proCEME, pGEX-CEMA, pGEX-HNP-1 or pGEX-proHNP-1 were grown at 37°C to mid log phase and IPTG added to a final concentration of 0.2 mM to induce fusion protein expression. Growth was allowed to continue for 3 h before cells were harvested. To test for fusion protein production, 500 μL of cells were pelleted in a microfuge for 1 min and resuspended in 30-50 μL of water. An equal volume of 2X SDS-PAGE whole cell lysing buffer (2X WCLB; 0.125 M Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS and 10% (v/v) 2-mercaptoethanol) was added, the sample heated at 100°C for 10 min and loaded onto an SDS-PAGE gel.

3. Protein A Fusions.

Initially, expression of PA/CEME fusion protein was attempted in *E. coli* DH5α cells harboring pRIT5 or pPA-CEME were grown at 37°C until mid-log phase. Samples of whole cells and culture supernatants were analyzed by SDS-PAGE and Western immunoblotting for the presence of fusion protein. Fusion proteins were detected on Western immunoblots using an IgG antiserum which bound to the carrier portion of the molecule. Plasmids pRIT5, pPA-CEME, pPA-

HNP-1, and pPA-proHNP-1 were also expressed in S. *aureus* strain RN4220. In this case, S. *aureus* cells harboring the various plasmids were grown at 37°C to an OD_{600} of between 1.5-1.8 before the cells were removed by centrifugation. Culture supernatants (20 μL) were analyzed as above by Western immunoblotting. Large scale (20 L or 60 L) expression of pPA-CEME was done in a LH fermentor (100L working volume, 5000 series) and the cells removed by a Sharples centrifuge T-1P. After the addition of 1 mM sodium azide, the supernatant was clarified using a 0.45 μM membrane in a Pellicon Cassette System with tangential flow filtration (Millipore). The supernatant was then concentrated about 10-fold by ultrafiltration using a 1 kDa Omega Filtron Centrasett Screen Channel. Large scale growth of RN4220(pPA-CEME) and the clarification and concentration of the culture supernatant were carried out by L. Robillo and G. Lesnicki (Biotechnology Laboratory, University of British Columbia)

G. Fusion Protein Purification.

1. GST Fusion Proteins.

Cell pellets were resuspended in 1/100 volume of 50 mM Tris-HCl pH 8.0, 50 mM NaCl and 1 mM EDTA and passed twice through a French pressure cell at 15,000 psi. The lysate was fractionated by centrifugation at 2500 g for 5 min and the fusion protein recovered either in the supernatant fraction or in the pellet as insoluble inclusion bodies. In the former situation, the supernatant was incubated for 1 h at 4°C with glutathione agarose beads (sulfur linkage, Sigma, St. Louis, Missouri) previously swelled and equilibrated in mouse tonicity phosphate buffered saline (MTPBS; 150 mM NaCl, 16 mM Na_2HPO_4 and 4 mM NaH_2PO_4, pH 7.2). The beads were washed three times

with one volume of MTPBS by resuspension and centrifugation. The fusion protein was eluted with 5 mM reduced glutathione in 50 mM Tris-HCl pH 8.0. The cellular production of inclusion bodies prevented the use of affinity chromatography to purify the fusion protein. Therefore, two methods of differential solubilization were employed to purify the inclusion bodies. For GST/HNP-1, the insoluble pellet was washed sequentially with: 1) 10 mM Tris-HCl pH 8.0, 10 mM EDTA and 1% Triton X-100; 2) H_2O; 3) 3 M urea (ARISTAR quality, BDH Chemicals, Vancouver, Canada; previously deionized with AG 501-X8 mixed bed resin, Bio-Rad) in 5 mM Tris-HCl pH 8.0; and 4) H_2O. Each wash consisted of resuspending the pellet thoroughly with a pipet and immediately pelleting it at 20 K for 15 min in an ultracentrifuge using a Ti50 or 60 rotor. Finally, the inclusion bodies were solubilized with 8 M urea in 5 mM Tris-HCl pH 8.0. For GST/proCEME, the insoluble pellet was extracted using a different procedure. After washing the pellet with 10 mM Tris-HCl pH 8.0, 10 mM EDTA and 1% Triton X-100, it was sequentially extracted twice with 10 mM Tris-HCl pH 8.0, 10 mM EDTA and 3% (v/v) octyl-polyoxyethylene (O-POE; Bachem Bioscience, Philadelphia), and twice with 10 mM Tris-HCl pH 8.0. This procedure removed all the contaminating membrane proteins and resulted in a reasonably pure inclusion body preparation.

2. Protein A Fusion Proteins.

PA/CEME and PA/CEMA were purified as previously described (Moks et al., 1987a). Briefly, clarified culture supernatants were adjusted to pH 7.6 with NaOH and passed over an IgG-Sepharose column (bed volume of 30 mL, Pharmacia), previously equilibrated with TST buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.05% Tween 20). The column was washed sequentially with

10 volumes of TST and 5 volumes of 5 mM ammonium acetate pH 5.0. Sample loading and column washing was done at a flow rate of 100-150 mL/hr which was controlled by a Pharmacia P-1 peristaltic pump. The fusion protein was eluted at a flow rate of 50 mL/hr with 0.5 M acetic acid pH 3.4 and lyophilized.

H. Peptide Release.

1. Factor X_a.

Inclusion body preparations of GST/HNP-1 previously solubilized in 8 M urea and 5 mM Tris-HCl pH 8.0 were placed in Spectra/Por 3 dialysis tubing (Spectrum Medical Industries, Los Angeles, California) and sequentially dialyzed overnight against 1 M urea in 5 mM Tris-HCl pH 8.0, and factor X_a cleavage buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl and 1 mM $CaCl_2$). If necessary, the sample was concentrated using a Centricon 10 (Amicon, Oakville, Canada). Factor X_a (Boehringer Mannheim) was added at an enzyme to substrate ratio of approximately 1:25, and the reaction was allowed to proceed at 37°C for 60 h. Samples were taken at 12 h intervals and analyzed on a 12-25% gradient SDS-PAGE gel.

2. Cyanogen Bromide.

Whether the sample was an inclusion body preparation of GST/proCEME or purified, lyophilized PA/CEME or PA/CEMA, the fusion proteins were solubilized in 70% formic acid to which 1 M CNBr was added. Protein concentrations in these reaction were such that the molar excess of CNBr to methionine residues in the fusion protein was at least 2000. The sample was flushed with nitrogen, tightly capped, and incubated in the dark at 25°C for 18-

24 h. The reaction was quenched by diluting to 5% formic acid with H_2O followed by lyophilization.

I. Peptide Purification.

The CNBr cleaved PA/CEME or PA/CEMA fusion protein was resuspended in 5% formic acid, and approximately 100 mg loaded onto a Bio-Gel P100 column (bed volume of 300 mL, flow rate of 10 mL/h, Bio-Rad), and eluted with 1% acetic acid. Two mL fractions were collected, lyophilized and analyzed by AU-PAGE. Fractions containing the peptide were pooled, lyophilized and resuspended in 0.1% TFA. In the case of a large scale preparation, a 15 mg sample was loaded onto an FPLC Bio-Sil C_{18} reverse phase column (bed volume of 50 mL, flow rate of 2 mL/min, Bio-Rad), previously equilibrated with 0.1% TFA. The sample was eluted with a 60 mL gradient of 50% acetonitrile in 0.1% TFA. Samples containing the peptide of interest were pooled, lyophilized and again resuspended in 0.1% TFA. Final purification of the peptide was performed on a Pharmacia PepRPC HR 5/5 column (bed volume of 1 mL, flow rate of 0.7 mL/min). One mg of protein was loaded onto the column and the peptide was eluted with the following gradient of acetonitrile in 0.1% TFA: 3 mL, 0-30%; 5 mL, 30%; 20 mL, 30-50%. Fractions (0.5 mL) were lyophilized and analyzed by AU-PAGE to confirm homogeneity.

J. Gel Overlay Assay.

Detection of antibacterial activity of proteins separated by AU-PAGE was described previously (Hultmark et al., 1980). Briefly, purified or partially

purified samples of antibacterial peptides were electrophoresed on a 15% acid urea gel which was then incubated in Mueller-Hinton broth containing 0.2 M sodium phosphate buffer pH 7.4 for 1 h. The gel was overlaid with 5 mL of the same media containing 0.6% agar and about 10^5 colony forming units of *E. coli* DC2, and then again with another 5 mL of agar. The gel was incubated overnight at 37°C which resulted in zones of lysis that corresponded to the migration site of the antibacterial peptide.

K. Peptide Analysis.

1. Preparation of Samples.

Peptides were subjected to AU-PAGE on a 15% gel and electroblotted onto Immobilon membrane (Millipore, Bedford, Massachusetts) using the manufacturer's method. Briefly, the transfer membrane was wetted with 100% methanol for a few seconds, rinsed in water, and equilibrated in transfer buffer (0.7% acetic acid, 10% methanol). Electroblotting was carried out in a Bio-Rad Mini Trans-Blot electrophoretic apparatus in the direction of the cathode for 2 h at 150 V. The membrane was stained with 0.5% (w/v) Ponceau S in 1% acetic acid for 2 min and destained with water until protein bands were visible (Ausubel et al., 1987).

2. Amino Acid Sequencing and Analysis.

The band containing the peptide of interest was excised and analyzed for amino acid content using an Applied Biosystems amino acid analyzer (model 470) and the N-terminal sequence determined using an Applied Biosystems sequencer (model 420). The amino acid analysis and peptide sequencing was

carried out by S. Keiland (Department of Biochemistry and Microbiology, University of Victoria, B.C.).

L. Assays.

1. Protein Concentration Estimation.

a. Lowry.

Most protein concentrations were estimated with a modified Lowry assay (Sandermann and Strominger, 1972). A 1 mg/mL solution of bovine serum albumin was used as a standard.

b. Dinitrophenylation.

Since CEME and CEMA only have one aromatic residue in their primary structures, a protein assay that measures the presence of free amino groups (Bader and Teuber, 1973) was used instead of the modified Lowry assay. A 1 mg/mL solution of polymyxin B was used as the standard. Briefly, a 50 µL sample of peptide was mixed with $Na_2B_4O_7 \cdot 10H_2O$ (final concentration 0.8%) and 1-fluoro 2,4 dinitrobenzene (final concentration 10 mM), and incubated at 37°C for 1 h. One mL of 2 N HCl and 1 mL of n-butanol were added, and the mixture vortexed. After centrifugation at 100 rpm for five minutes, the absorbance of the butanol phase was read in a spectrophotometer at 420 nm. The values obtained needed to be adjusted to reflect the number of free amino groups in the respective peptides compared to the standard (CEME had 6, CEMA had 8, and polymyxin B had 5).

2. Killing Assay.

This assay was performed as described previously (Lehrer et al., 1983). Briefly, reactions were carried out in 100 µL volumes and contained 10^6 CFU of *P. aeruginosa* H187 in a low ionic strength buffer (10 mM potassium phosphate, pH 7.4), and either 2.5 µg/mL or 5.0 µg/mL of CEME. After either 20 or 60 min at 37°C, samples of the bacteria were removed, diluted and plated to obtain a viable count.

3. Lysozyme Lysis.

The uptake of lysozyme into whole cells due to membrane permeabilization by various compounds was previously described by Hancock et al. (1981). Overnight cultures of *P. aeruginosa* H309 or *E. cloacae* 218R1 grown in LB-S were diluted 1 in 50 in fresh medium and grown to an OD_{600} of 0.5-0.6. The cells were harvested in a Silencer H-103N clinical centrifuge at 1800 g for 10 min, washed once with one volume of assay buffer (5 mM HEPES pH 7.2, 5 mM KCN), and resuspended in the same buffer to an OD_{600} of 0.5. Assays consisted of 600 µL of cells with 50 µg/mL of chicken egg white lysozyme and varying concentrations of cationic compounds. Cell lysis was measured as a decrease in the OD_{600} in a Perkin-Elmer dual beam spectrophotometer. Parallel experiments performed without lysozyme enabled the measurement of the lytic activity of the compounds themselves. To test whether or not permeability to lysozyme could be inhibited by divalent cations, various concentrations of $MgCl_2$ were added to the assay after the addition of lysozyme and before the addition of the test compound.

4. 1-*N*-phenylnaphthylamine Uptake.

This assay was previously described by Loh et al. (1984). Cells were prepared exactly the same as for the lysozyme lysis assay. 1-*N*-phenylnaphthylamine (NPN) was dissolved in acetone at a concentration of 500 μM. NPN fluorescence was measured in a Perkin-Elmer 650-10S fluorescence spectrophotometer using excitation and emission wavelengths set to 350 nm and 420 nm respectively, with slit widths of 5 nm. The assay was standardized by adding 20 μL of NPN (final concentration of 10 μM) and 10 μL of a 0.64 mg/mL solution of polymyxin B (final concentration of 6.4 μg/mL) into 1 mL of cells, and adjusting the resulting fluorescence to read 90% deflection on the chart recorder (90 arbitrary units). Various compounds were tested by adding 10 μL of different concentrations to a cuvette containing 1 mL of cells and 10 μM NPN. Permeabilizing activity was designated as the total fluorescence minus the fluorescence due to NPN alone. Following the fluorescence measurement, the OD_{600} of the cells was measured to ensure no significant cell lysis had occurred. Control experiments showed that neither acetone nor test compounds alone resulted in an increase in fluorescence in the absence of NPN.

5. Dansyl Polymyxin B Displacement.

a. Lipopolysaccharide Isolation.

P. aeruginosa LPS was isolated as previously described (Darveau and Hancock, 1983). The procedure was performed by Susan Farmer.

b. Dansyl Polymyxin B Synthesis.

Dansyl polymyxin B was synthesized as described by Schindler and Teuber (1975). Briefly, 40 mg of polymyxin B and 10 mg of dansyl chloride were mixed in 2 mL of 60 mM $NaHCO_3$ and 40% acetone and incubated in the dark for 90 min. The unreacted dansyl chloride was separated from the dansyl polymyxin B by gel filtration on a Sephadex G-50 column. The fractions containing dansyl polymyxin were extracted with 1/2 volume of n-butanol and evaporated to dryness in a desiccator at 37°C. The dansyl polymyxin B was resuspended in 5 mM HEPES pH 7.0, quantified by dinitrophenylation and stored in aliquots at -20°C. This procedure was carried out by Susan Farmer.

c. Assay.

The method of Moore et al. (1986) was used to test how much dansyl polymyxin B was needed to saturate the binding sites on LPS. Briefly, 5 µL samples of 100 µM dansyl polymyxin B were titrated into 1 mL of 3 µg/mL of LPS until a maximum fluorescence was reached. The fluorescence was measured in a Perkin-Elmer 650-10S fluorescence spectrophotometer with an excitation wavelength of 340 nm and an emission wavelength of 485 nm using slit widths of 5 nm. A final concentration of dansyl polymyxin B giving 90-100% maximum fluorescence (2.5 µM) was chosen and used in all subsequent experiments. For the binding displacement assays (Moore et al., 1986), 2.5 µM dansyl polymyxin B was added to 3 µg/mL of H103 LPS in 5 mM HEPES pH 7.2. Samples (5 µL) of the test compounds were added and the decrease in fluorescence due to displacement of the dansyl polymyxin B from the LPS was recorded. The addition of the compound was continued until it resulted in only a small (<5%) decrease in fluorescence. The data were plotted as the fraction of

dansyl polymyxin B bound as a function of the concentration of compound (I). The relative affinities of the compounds for the binding sites on LPS were determined by calculating the I_{50} values directly from the graph. I_{50} represented the concentration of compound that resulted in 50% maximal displacement of dansyl polymyxin B from the LPS. All experiments were performed a minimum of three times.

For dansyl polymyxin B binding inhibition assays using whole cells instead of purified LPS, H309 cells were prepared in the same way as for the lysozyme lysis assay. The assay consisted of 10 µL of cells at an OD_{600} of 0.5, 990 µL of 5 mM HEPES pH 7.2 and 5 mM KCN, and a concentration of dansyl polymyxin B that had been determined to result in 90-100% binding saturation. This concentration varied from day to day but usually was between 2.5 µM and 3.5 µM. Compounds were titrated in, and I_{50} values determined as described above.

6. Minimum Inhibitory Concentration.

These assays were done according to the broth dilution method (Amsterdam, 1991). Briefly, cells were grown overnight at 37°C in LB-S and diluted 1 in 10 000 in the same medium to give concentrations of about 10^4 to 10^5 CFU/mL. Serial dilutions of the antimicrobial substances in LB–S were performed in a 96 well microtitre plate. Subsequently, 10 µL of bacteria were pipetted into 100 µL volume of the diluted antibiotic, and the plates incubated overnight at 37°C. Samples of the bacterial inoculum were plated to ensure they were within the proper inoculum range. The next day the microtitre plates were scored for growth in the wells, and the MIC determined as the lowest antibiotic concentration that inhibited growth. To determine the effect of

cations on the MIC values of the various compounds, either 5 mM $MgCl_2$ or 80 mM NaCl were included in the LB-S medium. In the synergy MIC studies, the given sub-MIC concentrations of the peptides were included in the LB-S medium.

RESULTS

CHAPTER ONE. The Production of Cationic Peptides as GST Fusion Proteins.

A. Introduction: Why Fusion Proteins Are Necessary.

The HNP-1 gene was initially cloned into the T7 RNA polymerase expression vectors pT7-5 and pT7-7 (Materials and Methods, section C.1.). Attempts at producing HNP-1 from either of these two constructs ((Materials and Methods, section C.1.) were unsuccessful as determined by SDS-PAGE and AU-PAGE (data not shown). To ensure that the lack of peptide production was not a transcriptional problem, Northern blots were performed with uninduced and induced cultures of K38 (pGP1-2)(pKP160) using oligonucleotide D (Table VI) as a probe. The results showed a small transcript being produced in the induced cultures that was not present in the uninduced culture (data not shown). Based on these experiments, it was concluded that although the transcription of the gene was occurring, the protein was either not being produced at all, or was being produced and subsequently degraded by the host organism.

The inability to produce HNP-1 by direct expression of the gene in *E. coli* led to attempts to produce it as a fusion protein. Certain requirements of a fusion protein expression system had to be met if it were to be useful for producing cationic peptides. First, the carrier protein had to have a high affinity for a specific ligand to provide a simple means of purification. Second, the affinity tag had to contain a site specific cleavage sequence (either enzymatic or chemical) to allow the release of the cationic peptide. Third, the vector had to possess restriction enzyme sites that would enable the insertion of the gene immediately adjacent to the specific cleavage site. This would allow

the release of the peptide from the carrier protein without any extra amino acids at the N-terminus of the peptide. This is critical in the case of cationic peptides since it has been shown that the addition of one or more amino acids to the peptide can alter its biological activity (Bessalle et al., 1992). Since the GST fusion protein system, specifically the vector pGEX-3X, fulfilled these criteria, it was used to express the genes encoding HNP-1 and CEME.

B. Construction of the pGEX-KP Vector.

The vector pGEX-3X did not have the restriction enzyme sites that were compatible with the ends of the HNP-1 gene. Therefore, the multiple cloning site (*Bam*HI/*Sma*I/*Eco*RI) was changed to include the appropriate sites (*Sph*I/*Hin*dIII/*Eco*RI) necessary for the insertion of the HNP-1 gene. This was accomplished using a series of genetic manipulations (Materials and Methods, sections B.3. and C.2.; Figure 9). When the HNP-1 gene (Figure 10B) was cloned into the resulting vector, pGEX-KP (Materials and Methods, section C.2.; Figure 10A), the first amino acid codon of the HNP-1 gene (Ala) was immediately 3' to the last amino acid codon of the factor X_a recognition site (Arg)(Figure 9). Theoretically, therefore, when the fusion protein (GST/HNP-1) produced by this construct (pGEX-HNP-1) was purified and cleaved with factor X_a, there would be no extraneous N-terminal amino acids on the HNP-1 peptide.

C. The Production and Purification of GST/HNP-1.

The production of the GST/HNP-1 fusion protein was investigated by inducing the expression of the gene with IPTG for varying amounts of time (Figure 11). The vector pGEX-3X was used as a control, and pGEX-KP was

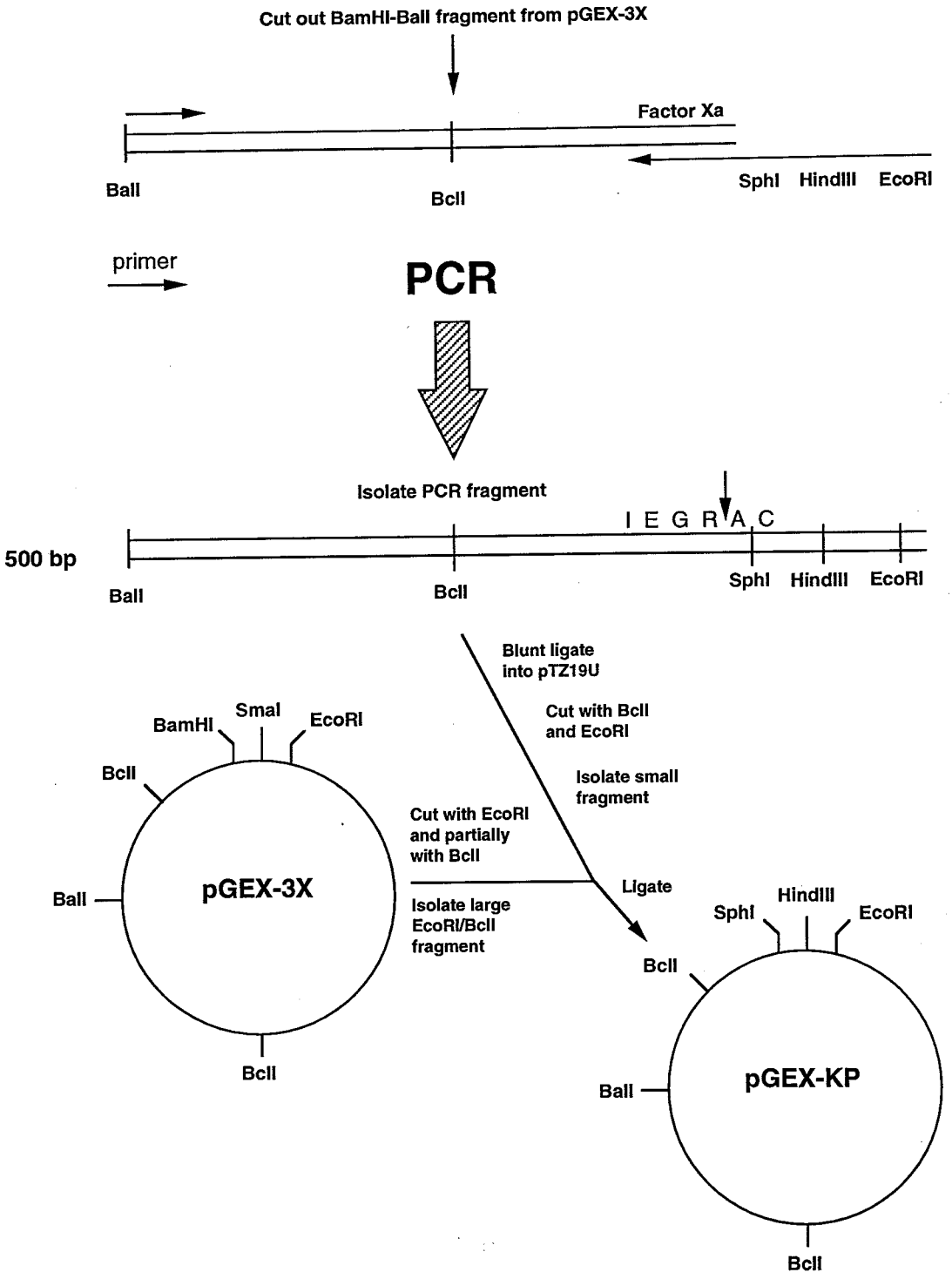

Figure 9: Construction of pGEX-KP.

All genetic manipulations are described in Materials and Methods, sections B.3. and C.2. The I-E-G-R sequence is the factor X_a recognition site and the A-C residues are encoded by the *Sph*I site and represent the first two amino acids of HNP-1. The arrow between them shows the site of factor X_a cleavage.

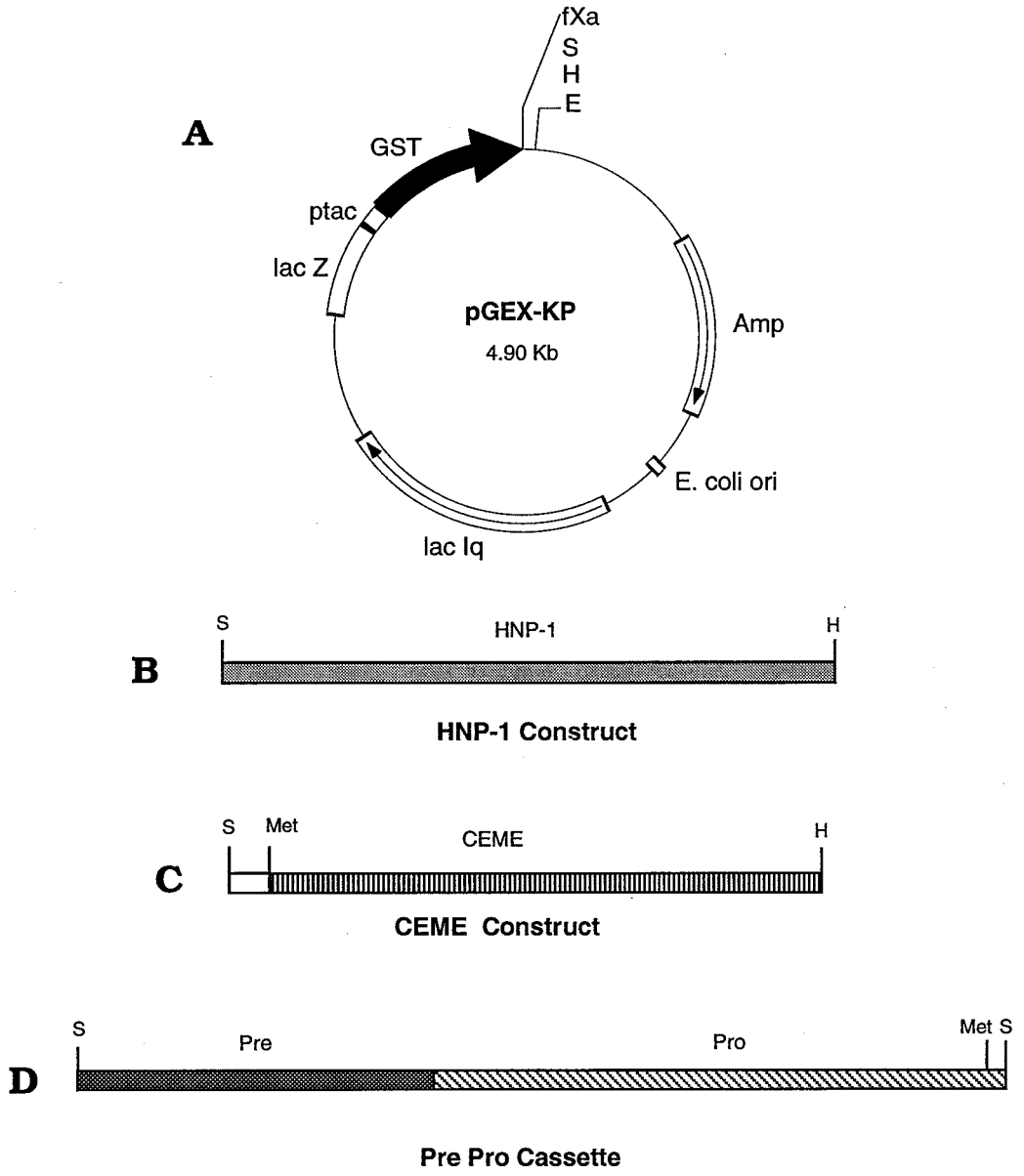

Figure 10: Construction of GST/cationic peptide fusion proteins.

A, The pGEX-KP cloning vector. **B**, DNA fragment containing the HNP-1 gene which was inserted into pGEX-KP using the *Sph*I and *Hin*dIII sites to form pGEX-HNP-1. **C**, DNA fragment containing the CEME gene, preceded by a methionine codon, which was cloned into pGEX-KP using *Sph*I and *Hin*dIII to form pGEX-CEME. **D**, DNA fragment encoding the mammalian defensin prepro region which was inserted into the *Sph*I sites of both pGEX-HNP-1 and pGEX-CEME to create pGEX-proHNP-1 and pGEX-proCEME respectively. GST, glutathione-S-transferase; ptac, tac promotor; Amp, β-lactamase gene; ori, origin of replication; fX_a, factor X_a recognition site; S, *Sph*I; H, *Hin*dIII; E, *Eco*RI; Met, methionine codon.

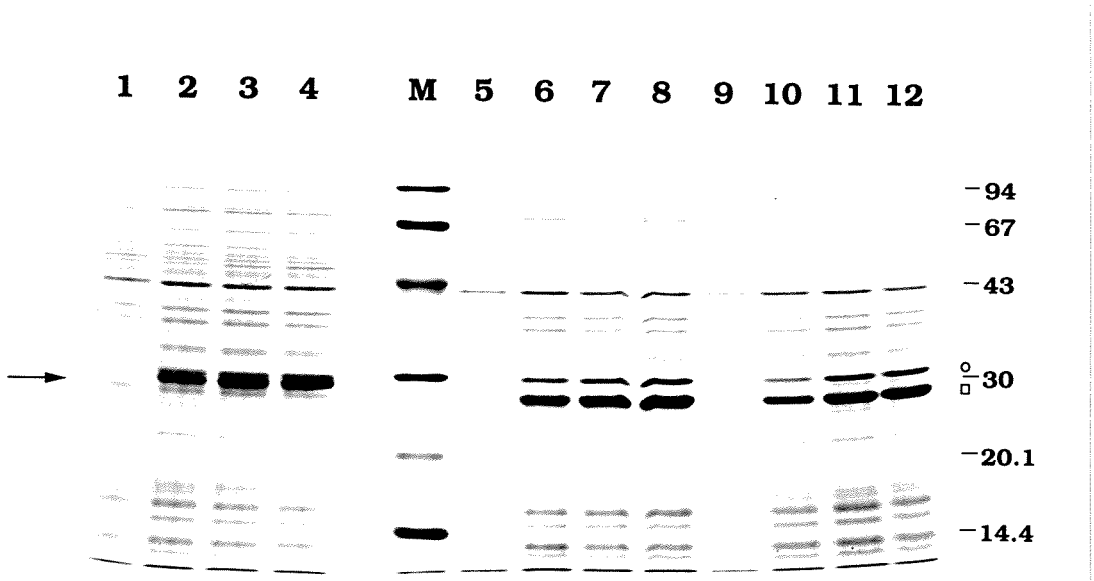

Figure 11: Production of GST/HNP-1.

Whole cell lysates of DH5α(pGEX-HNP-1)(lanes 1-4), DH5α(pGEX-KP)(lanes 5-8), and DH5α(pGEX-3X)(lanes 9-12) were electrophoresed through an 11% SDS polyacrylamide gel and stained for protein with Coomassie blue. Approximately 15 μg of protein were loaded in each lane except lane 5 and 9 (5 μg). Cells were grown to an OD_{600} of approximately 0.5 and induced with 0.2 mM IPTG for: 0 h (lanes 1,5 and 9); 1 h (lanes 2,6 and 10); 2 h (lanes 3,7 and 11); or 3 h (lanes 4,8 and 12). Lane M, molecular weight standards (in kDa). Arrow, GST/HNP-1; open circle, induced 30 kDa β-lactamase; open square, native GST.

included to ensure that the genetic changes made to the vector had no negative effects on gene expression. The results showed that pGEX-3X and pGEX-KP both produced a protein with an apparent molecular weight of 26 kDa (Figure 11, lanes 6-8 and 10-12). The vector pGEX-HNP-1 produced a heterologous protein of 29 kDa which migrated just below the induced β-lactamase protein (lanes 2-4). Maximal expression seemed to occur between 2 and 3 h. Other studies showed that if expression were allowed to continue, the fusion protein began to be degraded after 6 h and was no longer detectable after overnight growth (data not shown).

Once stable expression had been demonstrated, the next step was to purify the fusion protein. The affinity of GST for glutathione attached to an agarose matrix was utilized as a purification technique. The scheme for such a purification (Figure 12) contained all the basic elements of affinity tag purification: the specific binding of the desired fusion protein to the matrix (usually from a heterogeneous population of proteins such as a cell extract), the release of this protein from the matrix by competitive displacement, the specific release of the peptide from the carrier protein, and the final purification of the peptide using a second passage over the affinity column to remove the contaminating affinity tag. In this case, a soluble cell extract from a culture induced for GST/HNP-1 production was passed over a glutathione agarose column, and the fusion protein subsequently eluted using reduced glutathione (Materials and Methods, section G.1.).

The results of this first stage in HNP-1 purification are found in Figure 13. Various samples were retained throughout the purification procedure to monitor the state and location of the fusion protein. Lane 2 showed that the fusion protein was indeed being produced. The fact that lanes 3 and 4, the soluble cellular fractions before and after being incubated with glutathione

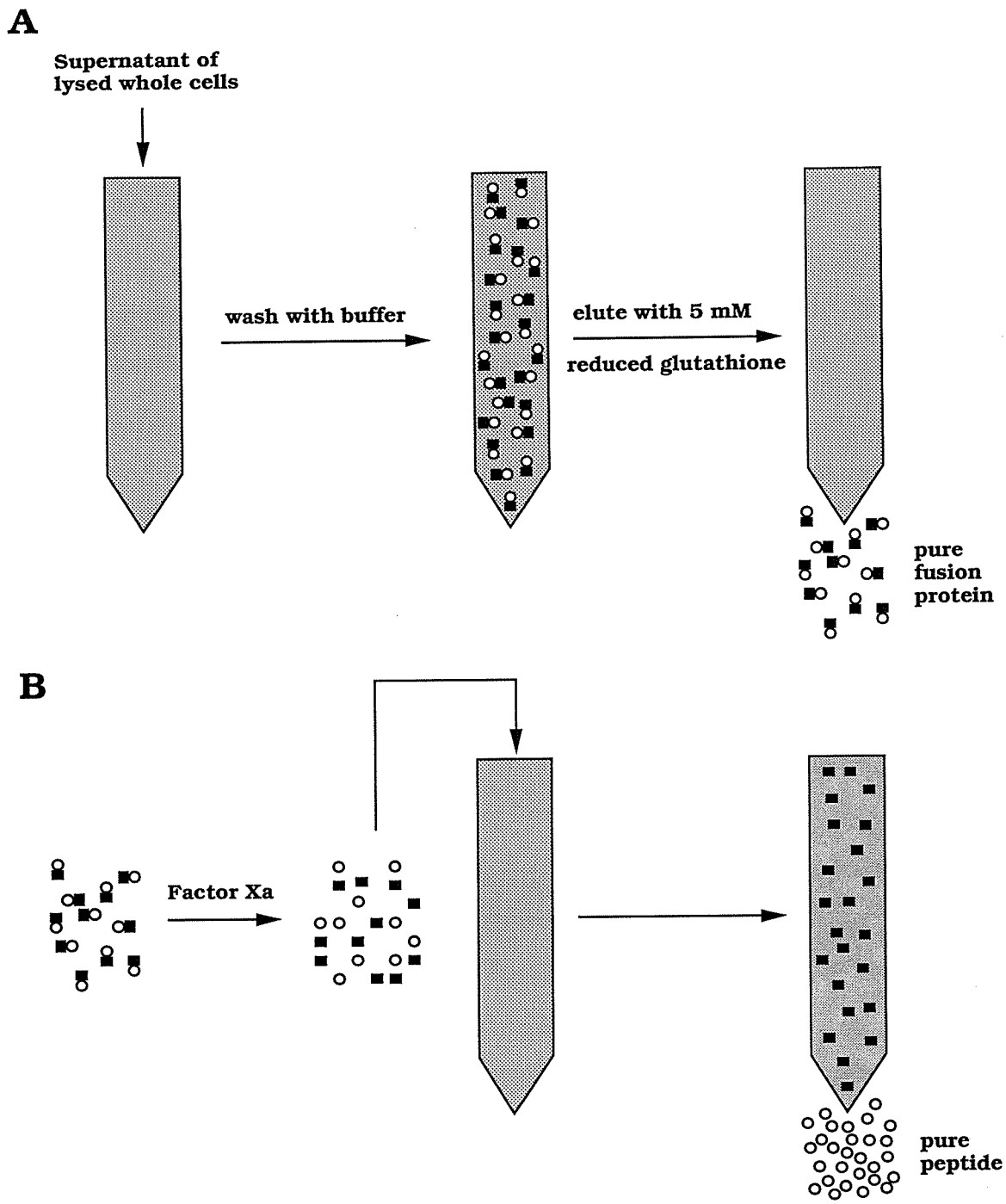

Figure 12: Schematic diagram of GST fusion protein affinity purification.

A, Purification of the fusion protein from the soluble fraction of whole cell lysates. **B**, Release of the target protein by factor X_a cleavage and its purification by a second passage over the affinity matrix. Closed squares, GST; open circles, target protein.

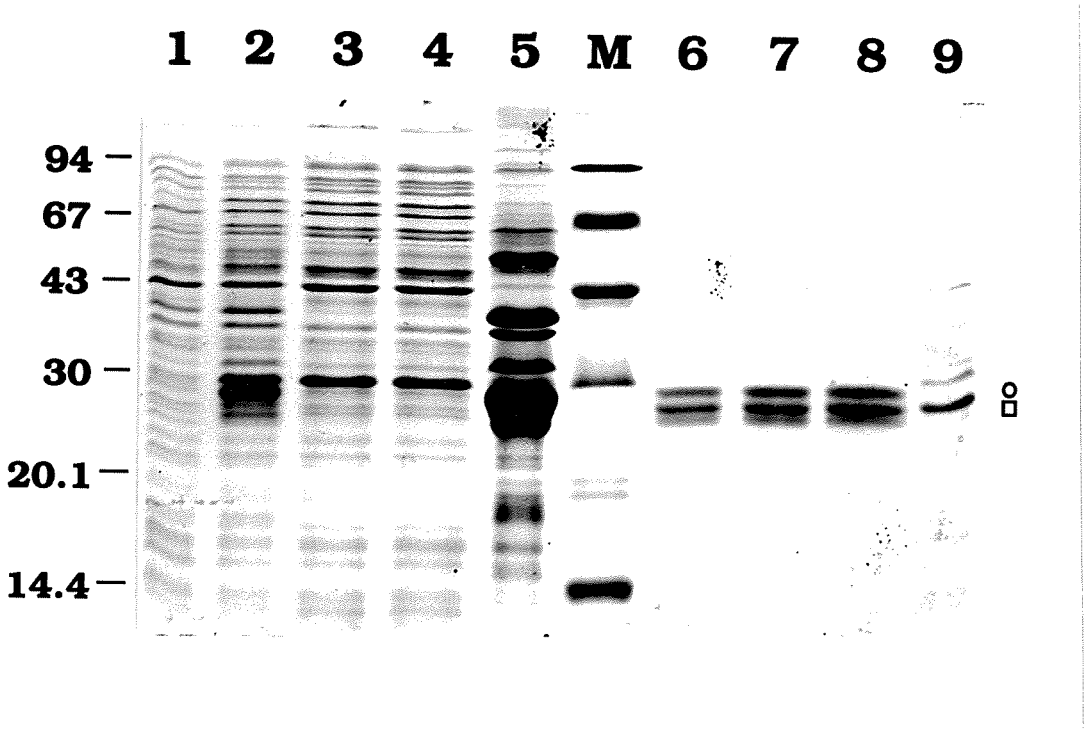

Figure 13: Affinity purification of GST/HNP-1.

A culture of DH5α(pGEX-HNP-1) was induced for fusion protein production (Materials and Methods, section F.2.) and the fusion protein purified by affinity chromatography (Materials and Methods, section G.1.). Samples were retained after various steps in the procedure and subjected to 12% SDS-PAGE prior to being stained for protein with Coomassie blue. Lanes: 1, uninduced whole cell lysate; 2, induced whole cell lysate; 3, soluble fraction before incubation with glutathione agarose beads; 4, soluble fraction after incubation with glutathione agarose beads; 5, insoluble pellet fraction; 6, glutathione agarose bead-bound fraction before elution with 5 mM reduced glutathione; 7, glutathione agarose bead-bound fraction after elution with 5 mM reduced glutathione; 8, eluted fraction; 9, whole cell lysate of induced DH5α(pGEX-KP); M, molecular weight standards (in kDa). Open circle, GST/HNP-1; open square, GST. Approximate protein content in each lane: 1-4, 15 μg; 5, 50 μg; 6-9, 10 μg.

agarose beads, showed virtually no difference suggested that there was very little soluble GST/HNP-1 in the cell. This was confirmed by the presence of a large protein band with the apparent molecular weight of GST/HNP-1 in the insoluble pellet fraction (lane 5), which suggested that most of the fusion protein was produced as inclusion bodies (see below). Nonetheless, some GST/HNP-1 was produced as soluble protein that bound to glutathione agarose beads (lane 6) and was released by competitive displacement with free glutathione (lane 8). When samples of the protein that bound to and were eluted from the affinity column were analyzed by SDS-PAGE, two distinct protein bands were detected (Figure 13, lanes 6-8). The migration distance of these two bands corresponded to those of GST/HNP-1 (compare with lane 2) and GST alone (compare to lane 9), which suggested that the fusion protein was susceptible to proteolytic degradation, particularly at the fusion junction between the carrier molecule and the peptide. Different conditions throughout the purification scheme were employed in an attempt to reduce the breakdown of this fusion protein. Neither lower temperatures nor the use of protease inhibitors such as PMSF, TLCK, TPCK or EDTA were able to enhance the stability of the protein (data not shown). The pGEX-HNP-1 vector was transformed into a number of different strains that were either mutants that prevented the expression of certain proteases or the parents of these mutant strains to investigate whether such hosts could produce stable GST/HNP-1 fusion protein. Table VII summarizes the data from these strains with respect to protein production and stability. Although several strains showed better expression of the fusion protein than DH5α, none of them, including the protease-deficient strains, were able to prevent the subsequent degradation of the fusion protein.

Table VII: Summary of Strains Used to Prevent Proteolytic Degradation of GST/HNP1.

Strain	Relevant Phenotype	Expression[a]	Degradation[b]	
			In Whole Cell Lysates	Purified Protein
DH5α	Original host used	+	—	++
BL21	Host for T7 RNA polymerase expression	+++	+	+++
HMS174	Host for T7 RNA polymerase expression	+++	+	+++
UT5600	*ompT*$^-$	—	ND	ND
C474	Parent of C475 and C476	++	—	++
C475	*lon*$^-$	++	+	++
C476	*htpR*$^-$	—	ND	ND

[a] +++, very strong; ++, strong; +, weak; —, not detected

[b] +++, >70% degraded; ++, 30-70% degraded; +, <30% degraded; —, no degradation detected; ND, not determined

As indicated above, the induction of GST/HNP-1 expression led to the formation of insoluble inclusion bodies (Figure 13, lane 5). This phenomenon has been documented for many recombinant proteins (Kane and Hartley, 1988) and, in fact, can be useful in a purification scheme (Marston, 1986). Since inclusion bodies are quite resistant to mild conditions of solubilization, many proteins in a heterogeneous pellet can be removed by such treatment before the inclusion bodies are solubilized using harsher conditions. Many different selective solubilization conditions were tested on an inclusion body preparation of GST/HNP-1 before a final purification scheme was established (Materials and Methods, section G.1.). This procedure used a lower induction temperature (30°C), which allowed the eventual solubilization of the inclusion bodies with milder conditions (Stein and Noteborn, 1988), and differential urea solubilizations to obtain a pure preparation of GST/HNP-1. Figure 14 shows that many of the contaminating proteins were removed through the procedure (lanes 2-4) and indicates the purity of the solubilized inclusion bodies (lane 5). The loss of some of the fusion protein which was either solubilized by 3 M urea (lane 4) or left in the pellet after the 8 M urea treatment (lane 6) was expected due to the heterogeneous nature of the inclusion bodies. The protein that was solubilized by the 8 M urea migrated slightly faster in SDS-PAGE (lane 5) possibly due to carbamylation which neutralizes positive charges (Stark, 1965; Marston and Hartley, 1990). This carbamylation occurred despite the use of deionized urea and the inclusion of 5 mM Tris in the solubilization buffer to minimize the amount of cyanate ions present.

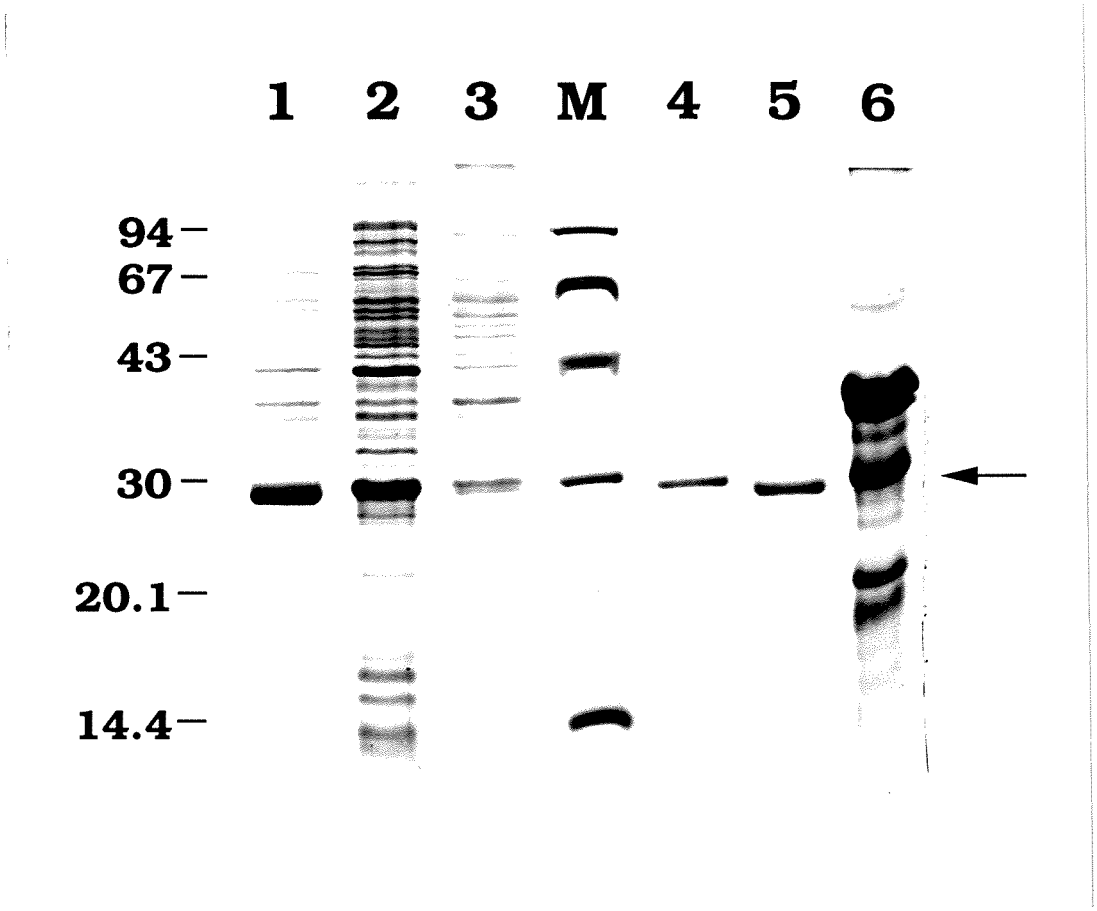

Figure 14: Purification of GST/HNP-1 inclusion bodies.

An induced culture of DH5α(pGEX-HNP-1) was lysed and the insoluble pellet extracted with a series of different solutions (Materials and Methods, section G.1.). Samples were electrophoresed on a 12% SDS polyacrylamide gel and stained for protein with Coomassie blue. Lanes: 1, induced whole cell lysate; 2, soluble fraction from lysed cells; 3, soluble extract after 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% Triton X-100 wash; 4, soluble extract after 3 M urea wash; 5, soluble extract after 8 M urea solubilization; 6, insoluble pellet after all washes and solubilizations; M, molecular weight standards (in kDa). Arrow indicates GST/HNP-1. Approximate protein content in each lane: 1-3, 15 μg; 4-5, 3 μg; 6, 50 μg.

D. Factor X_a Cleavage of GST/HNP-1.

Inclusion bodies solubilized by differential urea extraction were dialyzed against factor X_a cleavage buffer using a two step procedure (Materials and Methods, section G.1.) to avoid the precipitation of the protein that can occur if the urea was removed too quickly. The protein was subjected to factor X_a cleavage using a number of different incubation temperatures and times (Figure 15). Although incubation at 4°C can be used to prevent non-specific proteolysis of the fusion protein, this condition did not result in the cleavage of any GST/HNP-1 (data not shown). Incubation at 23°C resulted in some fusion protein cleavage as indicated by the reduction of the GST/HNP-1 band in lanes 3-5; however it led to very little, if any, protein corresponding to the size of HNP-1, even after long incubation times (lane 5). Increasing the temperature to 37°C improved the cleavage of the fusion protein and resulted in the appearance of a protein band of approximately 3500 daltons, the predicted molecular weight of HNP-1. This protein band was electroblotted onto an Immobilon transfer membrane and analyzed for its N-terminal amino acid sequence. The analysis showed that the HNP-1 peptide was present in this band. Therefore, the HNP-1 peptide was released from the GST/HNP-1 fusion protein by factor X_a but only under conditions (37°C for 60 h with an enzyme to substrate ratio of 1:25; Figure 16) that are much harsher than normally required (Nagai and Thøgersen, 1984; Smith and Johnson, 1988). The reason for this may be because the factor X_a recognition site was inaccessible to the large (55 kDa) factor X_a molecule. This inaccessibility may arise because of the highly constrained and compact structure of the defensin molecule, which might appear as a bulky addition to the GST protein. In addition, the denaturation/renaturation step of inclusion body purification may have led to

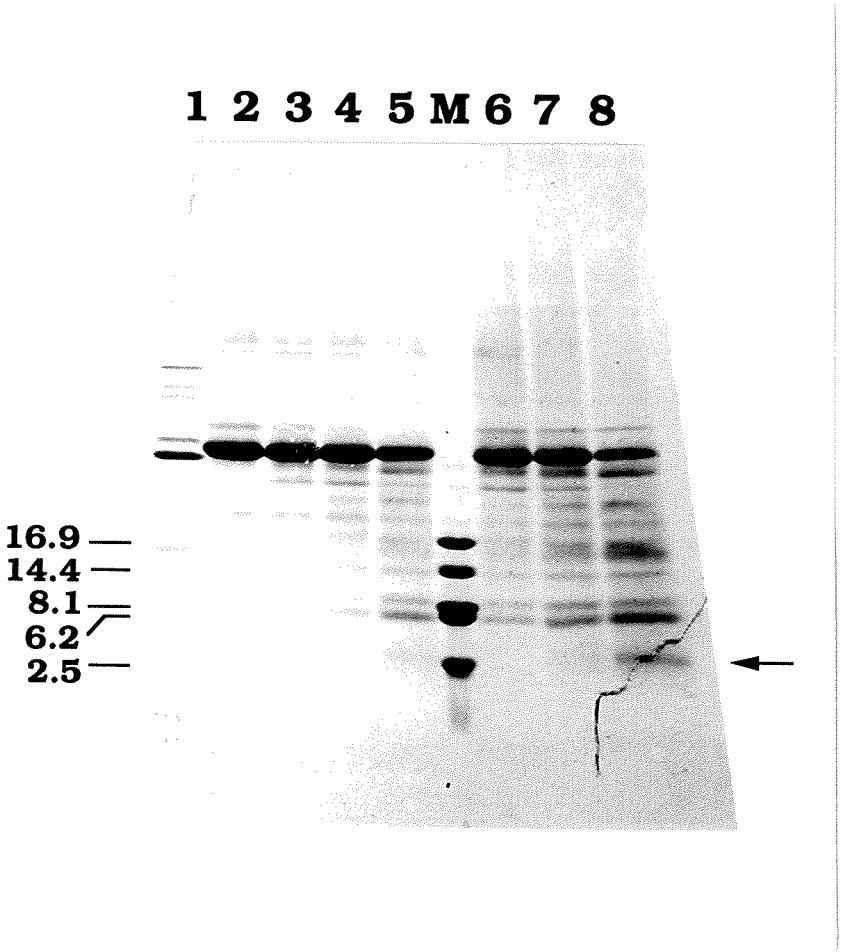

Figure 15: Factor X_a cleavage of GST/HNP-1.

Solubilized GST/HNP-1 inclusion bodies were dialyzed against factor X_a cleavage buffer (Materials and Methods, section H.1.) and subjected to factor X_a cleavage at 23°C (lanes 3-5) or 37°C (lanes 6-8). Samples were removed at 2 h (lanes 3 and 6), 6 h (lanes 4 and 7), and 24 h (lanes 5 and 8) and electrophoresed on a 12-25% gradient SDS polyacrylamide gel which was then stained for protein with Coomassie blue. Other lanes: 1, induced whole cell lysate of DH5α(pGEX-KP); 2, uncleaved, solubilized GST/HNP-1 inclusion bodies; M, molecular weight standards (in kDa). The arrow indicates the protein band in which HNP-1 was found. Equal volumes of the reaction mixture were loaded for lanes 2-8.

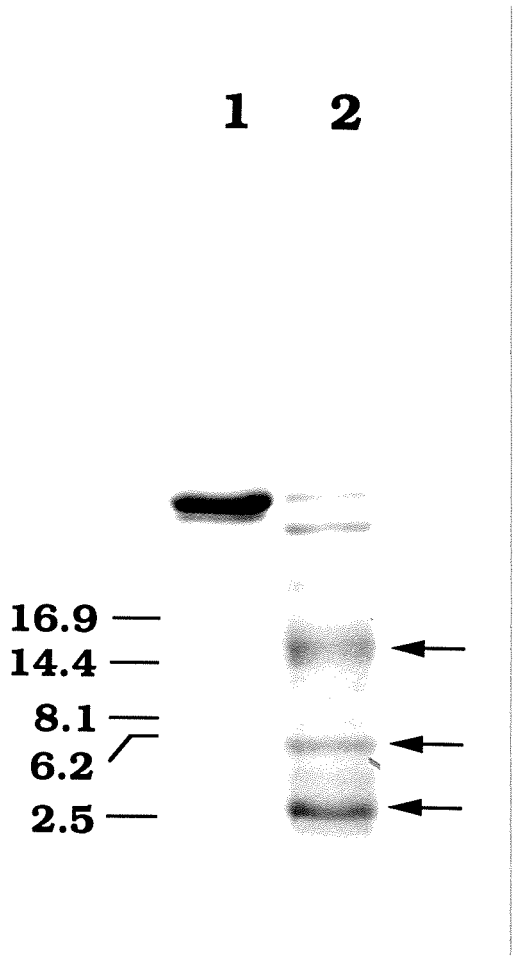

Figure 16: Optimal factor X_a cleavage of GST/HNP-1.

Samples of solubilized GST/HNP-1 inclusion bodies were incubated at 37°C for 60 h with (lane 2) or without (lane 1) factor X_a at an enzyme to substrate ratio of 1:25. Samples were then subjected to 12-25% gradient SDS-PAGE and stained for protein with Coomassie blue. The arrows indicate the major GST cleavage products (see text). 5 μg of protein was loaded in lane 1 and 10 μg of protein was loaded in lane 2.

incorrect disulfide bond formation and inaccurate protein folding and thus contributed to the inaccessibility of the target cleavage site.

The sequencing analysis of the 3500 dalton molecular weight band revealed the presence of another N-terminal sequence that started with Asn-Lys-Lys-Phe-Glu-Leu-Gly. Upon examination of the GST amino acid sequence (Smith et al., 1986), it was discovered that this contaminating peptide corresponded to an internal peptide fragment (Figure 17), which suggested it arose from the non-specific factor X_a cleavage of GST. This suggestion was supported by the fact that this peptide sequence in GST is preceded directly by an arginine residue, which is the amino acid found at the end of the factor X_a recognition site. Further N-terminal amino acid sequence investigation of the other two major bands produced in the factor X_a digest of GST/HNP-1 (Figure 16, lane 2, indicated by arrows), revealed that they also were proteolytic fragments of GST which corresponded to its first 42 ($\approx$ 5.0 kDa) and last 145 ($\approx$ 16 kDa) amino acids (Figure 17). The latter peptide, as with the 31 amino acid proteolytic fragment, was produced by cleavage of the protein after an arginine residue, which suggested that there were two sites in the GST/HNP-1 fusion protein, other than the authentic recognition site, that were susceptible to cleavage by factor X_a. Since GST is not usually cleaved internally by factor X_a (Smith and Johnson, 1988), the possibility existed that these sites became susceptible due to improper refolding of the GST moiety during the renaturation of the GST/HNP-1 fusion protein after inclusion body solubilization with 8 M urea. To test this hypothesis, purified GST was denatured using 8 M urea, and renatured using the same conditions as for GST/HNP-1 (sequential dialysis against 1 M urea in 5 mM Tris-HCl pH 8.0, and factor X_a cleavage buffer). This denatured/renatured GST was subjected to factor X_a cleavage as described for GST/HNP-1 and analyzed by gradient SDS-PAGE (Figure 18). The results

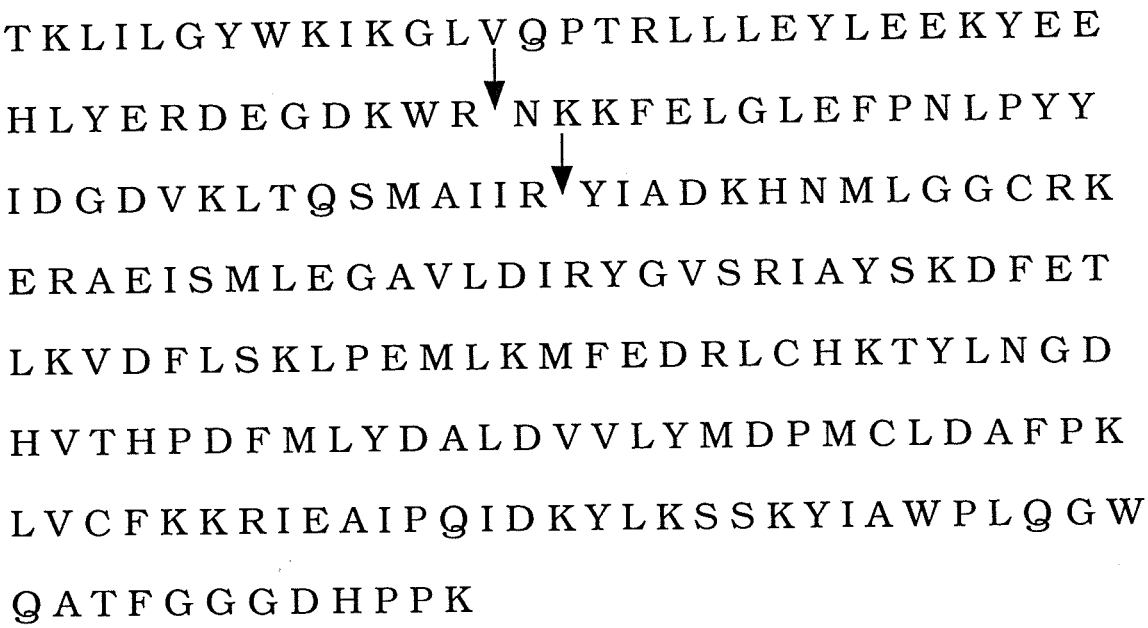

Figure 17: Amino acid sequence of GST.

The arrows indicate non-specific factor X_a cleavage sites in denatured/renatured GST/HNP-1 and GST.

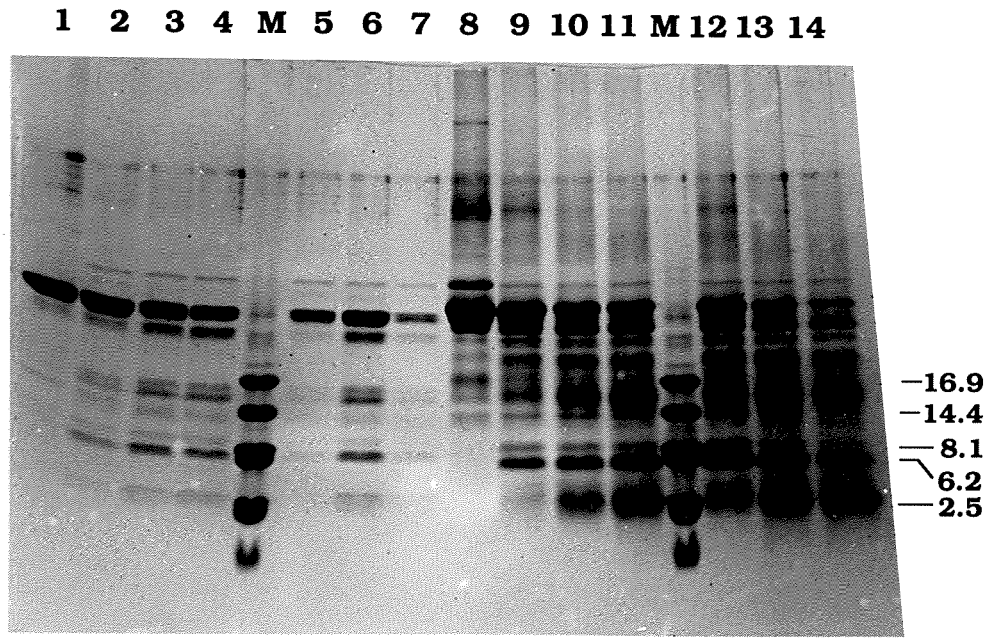

Figure 18: Cleavage of denatured/renatured GST by factor X_a.

GST that was denatured by 8 M urea then renatured (lanes 1-7), and solubilized GST/HNP-1 inclusion bodies (lanes 8-14) were cleaved with factor X_a at 37°C using different enzyme to substrate ratios and incubation times. Samples were and electrophoresed on a 12-25% gradient SDS polyacrylamide gel which was then stained for protein with Coomassie blue. Conditions: enzyme to substrate ratio, 1:25 (lanes 2-4 and 9-11) and 1:12 (lanes 5-7 and 12-14); time, 8 h (lanes 2,5,9 and 12), 24 h (lanes 3,6,10 and 13), and 60 h (lanes 4,7,11 and 14). Other lanes: 1, uncleaved GST; 8, uncleaved GST/HNP-1; M, molecular weight standards (in kDa). Equal volumes of the GST cleavage reaction were loaded in lanes 1-7. Equal volumes of the GST/HNP-1 cleavage reaction were loaded in lanes 8-14.

showed that GST treated in this manner was indeed susceptible to non-specific factor X_a cleavage, yielding the three proteolytic fragments found in factor X_a digests of GST/HNP-1 (Figure 18, lanes 6 and 7, 13 and 14). The fact that the cleavage of GST was not as extensive as GST/HNP-1 may indicate that the presence of the HNP-1 moiety contributed to the improper refolding of the GST molecule in GST/HNP-1.

E. The Production of GST/proHNP-1.

Although HNP-1 was successfully produced and released in the GST fusion protein system, further purification would have been difficult considering the small amount of peptide released and the contaminating GST fragment of identical molecular weight. The formation of GST/HNP-1 inclusion bodies also prevented the use of affinity chromatography to purify the fusion protein, although recent studies have been able to accomplish the affinity purification of other GST fusion proteins that form inclusion bodies (Hartman et al., 1992). It is not entirely understood why some recombinant proteins form inclusion bodies while others do not (Kane and Hartley, 1988), but one hypothesis is that inclusion bodies are formed due to improper folding of protein intermediates (Mitraki and King, 1989). Therefore, in the case of fusion proteins, any properties of the target sequence that contribute to the misfolding of the carrier protein can be the cause of inclusion body formation. Some of the properties which could apply to HNP-1 include a high charge density or any number of cysteine residues that could result in improper intra- and interchain disulfide bond formation. Although little could be done about incorrect disulfide bond formation, an attempt was made to minimize the effect of the high positive charge density of the HNP-1 molecule on proper fusion protein folding. *In vivo*,

HNP-1 is produced in the neutrophils as a prepro molecule (Harwig et al., 1992). The pro region of this molecule is highly negatively charged (Figure 3) and is believed to neutralize and stabilize the positively charged defensin molecule until it is properly compartmentalized into primary granules (Michailson et al., 1992) during which the pro peptide is removed and the defensin forms its native configuration. It was hypothesized that the inclusion of a prepro defensin cartridge between the GST and HNP-1 genes might result in the stabilization of the fusion protein as well as enhance its solubility by neutralizing the positive charges on the HNP-1 moiety. Therefore, four oligonucleotides (M-P, Table VI) encoding the prepro defensin sequence (Figure 3) on an *Sph*I fragment (Figure 10D) were synthesized, annealed and inserted into the *Sph*I site of pGEX-HNP-1 to form pGEX-proHNP-1. Proper insertion orientation was confirmed by restriction endonuclease digestion analysis. Production of GST/proHNP-1 was performed (Materials and Methods, sections F.2. and G.1.) to determine if the presence of the prepro sequence prevented proteolysis of the fusion protein. The results indicated that GST/proHNP-1 was produced in *E. coli* (Figure 19, lane 2). Although some of the protein was soluble as indicated by the difference between the soluble fraction before (lane 3) and after being incubated with glutathione agarose beads (lane 4), much of the protein was still found in the insoluble pellet as inclusion bodies (lane 5). When the soluble fusion protein was purified on glutathione agarose beads, it was again found to be proteolytically degraded (lanes 6-8) although apparently not to the same degree as GST/HNP-1 (Figure 13, lanes 6-8). It was determined from this experiment that the presence of the prepro defensin cartridge in pGEX-proHNP-1 had a slight effect on the stability but not on the solubility of the fusion protein.

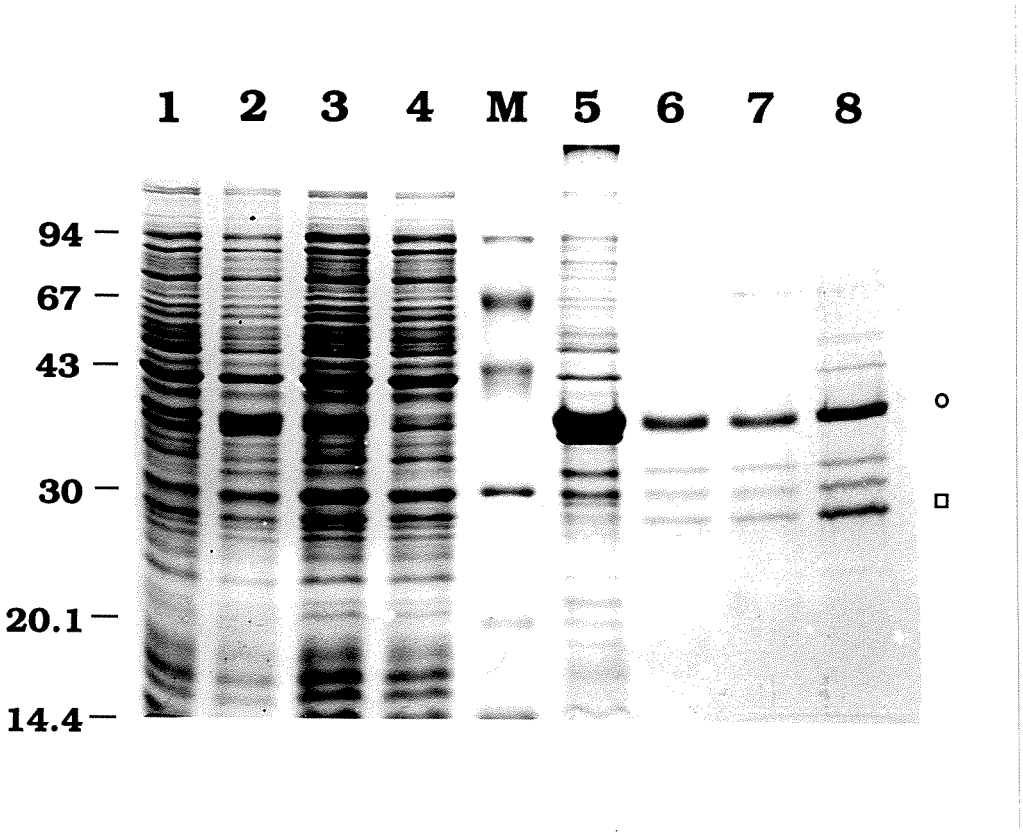

Figure 19: Production and affinity purification of GST/proHNP-1.

A culture of DH5α(pGEX-proHNP-1) was induced for fusion protein production (Materials and Methods, section F.2.) and the fusion protein purified by affinity chromatography (Materials and Methods, section G.1.). Samples were retained after various steps in the procedure and subjected to 12% SDS-PAGE prior to being stained for protein with Coomassie blue. Lanes: 1, uninduced whole cell lysate; 2, induced whole cell lysate; 3, soluble fraction before incubation with glutathione agarose beads; 4, soluble fraction after incubation with glutathione agarose beads; 5, insoluble pellet fraction; 6, glutathione agarose bead-bound fraction before elution with 5 mM reduced glutathione; 7, glutathione agarose bead-bound fraction after elution with 5 mM reduced glutathione; 8, eluted fraction; M, molecular weight standards (in kDa). Open circle, GST/proHNP-1; open square, GST. Approximate protein content in each lane: 1-5, 20 μg; 6-8, 5 μg.

F. The Production of GST/CEME.

The cecropin A/melittin hybrid peptide CEME was chosen for this study because it represented the α-helical (non-defensin-like) family of cationic peptides and it possessed potent antibacterial activity against *P. aeruginosa* (Wade et al., 1990). The hypothesis that this peptide would be less likely to form inclusion bodies in the GST fusion protein system than HNP-1 came from the fact that it contained no cysteine residues and consequently no disulfide bonds. The gene encoding CEME was created by a two step process (Materials and Methods, section C.2.) because of a two base pair deletion in the original gene. It should be noted that although a factor X_a cleavage site was present in this construct, the mature CEME peptide was preceded directly by a methionine residue (Figure 10C). Thus CNBr cleavage would ensure that no extra amino acids were present at its N-terminal end. The plasmid pGEX-CEME, was expressed in *E. coli* as described for both pGEX-HNP-1 and pGEX-proHNP-1 (Materials and Methods, section F.2.). The samples prepared from the production and purification of GST/CEME were run in duplicate on SDS-PAGE (Figure 20A) and one set of samples was electroblotted onto nitrocellulose for Western immunoblotting (Figure 20B). The primary antibody used to detect the fusion protein was a rabbit polyclonal serum raised against GST/CEMA (Materials and Methods, section D.1.) As predicted, virtually all of the protein produced by this vector was found in the soluble cellular fraction (Figure 20B, lane 3) while little, if any, was detected, even with the αGST/CEMA antibody, in the insoluble pellet fraction (Figure 20B, lane 5). As with GST/HNP-1, however, GST/CEME fusion protein was highly susceptible to proteolytic degradation. SDS-PAGE of a sample of GST/CEME that was purified using glutathione agarose beads revealed that almost all of the protein was degraded to the

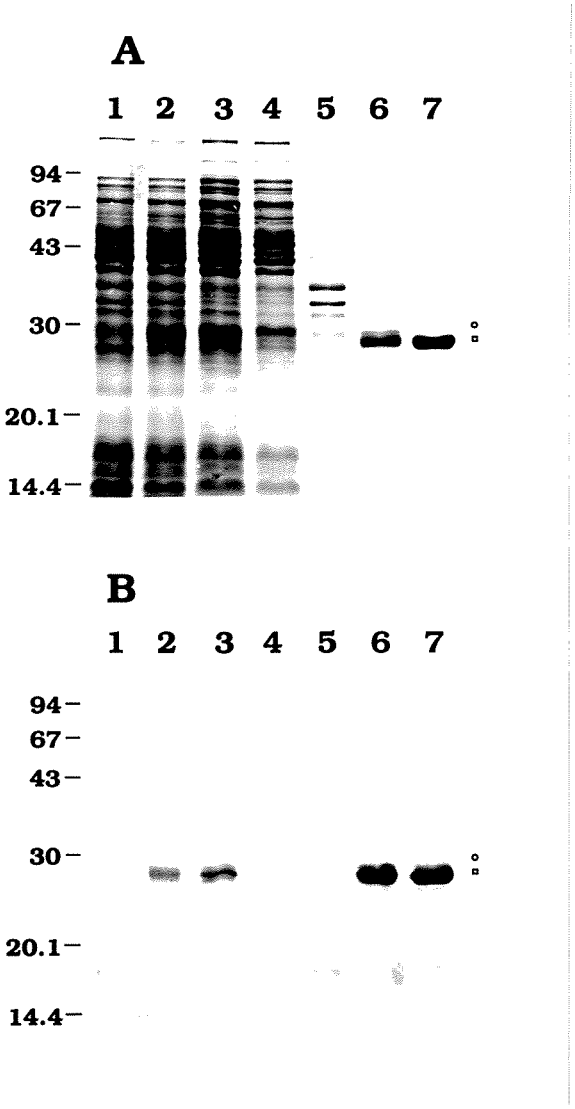

Figure 20: Production and affinity purification of GST/CEME.

A culture of DH5α(pGEX-CEME) was induced for fusion protein production (Materials and Methods, section F.2.) and the fusion protein purified by affinity chromatography (Materials and Methods, section G.1.). Samples retained throughout the procedure were run in duplicate on an 11% SDS polyacrylamide gel and either **A**, stained for protein with Coomassie blue, or **B**, electroblotted onto nitrocellulose for Western immunoblotting. Lanes: 1, uninduced whole cell lysate; 2, induced whole cell lysate; 3, soluble fraction before incubation with glutathione agarose beads; 4, soluble fraction after incubation with glutathione agarose beads; 5, insoluble pellet fraction; 6, glutathione agarose bead-bound fraction before elution with 5 mM reduced glutathione; 7, purified GST. The primary antibody used in **B** was a rabbit polyclonal αGST/CEMA serum. Open circle, GST/CEME; open square, GST. Molecular weight standards are indicated in kDa. Approximate protein content in each lane: 1-4, 25 μg; 5-7, 5 μg.

molecular weight of GST (Figure 20A and B, lanes 6 and 7).This is in contrast to the sample of GST/HNP-1 which showed that almost half of the purified fusion protein remained intact throughout the purification procedure (Figure 13, lanes 6-8).

G. The Production and Purification of GST/proCEME.

In an attempt to stabilize the GST/CEME fusion protein, an oligonucleotide cartridge encoding the anionic prepro defensin sequence (Figure 3) was cloned into the *Sph*I site of pGEX-CEME to form pGEX-proCEME. This plasmid was transformed into DH5α and the gene expressed as previously described (Materials and Methods, section F.2.). Samples subjected to SDS-PAGE were either stained for protein content with Coomassie blue or Western immunoblotted to detect the fusion protein (Figure 21). Although the GST/proCEME fusion protein was only weakly detected in a sample from an induced culture stained with Coomassie blue (Figure 21A, lane 2), its production was confirmed with the Western blot (Figure 21B, lane 2). It was interesting to note that the fusion protein in this sample was already partially degraded. The addition of the prepro sequence to this fusion protein resulted in a shift from soluble protein (very little GST/proCEME in lanes 3 and 4), as was the case for GST/CEME, to insoluble inclusion bodies (lane 5). The small amount of protein that was produced in soluble form (lane 8) was almost completely degraded to the size of GST (lane 9). Therefore, although the inclusion of the prepro region did not stabilize the soluble form of the fusion protein, it did result in the formation of insoluble inclusion bodies which protected the heterologous protein from extensive proteolysis.

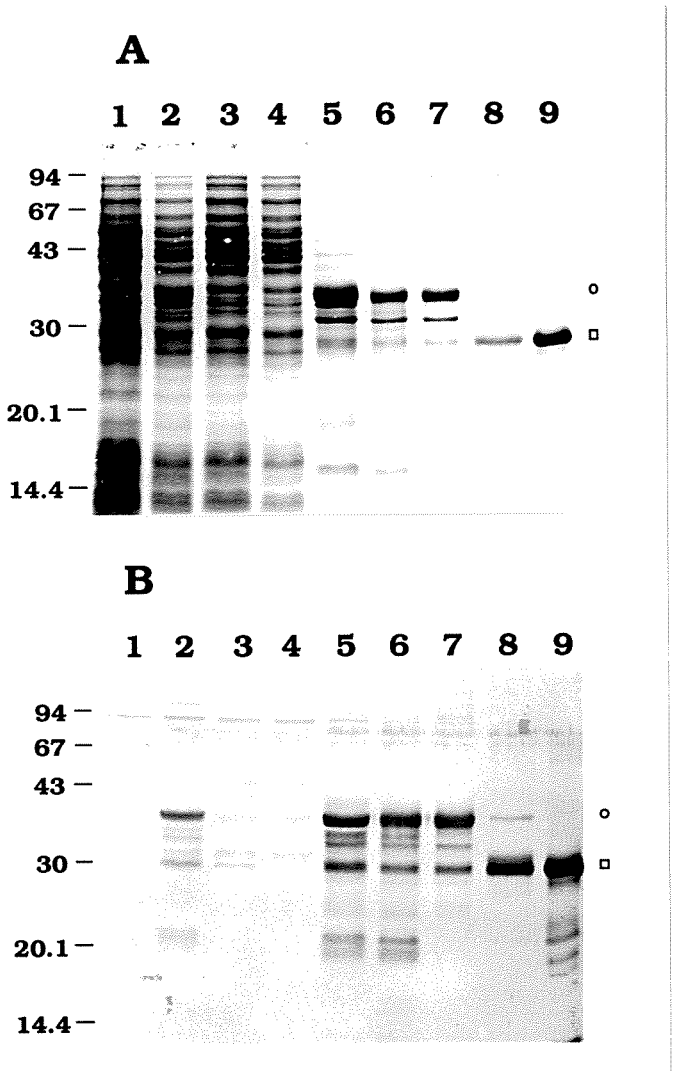

Figure 21: Production and purification of soluble and insoluble GST/proCEME.

A culture of DH5α(pGEX-proCEME) was induced for fusion protein production (Materials and Methods, section F.2.) and the fusion protein purified either by affinity chromatography or differential solubilization of the insoluble pellet fraction (Materials and Methods, section G.1.). Samples retained throughout the procedure were run in duplicate on an 11% SDS polyacrylamide gel and either **A**, stained for protein with Coomassie blue, or **B**, electroblotted onto nitrocellulose for Western immunoblotting. Lanes: 1, uninduced whole cell lysate; 2, induced whole cell lysate; 3, soluble fraction before incubation with glutathione agarose beads; 4, soluble fraction after incubation with glutathione agarose beads; 5, insoluble pellet fraction; 6, heated and 7, unheated samples of insoluble pellet fraction after 3% octyl -POE extraction; 8, glutathione agarose bead-bound fraction; 9, purified GST. The primary antibody used in **B** was a rabbit polyclonal αGST/CEMA serum. Open circle, GST/proCEME; open square, GST. Molecular weight standards are indicated in kDa. Approximate protein content in each lane: 1-4, 25 μg; 5-7, 15 μg; 8-9, 5 μg.

Since the CEME peptide had to be released from GST/proCEME using CNBr, the inclusion bodies eventually needed to be solubilized in 70% formic acid. Such conditions would solubilize most protein contained in the insoluble pellet fraction. Therefore, a novel method of purifying the GST/proCEME inclusion bodies was developed. The strategy in this protocol was to solubilize all the contaminating proteins, thus leaving only the inclusion bodies to be solubilized by 70% formic acid. Since outer membrane proteins are known to be the major contaminants in inclusion body preparations (Veeraragavan, 1989) the protocol used 3% O-POE, a detergent that has been shown to solubilize such proteins (Siehnel et al., 1992). The pellet was sequentially washed as described in Materials and Methods, section G.1. The selective extraction of outer membrane proteins was easily monitored by SDS-PAGE analysis of heated (100°C for 10 min) and non-heated samples, since outer membrane proteins are known to be heat-modifiable (Hancock and Carey, 1979). Both the heated and non-heated samples of post-3% O-POE extraction pellets were analyzed by SDS-PAGE and Western immunoblotting (Figure 21A and B, lanes 6 and 7). The results showed little contamination and little difference between the two samples, which indicated there were no detectable membrane protein contaminants in the pellet. Western blots of the 3% O-POE supernatant samples showed that no GST/proCEME inclusion bodies were solubilized under these conditions (data not shown).

H. The Release of CEME From GST/proCEME Using CNBr.

The GST/proCEME inclusion bodies were solubilized in 70% formic acid and subjected to CNBr cleavage (Materials and Methods, section H.2.). Analysis of the cleavage products by AU-PAGE revealed a complex array of peptides

(Figure 22A, lane 1). This was expected given the fact that the inclusion body preparation was not entirely homogeneous and that the GST protein contains 8 methionine residues (Figure 17). Nonetheless, a peptide band which corresponded to the migration of chemically synthesized CEME was detected (Figure 22A, lane 1, arrow) Identical samples were tested for antibacterial activity using a gel overlay assay (Materials and Methods, section J.; Figure 22B). The results showed a zone of bacterial lysis in the CNBr-cleaved GST/proCEME sample that corresponded to the zone produced by synthetic CEME. This confirmed that biologically active CEME could be produced in this system. However further purification of this peptide from the complicated mixture of CNBr cleavage fragments was presumed to be too difficult and potentially inefficient given the fact that the CEME band was a minor species.

I. Summary.

Both HNP-1 and CEME were produced as GST fusion proteins. When made as soluble proteins, they were highly susceptible to proteolytic degradation. GST/HNP-1 formed insoluble inclusion bodies which were purified using differential urea solubilization. Cleavage of this fusion protein with factor X_a released a peptide fragment that was confirmed by N-terminal amino acid sequencing to be HNP-1. The inclusion of the defensin prepro region in the fusion protein (GST/proHNP-1) rendered it only slightly more stable than GST/HNP-1. Compared to GST/HNP-1, GST/CEME was more soluble but also more susceptible to proteolysis. The presence of the prepro region in GST/proCEME dramatically increased the stability of this fusion protein, presumably in part because it resulted in the formation of inclusion bodies. These inclusion bodies were purified by removing contaminating

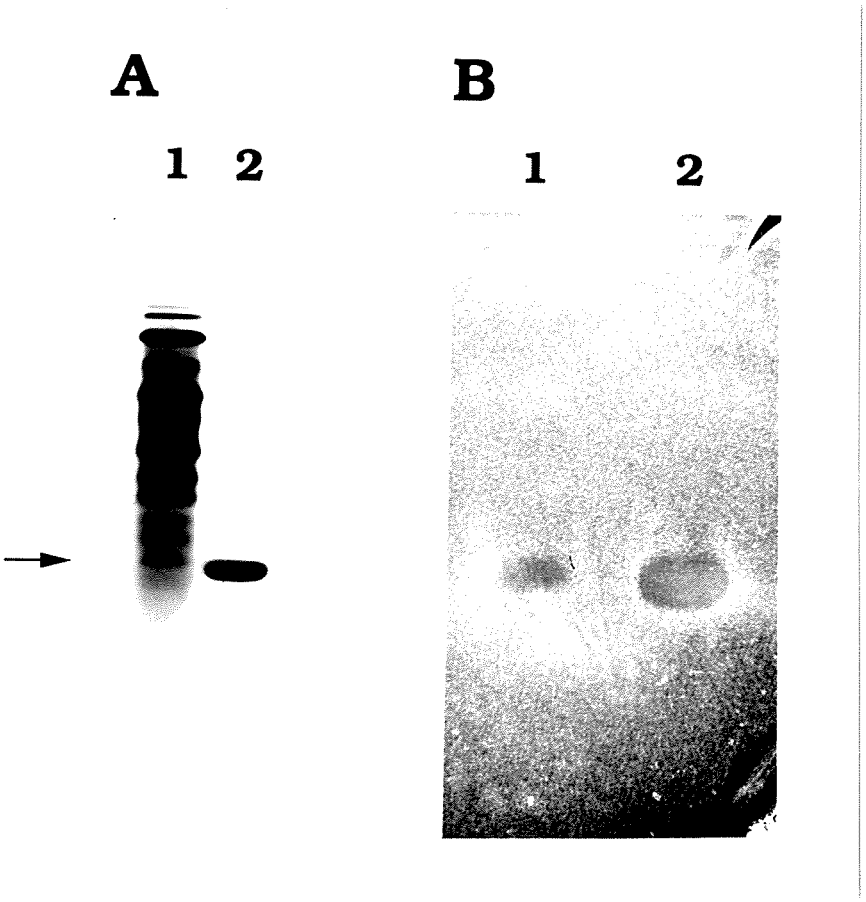

Figure 22: CNBr release of active CEME from GST/proCEME.

GST/proCEME inclusion bodies were solubilized and cleaved with CNBr (Materials and Methods, section H.2.). Samples of the CNBr digest (lane 1) and purified CEME obtained by chemical synthesis methods (lane 2) were electrophoresed in duplicate on a 15% acid urea polyacrylamide gel and either **A**, stained for protein with Coomassie blue, or **B**, tested for antibacterial activity using a gel overlay assay (Materials and Methods, section J.). Arrow indicates the CEME peptide. Approximate protein content in each lane: 1, 90 µg; 2, 10 µg.

proteins with 3% O-POE, and cleaved with CNBr to release active CEME peptide, as confirmed by a gel overlay assay.

CHAPTER TWO: The Production of Cationic Peptides as Protein A Fusion Proteins.

A. Introduction.

Protein A from *S. aureus* has a high binding affinity for the Fc portion of IgG molecules (Forsgren and Sjöquist, 1966). This property was the basis of a number of gene fusion vectors (Uhlén et al., 1983; Nilsson et al., 1985a) whose fusion proteins could be purified by IgG affinity chromatography. Several proteins have been produced in this system including β-galactosidase and alkaline phosphatase (Nilsson et al., 1985a) and human insulin growth factor (Moks et al., 1987a). This chapter describes the use of the protein A fusion protein system for producing cationic peptides. The vector pRIT5 (Figure 23A) was chosen for these studies because of its host range (it possesses both *E. coli* and *S. aureus* origins of replication) and the presence of the protein A signal sequence which directs export of the fusion protein to the periplasm when in *E. coli* or to the external medium when in *S. aureus*, and thus may serve to protect it from intracellular proteases.

B. Construction of the Vectors.

The restriction enzyme sites available on pRIT5 were not compatible with those of the gene cartridges that encoded HNP-1, CEME or CEMA. Rather than changing the multiple cloning site of the vector several times to create the necessary reading frames for the cationic peptide genes, the genes themselves were modified using a series of oligonucleotides (Materials and Methods, section C.3.). Figure 23A-C are schematic representations of the changes made to the genes (compare with Figure 8A-C). The genes encoding CEME and CEMA were

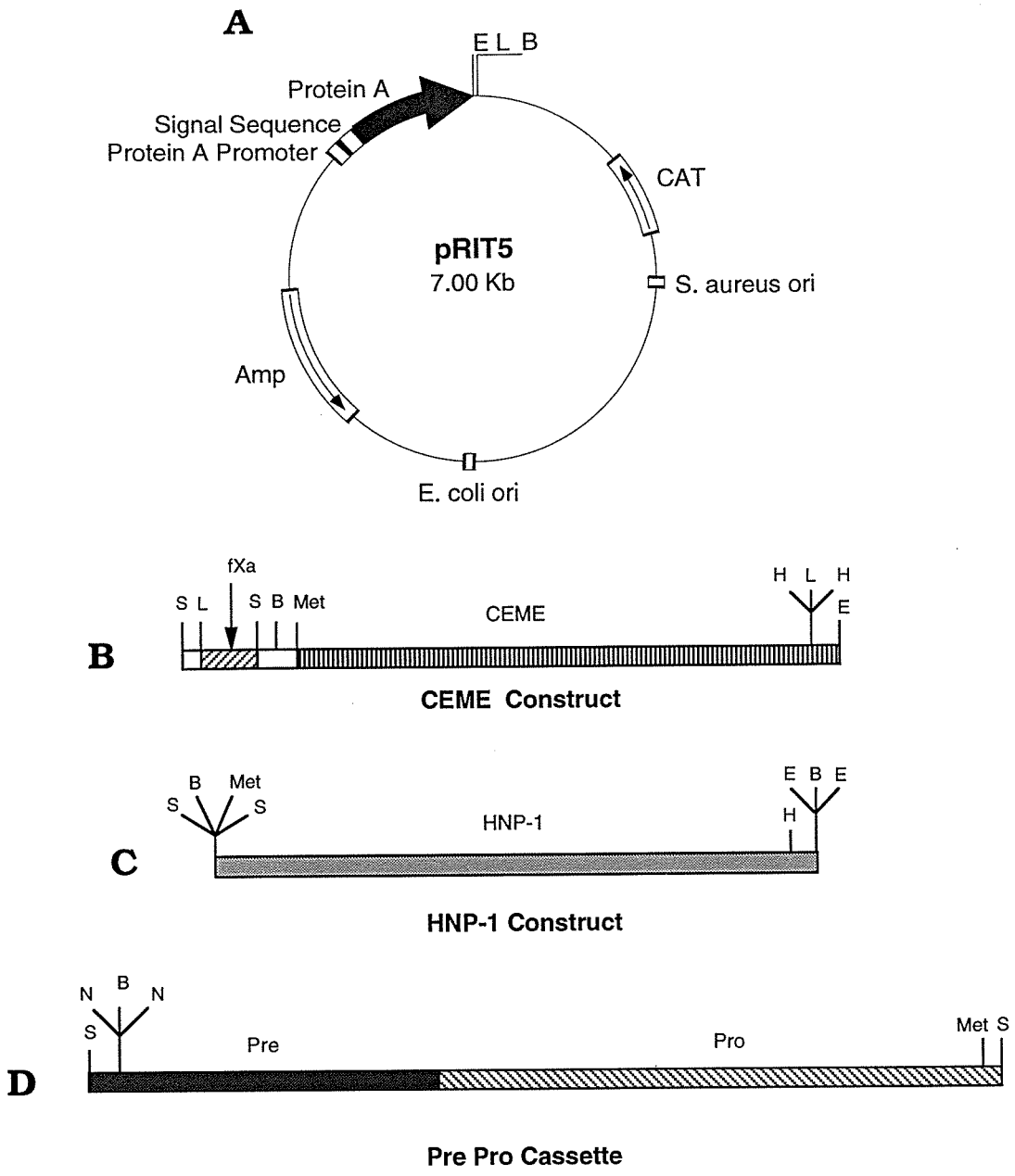

Figure 23: Construction of protein A/cationic peptide fusion proteins.

A, The pRIT5 vector; **B-D**, DNA fragments from Figure 10B-D encoding CEME, HNP-1 and the mammalian defensin prepro region. Their restriction endonuclease sites were altered using oligonucleotide adaptors. The genes were cloned into pRIT5 using a *Sal*I fragment for CEME and a *Bam*HI fragment for HNP-1 and preproHNP-1 (Materials and Methods, section C.3.). Protein A, truncated protein A gene of *S. aureus*; SS, protein A signal sequence; pPA, protein A promotor; Amp, β-lactamase gene; CAT, chloramphenicol acetyl-transferase; ori, origin of replication; S, *Sph*I; L, *Sal*I; B, *Bam*HI; H, *Hin*dIII; N, *Nde*I; E, *Eco*RI; f X_a, factor X_a recognition site; Met, methionine codon.

excised from pGEX-CEME-S and pGEX-CEMA-S using *Sal*I, and inserted into the *Sal*I site of pRIT5 to form pPA-CEME and pPA-CEMA respectively. Similarly, *Bam*HI fragments from pGEX-HNP-1-B and pGEX-proHNP-1-B encoding the HNP-1 and preproHNP-1 genes respectively were cloned into the *Bam*HI site of pRIT5 to form pPA-HNP-1 and pPA-proHNP-1.

C. Expression of pPA-CEME in *E. coli.*

Fusion proteins produced by pRIT5 in *E. coli* are transported to the periplasm. Both pRIT5 and pPA-CEME were transformed into DH5α and expressed in cells grown to mid-log phase. Samples of whole cells were analyzed by SDS-PAGE and Western immunoblot using IgG antibody to detect the protein A moiety (Figure 24). There was no detectable difference between the pRIT5 and pPA-CEME samples (Figure 24B, lanes 1 and 2), indicating extensive degradation of the PA/CEME fusion protein. In fact, it appears as if the protein A carrier molecule itself is subject to proteolysis in *E. coli* (compare lanes 1 and 3). This result is not unexpected since other researchers using the same vector system to produce human IGF-I found that using *E. coli* as a host organism led to the production of unstable fusion protein (Nilsson et al., 1985b; Moks et al., 1987a).

D. Expression and Purification of Protein A/Cationic Peptide Fusion Proteins in *S. aureus.*

Since no stable fusion protein was produced in the *E. coli* periplasm, all four plasmids, pRIT5, pPA-CEME. pPA-HNP-1, and pPA-proHNP-1, were electroporated into *S. aureus* (Materials and Methods, section B.4.) where the

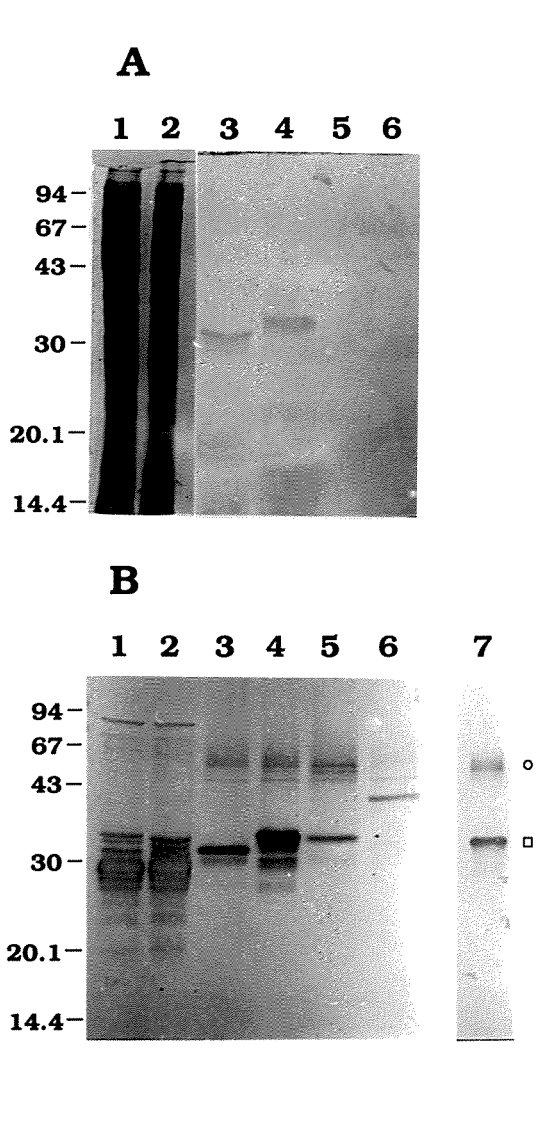

Figure 24: Production of protein A/cationic peptide fusion proteins.

Fusion proteins were produced in *E. coli* DH5α (lanes 1 and 2) or *S. aureus* RN4220 (lanes 3-6)(Materials and Methods, section F.3.). Whole cell lysates (lanes 1 and 2) and extracellular supernatants (lanes 3-6) were run in duplicate on an 11% SDS polyacrylamide gel and either **A**, stained for protein with Coomassie blue or **B**, transferred to nitrocellulose for Western immunoblotting. Lanes: 1, DH5α(pRIT5); 2, DH5α(pPA-CEME); 3, RN4220(pRIT5); 4, RN4220(pPA-CEME); 5, RN4220(pPA-HNP-1); 6, RN4220(pPA-proHNP-1). Lane **B**7 is a 5 μg sample of PA/CEME purified on an IgG Sepharose column (Materials and Methods, section G.2.). Open square, PA/CEME; open circle, native *S. aureus* protein A. Molecular weight standards are shown in kDa. Equal volumes of culture supernatants were loaded in lanes 3-6.

fusion protein would be exported to the external medium. The proteins were expressed (Materials and Methods, section F.3.) and samples of culture supernatant analyzed by SDS-PAGE and Western immunoblot (Figure 24). The protein A carrier molecule and the PA/CEME fusion protein were barely visible on a Coomassie-stained polyacrylamide gel (Figure 24A, lanes 3 and 4) but PA/HNP-1 and PA/proHNP-1 were not detected (lanes 5 and 6). The Western immunoblot, however, clearly showed that the proteins were produced and exported with little (Figure 24B, lanes 3 and 4) or no (lanes 5 and 6) proteolytic degradation. Once expression of the genes had been established, PA/CEME was chosen as the fusion protein to be purified. PA/CEME was affinity purified from culture supernatants of S. *aureus* RN4220(pPA-CEME) on an IgG-Sepharose column (Materials and Methods, section G.2.). A sample of the protein eluted off the column showed that only PA/CEME and native S. *aureus* protein A were present in the sample (Figure 24B, lane 7). These were the only two bands present in a Coomassie-stained SDS polyacrylamide gel (data not shown).

E. Release and Purification of CEME.

Purified PA/CEME fusion protein was pooled, lyophilized and treated with CNBr to release the CEME peptide (Materials and Methods, section H.2.). The protein A moiety of the fusion protein contains three methionine residues other than the one that precedes the CEME peptide (Uhlén et al., 1984; Nilsson et al., 1985a), which resulted in a total of five CNBr fragments including CEME. Native protein A present in the sample contained a total of six methionine residues (Uhlén et al., 1984) which gave rise to seven fragments, four of which were different from those produced by the protein A carrier molecule (which is a

truncated version of native protein A). Fortunately, all the CNBr fragments from the protein A carrier molecule had pI values that ranged from 3.9 to 4.7 compared to 11.3 for CEME. This difference in pI values was evident when a sample of CNBr-cleaved PA/CEME was analyzed by AU-PAGE (Figure 25, lane D) which separates proteins on the basis of charge and size. The CEME peptide was well separated from the major contaminating protein, indicating that these differences in pI values could be used in subsequent purification techniques.

The PA/CEME CNBr digest was resuspended in 5% formic acid and fractionated on a Bio-Gel P100 column by isocratic elution with 1% acetic acid (Materials and Methods, section I.). Protein content of the fractions was monitored by absorbance at 280 nm (Figure 25A). A sample from every third fraction, beginning with number 27, was electrophoresed on an acid urea polyacrylamide gel (Figure 25B) which revealed that fragments from the CNBr digest eluted from the column well ahead of CEME. Fractions which contained CEME (Figure 25A, indicated by bar) were pooled and lyophilized.

The final purification step was by reverse phase chromatography using a PepRPC FPLC column (Materials and Methods, section I.). If large samples were to be purified, an initial purification step was performed on a larger Bio-Sil C_{18} reverse phase column which possessed a higher loading capacity but resulted in poorer resolution (data not shown). Samples from Bio-Gel P100 or Bio-Sil C_{18} chromatography that contained CEME were dissolved in 0.1% TFA and applied in 1 mg quantities to the PepRPC column. Peptides were eluted from the column using a tri-phasic acetonitrile gradient (Figure 26; Materials and Methods, section I.). Under these conditions, CEME was eluted by approximately 44% acetonitrile as a homogeneously pure peptide that comigrated with chemically synthesized CEME on an acid urea polyacrylamide gel (Figure 26, inset). A sample of this purified CEME was electroblotted onto

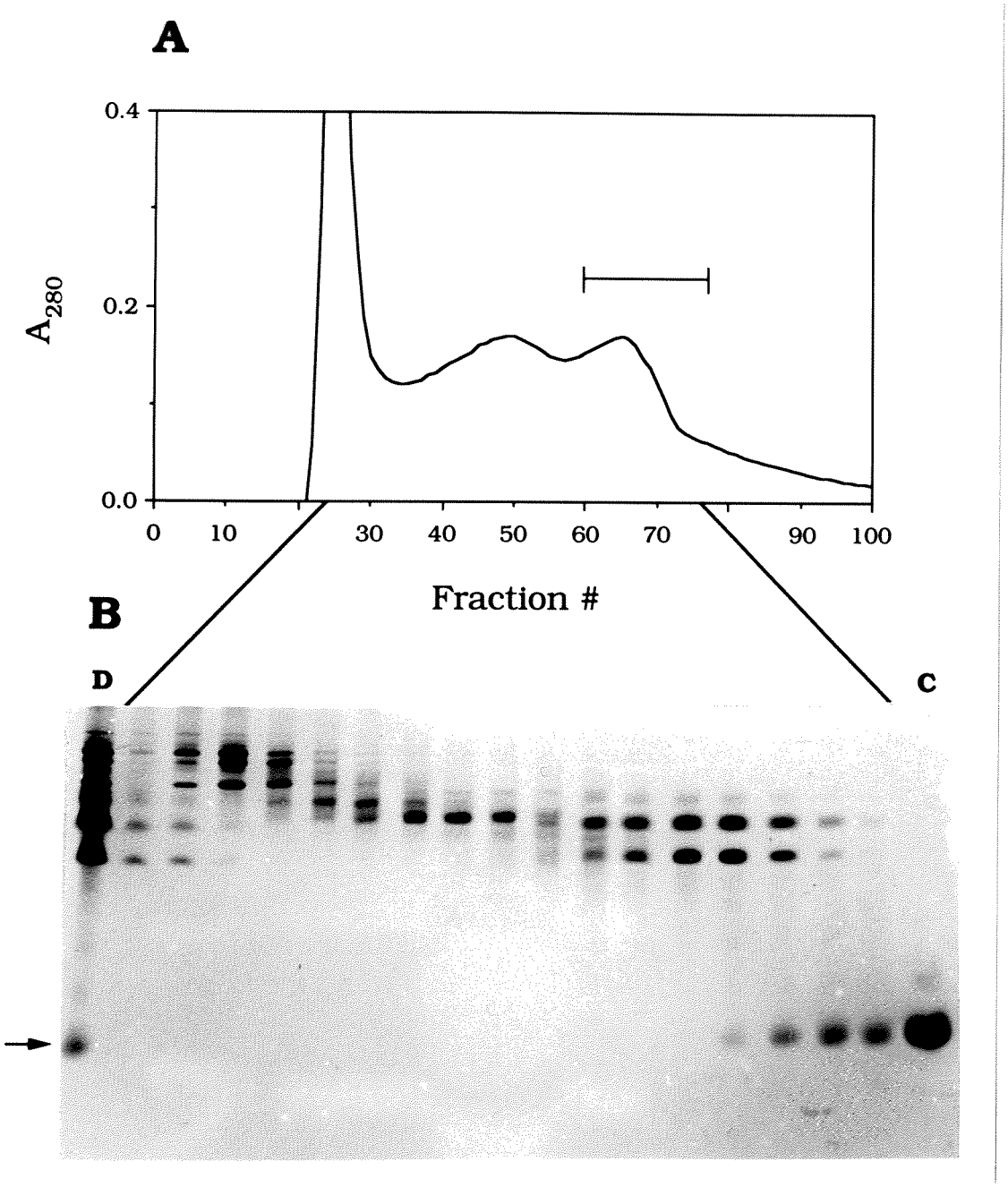

Figure 25: Bio-Gel P100 chromatography of CNBr-digested PA/CEME.

Purified PA/CEME was cleaved with CNBr, lyophilized, resuspended and subjected to gel exclusion chromatography (Materials and Methods, section I.). **A**, Profile of protein content in column fractions. The bar indicates the fractions that were pooled for RP-FPLC. **B**, 15% AU-PAGE in which every third fraction between 27 and 75 (inclusive) was analyzed and stained for protein with Coomassie blue. D, 50 µg of PA/CEME CNBr digest; C, 15 µg of synthetic CEME control; arrow, CEME. Approximately 5-10 µg from each column fraction was loaded.

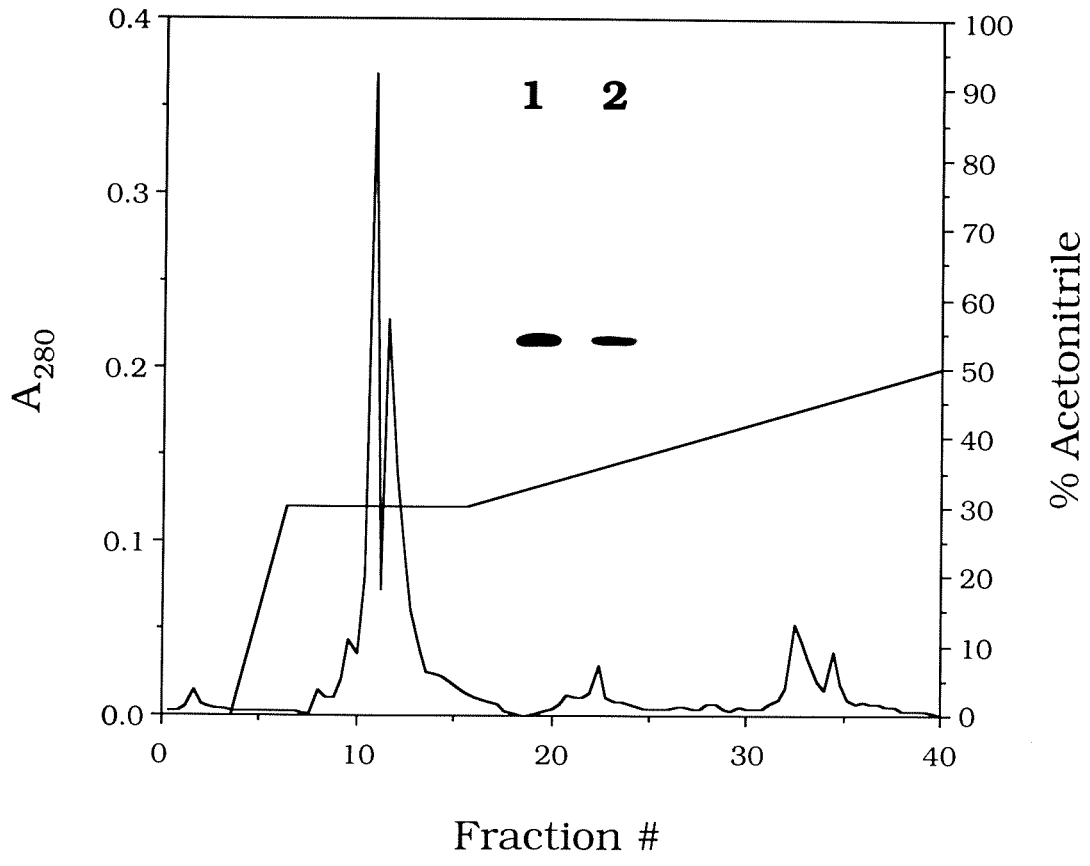

Figure 26: Reverse phase chromatography of CEME.

Pooled fractions from Bio-Gel P100 chromatography were applied to a PepRPC HR 5/5 column and eluted with an acetonitrile gradient (Materials and Methods, section I.). Fractions were monitored for protein content and analyzed by AU-PAGE. Inset: 15% AU-PAGE showing a 5 µg sample from fraction 33 (lane 1), and 5 µg of chemically synthesized CEME (lane 2).

an Immobilon transfer membrane (Materials and Methods, section K.1.) and analyzed for both amino acid content and N-terminal amino acid sequence. All the data was consistent with the amino acid sequence and content of authentic CEME. The yield of the CEME peptide from a 60 L fermentor run of RN4220(pPA-CEME) was not overly impressive (Table VIII). It should be noted, however, that this was the only attempt at large scale production of CEME. Although the bacterial production of cationic peptides was a goal of this study, optimization of production was not the focus, and therefore no attempt was made to improve the yield of the peptide in this system.

To determine whether antibacterial activity was retained during the purification process, samples of isolated PA/CEME fusion protein, CNBr digested PA/CEME, purified CEME from the PepRPC column, chemically synthesized CEME, and melittin were subjected to AU-PAGE (Figure 27A) and tested for activity using a bacterial overlay assay using *E. coli* DC2 as the test organism. Figure 27B shows that CEME produced by recombinant DNA techniques had antibacterial activity comparable to melittin and CEME produced by chemical synthesis, while the uncleaved PA-CEME showed no activity.

F. Purification of Other Cationic Peptides.

The CEMA peptide, which is the CEME analogue with two extra C-terminal amino acids and two additional positive charges (Figure 3), was purified to homogeneity from culture supernatants of RN4220(pPA-CEMA) using the same procedure as for CEME by Dr. M. Brown. Dr. M. Brown also used a similar scheme to purify PA/HNP-1. The fusion protein was isolated, cleaved with CNBr, and passed over a Bio-Gel P100 column. This resulted in a

Table VIII: Percent Yield of CEME from a 60 l Fermentor Run.

Protein Sample	Concentration (mg/mL)	Volume (mL)	Total Protein (mg)	Estimated amount of CEME (mg)	Yield (%)
Supernatent after 0.4 μm filtration	0.1[a]	50000	5000	N/A	N/A
Supernatent after 1 kDa cut-off concentration	0.26[a]	5000	1309	N/A	N/A
After IgG Sepharose purification	106[a]	6	636	22.3[c]	100
After CNBr treatment	32[a]	14	448	15.7[c]	70
After P100 gel filtration purification	10[a]	5	50	8[d]	36
After RP-FPLC using Bio-Sil	3.3[a]	1.5	5	1[d]	4.5
After RP-FPLC using PepRPC	0.54[b]	1.1	0.6	0.6[d]	2.7

[a] Determined by the modified Lowry assay.

[b] Determined by the dinitrophenylation assay.

[c] Estimation assuming that 50% of the protein bound to the IgG Sepharose column is native *S. aureus* protein A and that CEME is 7% of the PA-CEME fusion protein.

[d] Estimation based on AU-PAGE of the sample.

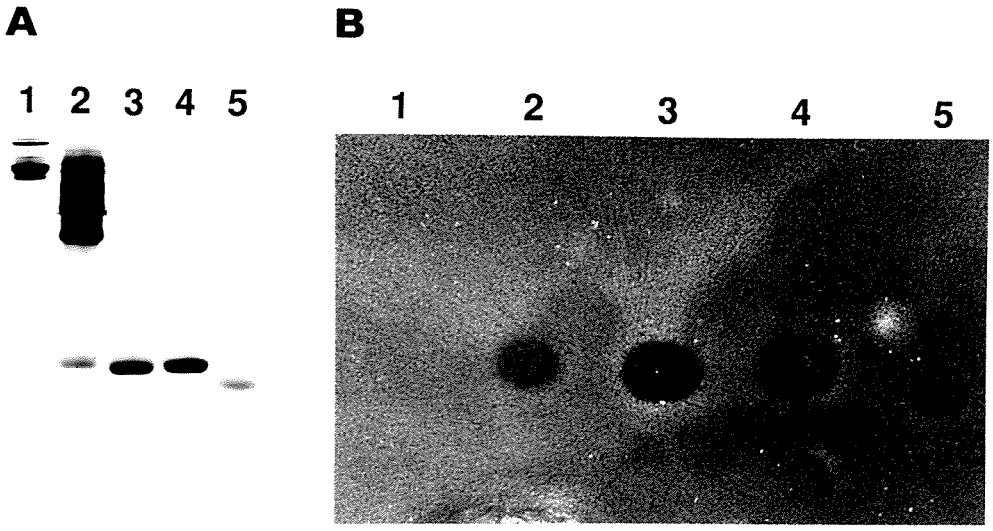

Figure 27: Antibacterial activity of CEME.

Various samples were electrophoresed in duplicate on a 15% acid urea polyacrylamide gel and either **A**, stained for protein with Coomassie blue, or **B**, tested for antibacterial activity using a gel overlay assay (Materials and Methods, section J.). Lanes: 1, 5 μg of purified, uncleaved PA/CEME; 2, 50 μg of CNBr-cleaved PA/CEME; 3, 5 μg of purified, recombinant CEME; 4, 5 μg of chemically synthesized CEME; 5, 5 μg of melittin.

partially purified preparation of HNP-1, as determined by comigration with purified HNP-1 (a gift from M. Selsted) on AU-PAGE (data not shown). This partially purified HNP-1 was also tested for antimicrobial activity using the gel overlay assay which showed that the HNP-1 preparation had no detectable antibacterial activity. There are a few possibilities as to why this peptide did not show any antibacterial activity in this assay. First, the assay may not be sensitive enough to detect HNP-1 activity, in part due to the high ionic strength of the buffer in which it is performed. Second, during the production of the fusion protein, proper disulfide bond formation in HNP-1 may not have occurred, which would result in an inactive peptide (Selsted and Harwig, 1989). It should be noted here that no attempt was made in this study to refold the disulfide bonds which may have been improperly formed throughout the purification procedure.

G. Summary.

The protein A fusion protein system was used to produce various cationic peptides. When *E. coli* was used as a host organism, both the PA/CEME fusion protein and the protein A carrier molecule were extensively degraded. However, all fusion proteins produced in *S. aureus* showed little or no proteolysis, which indicated that export to the culture supernatant was a critical step in the formation of stable fusion proteins. CEME was purified to homogeneity using a combination of affinity, gel exclusion and reverse phase chromatographic methods. The amino acid sequence of this peptide was confirmed and its biological activity demonstrated using a gel overlay assay. HNP-1 produced in the system was partially purified, but possessed no antibacterial activity.

CHAPTER THREE: Antimicrobial Activity of Cationic Peptides.

A. Introduction.

The antimicrobial properties of CEME and melittin against several Gram-positive and Gram-negative bacteria have been previously documented (Wade et al., 1990). These data showed that both peptides had a broad range of activity, with CEME being more active against Gram-negative bacteria and melittin being more active against Gram-positive bacteria. The key difference between these two is that melittin was lytic for erythrocytes which prohibited it from being considered as a therapeutic agent. Many attempts have been made to create cecropin/melittin hybrids peptides that have improved antibacterial activity but no hemolytic activity (Boman et al., 1989a; Andreu et al., 1992; Wade et al., 1992). These studies helped to define the structural requirements for antibacterial activity but did not address the actual mechanism of that activity.

This chapter focuses on the use of antibacterial activity assays as a means for investigating the mechanism of action of cationic peptides, particularly with regards to the self-promoted uptake model. This was done primarily through MIC assays using various mutant strains as test organisms, and different cations as antagonists. As well, synergy studies using other conventional antibiotics were undertaken to provide information on the mode of the uptake of these peptides, as well as their therapeutic potential. The peptides used in these studies were CEME, CEMA and melittin. CEMA was of particular interest because of its two extra positive charges, since it has been shown with magainins that extending the polypeptide chain with positively charged amino acids could augment antibacterial activity (Bessalle et al., 1992).

B. Killing of *P. aeruginosa* H187 by CEME.

The antimicrobial activity of CEME was initially quantified by a killing assay (Lehrer et al., 1983) in which 10^6 CFU of *P. aeruginosa* H187 were incubated with either 2.5 μg/mL or 5.0 μg/mL of peptide in a low ionic strength buffer (10 mM potassium phosphate, pH 7.4). After either 20 or 60 min, samples of the bacteria were diluted and plated to obtain a viable count. The data was plotted as the log of CFU/mL as a function of time (Figure 28). The results showed that *P. aeruginosa* H187 was highly susceptible to the action of CEME, with greater than 99.9% of the bacteria killed by 2.5 μg/mL of peptide in 20 min. Under these conditions, CEME was able to kill *P. aeruginosa* more effectively (a decrease in viability of 3.2 log orders) than the reported value for rabbit lung macrophage cationic protein 1 (2.1 log order decrease; Lehrer et al., 1983). Further incubation caused little or no decrease in viability compared to the control. This could be due to interference by bacterial components, such as LPS, released from initially lysed cells, or it may represent a less susceptible subpopulation of cells.

C. Minimum Inhibitory Concentrations.

The minimum inhibitory concentrations of CEME, CEMA and melittin were determined for a number of different organisms (Materials and Methods, section L.5.; Table IX). Generally, CEME and CEMA had similar MIC values which were consistently lower than those of melittin. The exception to this was *S. aureus*, which had an MIC value for CEME and CEMA that was equivalent to that observed for melittin. This is in agreement with data from Wade et al. (1990) who demonstrated that melittin had a lethal concentration which was

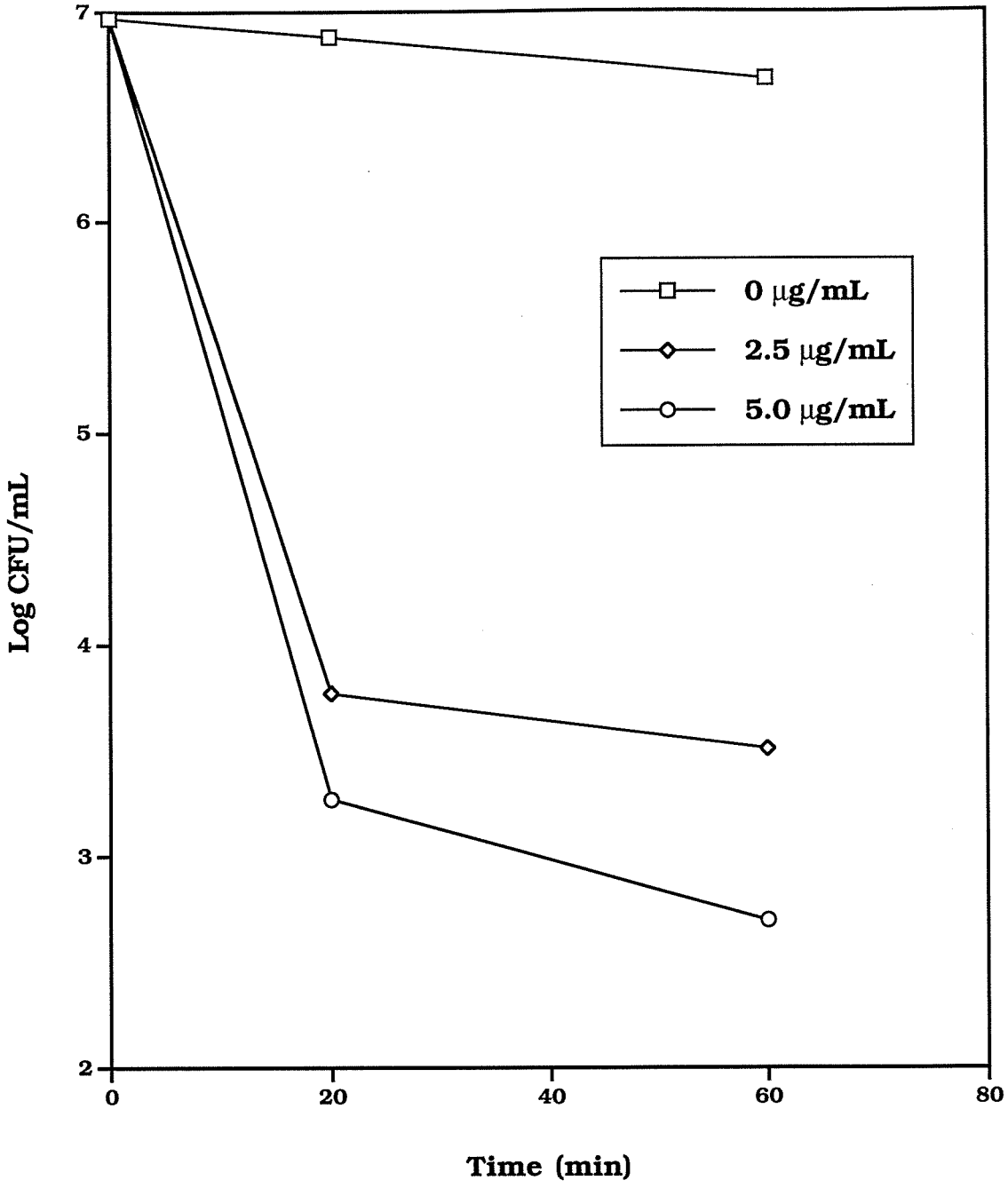

Figure 28: Killing of *P. aeruginosa* H187 by recombinant CEME.

P. aeruginosa H187 (10^7 CFU/mL) was incubated with 0, 2.5, or 5.0 μg/mL of CEME for 20 or 60 min before plating the bacteria out for viability counts (Materials and Methods, section L.2.). The data was plotted as the log of CFU/mL as a function of time.

Table IX: MIC Values of Various Antimicrobial Agents.

Bacterium	Strain	Relevant Phenotype	MIC (µg/mL)[a]					
			PX	GM	CEF	CEME	CEMA	MEL
P. aeruginosa	H309	Wildtype	0.5	1	1	2.4	2.8	8
	H187	Parent of H188	0.5	1	2	4.8	2.8	8
	H188	Antibiotic sensitive	0.06	0.25	0.03	1.2	1.4	8
E. coli	UB1005	Parent of DC2	0.5	1	0.5	2.4	2.8	8
	DC2	Polymyxin sensitive	0.06	0.5	0.06	0.6	0.7	4
	SC9251	Parent of SC9252	0.06	2	0.12	1.2	1.4	8
	SC9252	Polymyxin resistant	4	4	0.5	1.2	1.4	8
S. typhimurium	C587	Parent of C590	1	4	0.25	2.4	5.6	16
	C590	Defensin sensitive	0.25	2	0.25	0.6	1.4	8
E. cloacae	218S	Parent of 218R1	0.5	0.5	0.5	2.4	2.8	8
	218R1	ß-lactam resistant	0.5	0.5	>16	2.4	1.4	8
S. aureus	RN4220	Methicillin sensitive	>8	2	8	9.6	>5.6	8
	SAP0017	Methicillin resistant	>8	>8	>16	9.6	>5.6	8

[a] PX, polymyxin B; GM, gentamicin; CEF,ceftazidime; MEL, melittin.

lower than CEME for Gram-positive bacteria, but higher for Gram-negative bacteria. The MICs of the peptides were usually higher than those of polymyxin B, gentamicin (an aminoglycoside) and ceftazidime (a β-lactam), but this difference was deceiving due to the respective molecular weights of these compounds. For example, when the MICs for *P. aeruginosa* H309 were converted to μM, the MIC values (polymyxin B, 0.3 μM; gentamicin, 1.0 μM; ceftazidime, 1.8 μM; CEME, 0.9 μM; CEMA, 1.0 μM; melittin, 2.8 μM) showed that CEME and CEMA were equally, or more effective at killing this organism on a molar basis than the often utilized anti-pseudomonal antibiotics gentamicin and ceftazidime.

The MIC values of these compounds against certain mutant strains provided initial evidence regarding the uptake pathway of cationic peptides. Both antibiotic supersusceptible mutants (*P. aeruginosa* Z61 and *E. coli* DC2) were two- to four-fold more sensitive to the cationic peptides (the exception being melittin against Z61) as compared to the parental strains. This increased sensitivity for *E. coli* DC2 can be explained by the nature of its mutation. Studies on the outer leaflet of DC2 showed that there was a marked decrease in the esterification of the LPS molecules, which rendered them more negatively charged than the parent strain (Rocque et al., 1988). This could provide the cationic peptides with an increased number of accessible sites on the LPS with which to interact, thus increasing the susceptibility of the organism to these compounds. Strain Z61 has an outer membrane alteration resulting in increased susceptibility to most antibiotics (Angus et al., 1982). However, although analysis of the Z61 LPS is underway, no specific conclusions as to why this organism is susceptible to cationic peptides could be made.

The LPS of the polymyxin B resistance mutant SC9252, in contrast to DC2, was found to have increased esterification and consequently, fewer

negative charges (Peterson et al., 1987). SC9252 had increased resistance to polymyxin B which is known to interact at these sites (Schindler and Osborn, 1979). In contrast, SC9252 showed no increased resistance to any of the cationic peptides, despite the hypothesis that these molecules interact with negatively charged sites on the LPS. The discrepancy could be explained if the specific sites of esterification in SC9252 were not the primary sites of cationic peptide interaction. An indication of this came from the MIC values for the parental strain SC9251. Against this strain, polymyxin B had an MIC that was at least 20-fold lower than the cationic peptides. This suggested that the negatively charged sites in SC9251 that were esterified in SC9252 were targeted to a greater extent by polymyxin B than the cationic peptides. This site-specific interaction may reflect the accessibility of the sites to the different structures that polymyxin B and the various cationic peptides adopt in an aqueous environment. The importance of the relationship between the tertiary structure of compounds and their interaction with the outer membrane has been demonstrated by Vaara (1991) who showed that linear analogues of polymyxin B nonapeptide could not permeabilize the outer membrane.

The *S. typhimurium* defensin sensitive strain was an interesting organism to test, since it has a mutation in the *phoP/phoQ* two-component virulence regulon (Fields et al., 1989). The PhoQ protein is a transmembrane sensor kinase that contains an anionic domain predicted to be in the periplasmic region, and therefore it was hypothesized that in wild-type cells this domain would interact with, and neutralize defensins (Miller et al., 1989). Characterization of *phoP* and *phoQ* mutants revealed that this hypothesis was incorrect and that defensin sensitivity was conferred by the lack of synthesis of PhoP-activated genes (Miller et al., 1990). The defensin sensitive mutant was two- to four-fold more susceptible to the cationic peptides than the wild-type

parent. Only the MIC of ceftazidime, which is taken up across the outer membrane by a porin-mediated pathway different from that of the peptides, remained unchanged. These results suggested that the mutation was only affecting the self-promoted uptake pathway, possibly at the sites of initial antibiotic contact on the surface of the cell. This conclusion, however, remains speculative since little is known about the genes that are activated by PhoP.

The peptides were tested against a clinical isolate of *E. cloacae* (218S) and its β-lactam resistant mutant (218R1, a β-lactamase overproducer; Marchou et al., 1987). Not surprisingly, the compounds proposed to be taken up by the self-promoted uptake pathway were equally active against both of these, with ceftazidime being the only antibiotic that showed a higher MIC for the mutant. Similarly methicillin resistance in *S. aureus* had no apparent effect on cationic peptide susceptibility.

D. The Effect of Cations on the MIC of Cationic Peptides.

One of the key proposals of the self-promoted uptake hypothesis is the initial interaction between the cationic antibiotic and the negatively charged sites on the surface of the outer membrane (Hancock et al., 1981). In cells grown under physiological conditions, these sites are occupied by divalent cations. Therefore, one can envision that, in the presence of Mg^{2+} ions, the MIC values of compounds proposed to be taken up by the self-promoted uptake pathway would increase due to the Mg^{2+} ions competing for the negatively charged binding sites. Indeed it has been demonstrated that the presence of 5 mM $MgCl_2$ can increase the MIC of polymyxin B four-fold (Nicas and Hancock, 1980).

The MIC determination was repeated for strain H309 in the presence of 5 mM Mg^{2+} or 80 mM Na^{+} to determine whether or not the antibacterial activity of the cationic peptides would be inhibited (Materials and Methods, section L.5.; Table X). The results showed that the MIC of all three peptides were dramatically increased in the presence of Mg^{2+} but only minimally in the presence of a higher concentration of Na^{+}. Other antibiotics were also affected by the presence of Mg^{2+}, albeit to a much lesser extent. The differences between the effects of Mg^{2+} on the MIC of cationic peptides, and polymyxin B and gentamicin (which are all proposed to be taken up via the self-promoted uptake pathway), could reflect a difference in the nature of the initial contact of these compounds with the outer membrane. Nonetheless, this evidence was consistent with the hypothesis that the initial step in the antibacterial mechanism of cationic peptides is an association with the negatively charged Mg^{2+} binding sites on LPS molecules.

E. Synergy Studies with Cationic Peptides and Other Antibiotics.

The ability of the cationic peptides to augment the activity of different antibiotics was tested. Previous studies have shown that some cationic membrane permeabilizing agents such as PMBN and $lysine_{20}$, at sub-MIC levels, were able to increase the sensitivity of bacteria to a number of different antibiotics (Vaara and Vaara, 1983). Therefore, MIC assays using various antibiotics were performed in the presence of 1/2 or 1/4 MIC levels of cationic peptides to determine whether any synergy existed between them (Materials and Methods, section L.5.; Table XI). Generally the peptides had very little effect on the MIC of antibiotics that are proposed to be taken up through porins (ceftazidime, imipenem, and tetracycline), although CEMA at 1/2 MIC levels did

Table X: Effects of Mg^{2+} and Na^{+} Cations on the MICs of Cationic Peptides Against *P. aeruginosa* H309.

Compound	MIC (μg/mL)		
	No addition	+ 5 mM Mg^{2+}	+ 80 mM Na^{+}
Polymyxin	0.5	1	0.5
Gentamicin	1	4	1
Ceftazidime	1	2	2
CEME	2.4	38.4	4.8
CEMA	2.8	22.4	5.6
Melittin	8	>64	16

Table XI: Effects of Sub-MIC Levels of Cationic Peptides on the MICs of Common Antibiotics.

Compound	MIC (μg/mL) in the presence of						
	No peptide	CEME (μg/mL)		CEMA (μg/mL)		Melittin (μg/mL)	
		0.6	1.2	0.7	1.4	2	4
Polymyxin	1	0.5	0.25	0.5	0.12	0.5	0.06
Ceftazidime	4	4	4	2	2	4	2
Imipenem	4	4	4	4	2	4	4
Tetracycline	8	8	8	8	4	8	8
Novobiocin	256	256	256	256	128	256	256
Fusidic Acid	1024	1024	1024	1024	512	1024	1024

reduce their MICs two-fold. This phenomenon was also observed for novobiocin and fusidic acid, two antibiotics that are believed to cross the membrane via the hydrophobic uptake pathway. Any compound possessing membrane permeabilizing activity at sub-lethal concentrations (which included CEME and melittin) would be expected to enhance the uptake of these hydrophobic antibiotics by disrupting the outer membrane. This, however, was not observed. It is possible that due to the high antimicrobial activity of CEME, concentrations of the peptide that are required to permeabilize the outer membrane to such antibiotics result in cell death and thus mask its permeabilizing activity. This has been shown for polymyxin B, which could not enhance the uptake of novobiocin or fusidic acid (Vaara and Vaara, 1983), despite evidence that it could permeabilize outer membranes at sub-lethal concentrations (this study; Hancock and Wong, 1984). In contrast, PMBN was able to augment hydrophobic antibiotic activity at sub-lethal concentrations probably because it had such a high MIC (>100 μg/mL; Vaara and Vaara, 1983). Therefore, one could test its ability to permeabilize the outer membrane to hydrophobic antibiotics at relatively high concentrations without killing the cells.

The sub-MIC levels of cationic peptides rendered strain H103 4- to 16-fold more susceptible to polymyxin B (Table XI). Given the fact that the cationic peptides and polymyxin B are both proposed to be taken up by the self-promoted uptake pathway, these results are consistent with additive effects of the membrane permeabilizing and killing activities of these two compounds. This suggestion, however, is incomplete since melittin showed the greatest influence on the polymyxin B MIC despite having the lowest permeabilizing activity of the three peptides tested (see below). This raised the question of which compound of the two was responsible for outer membrane

permeabilization and which one was responsible for killing by the disruption of the cytoplasmic membrane. In the cases of CEME and CEMA with polymyxin B, it was considered likely that both compounds contributed equally to permeabilization and killing, since their membrane permeabilization activities (see below) and MIC values against H309 were quite similar. However, since melittin showed greater synergy with polymyxin B, it was reasonable to conclude that polymyxin B, with its stronger outer membrane disrupting ability, was permeabilizing the outer membrane for melittin which would subsequently target the cytoplasmic membrane. This interpretation would imply that melittin was more effective once it reached its target site and that the rate limiting step in its antibacterial activity was its passage across the outer membrane.

F. Summary.

The antimicrobial activities of CEME, CEMA, and melittin were examined to provide initial information regarding their mechanism of action. All the cationic peptides, especially CEME and CEMA, were shown to kill a number of different organisms at molar concentrations that were comparable to some conventional antibiotics. MIC assays performed against some mutant strains gave initial, yet unconfirmed evidence that the cationic peptides were interacting with the divalent cation binding sites on LPS molecules. This was supported by experiments that demonstrated the inhibitory effects of Mg^{2+} on the MIC of cationic peptides. Finally, synergy studies with the cationic peptides and various conventional antibiotics showed that with the exception of polymyxin B, the peptides were unable to substantially augment antibiotic activity.

CHAPTER FOUR: Membrane Permeabilizing Activities of Cationic Peptides.

A. Introduction.

One of the proposed requirements for compounds taken up by the self-promoted uptake pathway is they must be able to permeabilize the outer membrane (Hancock et al., 1981). The outer membrane permeabilizing activities of many different compounds have been studied (Hancock and Wong, 1984; Vaara, 1992). With respect to cationic peptides, and defensins in particular, there has been some disagreement as to whether or not they possess the ability to disrupt membranes. Rabbit defensins (macrophage cationic proteins; Sawyer et al., 1988) and human defensins (Lehrer et al., 1989) were shown to permeabilize the outer membrane as measured by the uptake of NPN or the crypticity of periplasmic β-lactamase respectively. Viljanen et al. (1988) argued that sub-lethal concentrations of human defensins could not permeabilize the outer membrane to rifampicin as measured by fractional inhibitory concentration assays. Therefore, this chapter describes experiments that were performed to determine whether or not these cationic peptides have membrane permeabilizing activity and therefore fulfill one of the requirements of the self-promoted uptake model.

B. Lysozyme Lysis Assays.

Lysozyme is a 14 kDa basic protein that is unable to penetrate intact outer membranes, but can diffuse across ruptured membranes to exert its lytic activity (Hancock and Wong, 1984). Because of its large size, one would expect

that significant destabilization of the membrane would be required in order for it to penetrate through to the peptidoglycan.

The cationic peptides were tested for their ability to facilitate the uptake of lysozyme by permeabilizing the outer membrane (Materials and Methods, section L.2.). Briefly, the assay mixture contained prepared cells, lysozyme (which demonstrated no lytic activity by itself) and cationic peptides at various concentrations. Cell lysis due to the cationic peptide-enhanced uptake of lysozyme was measured as a decrease in OD_{600} (Figures 29 and 30, solid lines). The data showed that CEMA was a stronger permeabilizer for lysozyme than polymyxin B in either *P. aeruginosa* H309 or *E. cloacae* 218R1. At low concentrations, CEME demonstrated better activity than polymyxin B against *P. aeruginosa* H309 while with *E. cloacae* 218R1, these two compounds showed similar activities. The assays were also performed in the absence of lysozyme (Figures 29 and 30, dashed lines). These studies revealed that CEME and particularly melittin (*P. aeruginosa* data only) possessed significant lytic activity, compared to permeabilizing activity, at the concentrations tested. In light of this activity, melittin did not appear to be a good permeabilizer compared to the other peptides, yet it was still 5- to 10-fold better than gentamicin (Hancock et al., 1981; data not shown). These data supported earlier suggestions in this study that although melittin appeared to have difficulty penetrating the membrane barrier, it had strong lytic activity at its target site. In contrast, CEMA had little or no lytic activity at concentrations which resulted in extensive membrane permeabilization. Since lysozyme has been shown to bind LPS (Ohno and Morrison, 1989), it could be suggested that this binding may interfere with the interaction of the cationic peptides with LPS. If this was a significant factor in this assay, one might expect to see antagonism rather than a synergistic effect between lysozyme and the cationic peptides. At low

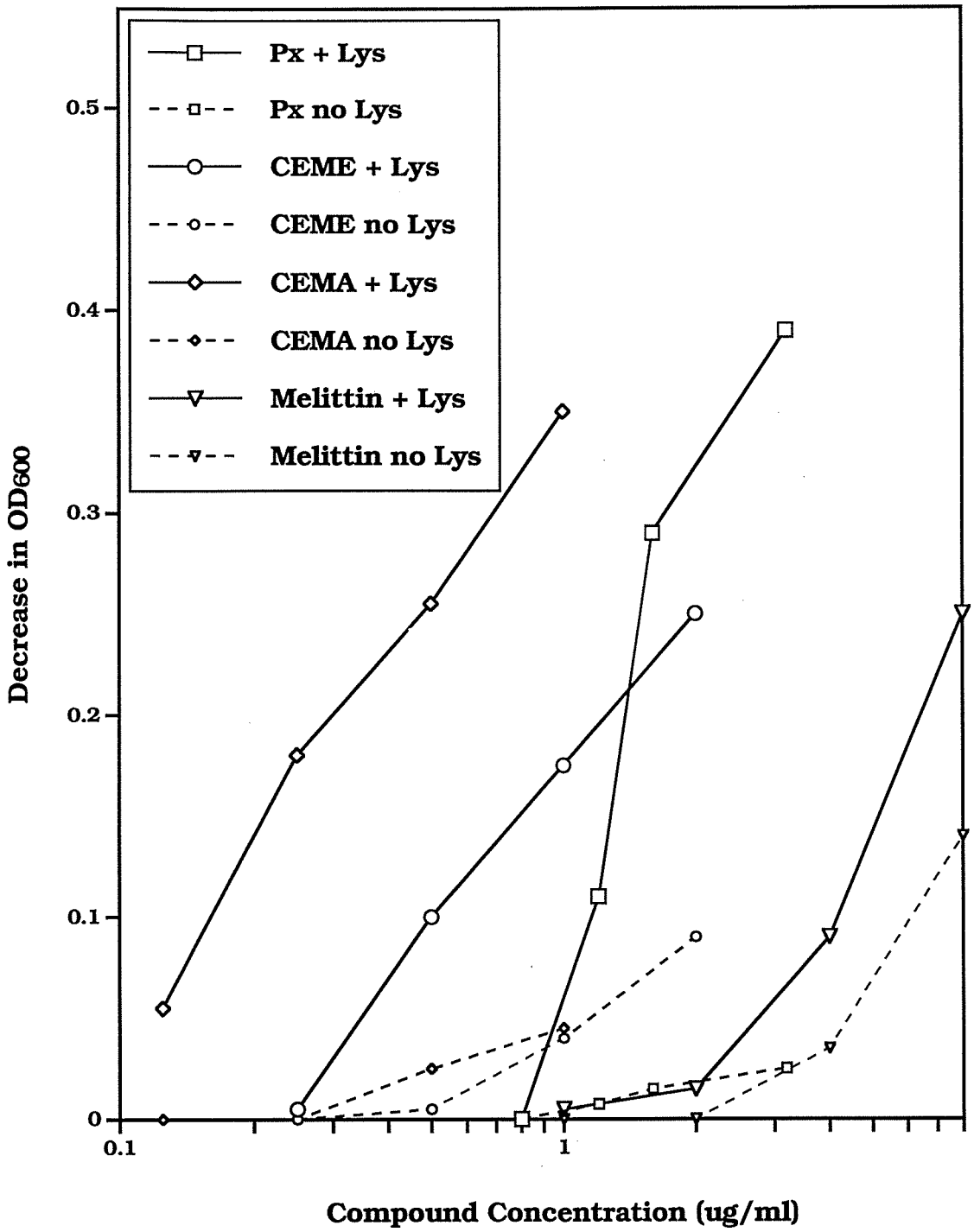

Figure 29: Peptide-mediated lysozyme lysis of *P. aeruginosa* H309.

Various peptide antibiotics were tested for their ability to permeabilize the outer membrane of *P. aeruginosa* H309 to lysozyme (Materials and Methods, section L.3.). Lysis was measured by a decrease in OD_{600} as a function of peptide concentration.

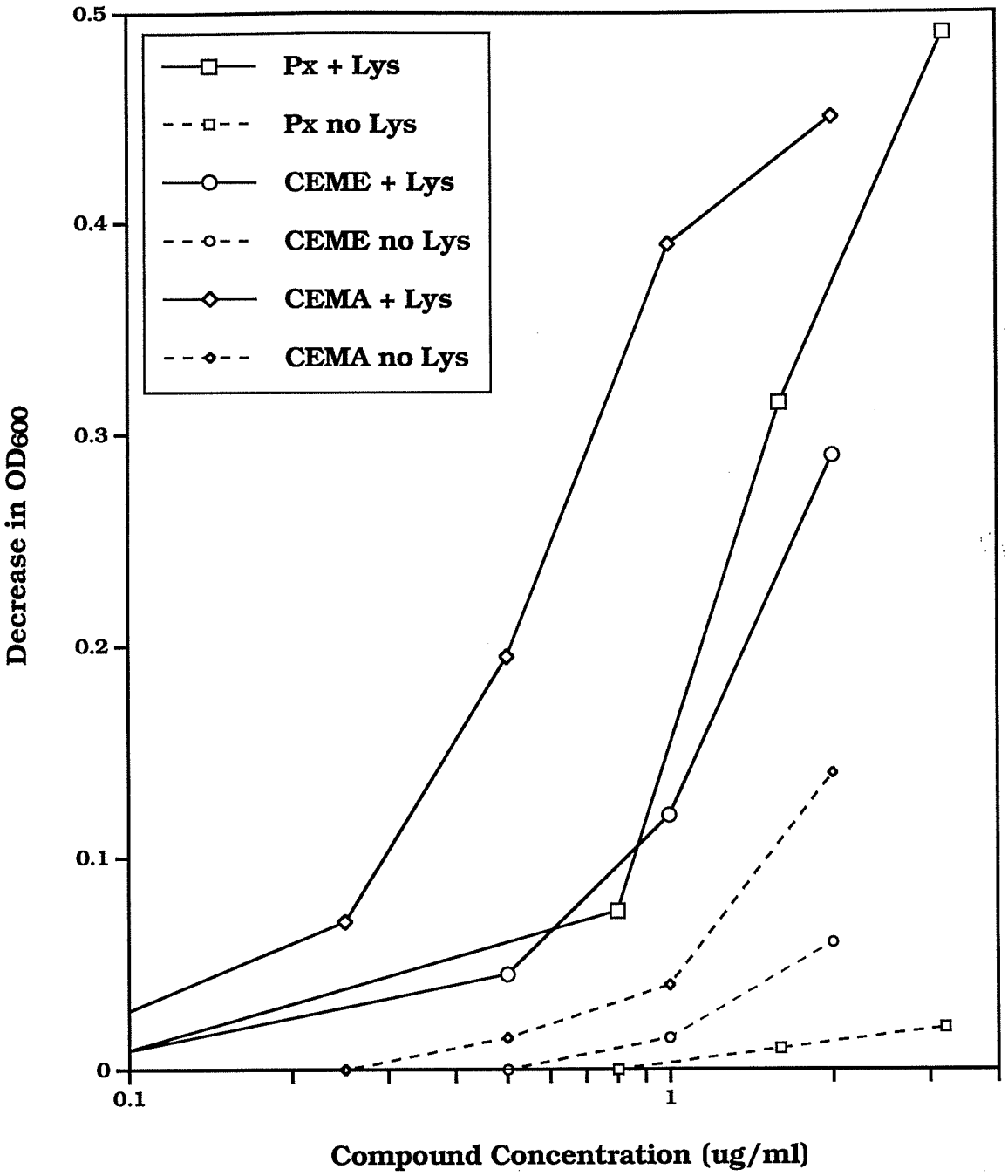

Figure 30: Peptide-mediated lysozyme lysis of *E. cloacae* 218R1.

Various peptide antibiotics were tested for their ability to permeabilize the outer membrane of *E. cloacae* 218R1 to lysozyme (Materials and Methods, section L.3.). Lysis was measured by a decrease in OD_{600} as a function of peptide concentration.

concentrations, neither the cationic peptides nor lysozyme were able to lyse cells, but when used simultaneously, lysis was observed. Therefore, it was concluded that lysozyme either did not bind appreciably to cellular LPS or it could be readily displaced by the cationic peptides, which possess high LPS binding affinities (see below). Additional experiments demonstrated that Mg^{2+} concentrations as low as 1 mM were able to prevent lysozyme lysis induced by the cationic peptides. This indicated that the cationic peptides interacted with divalent cation binding sites on the LPS to initiate outer membrane permeabilization.

C. 1-*N*-phenylnaphthylamine Uptake Assay.

1-*N*-phenylnaphthylamine (NPN) is an uncharged, hydrophobic fluorescent probe that has been used to study outer membrane permeabilization (Loh et al., 1984; Sawyer et al., 1988). When NPN is mixed with cells, it fluoresces weakly since it is unable to breach the outer membrane permeability barrier. Upon membrane destabilization, however, it can partition into the hydrophobic environment of the membrane where it emits a bright fluorescence. One advantage of NPN is that it is uncharged and therefore is not expected to bind to anionic sites on LPS and interfere with the activity of the cationic peptides. In addition, its small size and hydrophobicity enable it to insert into membranes more easily and therefore may be used to detect more subtle disruptions of the outer membrane.

NPN uptake assays were performed on *P. aeruginosa* H309 and *E. cloacae* 218R1 (Materials and Methods, section L.3.). Various concentrations of cationic peptides were added to cuvettes containing cells and NPN. The resulting increases in fluorescence (measured in arbitrary units) were plotted as a

function of the compound concentration (Figures 31 and 32). For *P. aeruginosa* H309, CEMA and polymyxin B were found to have virtually identical membrane permeabilizing activities. CEME and melittin also had similar activities, but were weaker permeabilizers than CEMA and polymyxin B. These results (CEMA ≈ polymyxin B > CEME ≈ melittin) were different to those obtained in the lysozyme lysis assays (Figure 29; CEMA > CEME > polymyxin B > melittin). Analysis of these data revealed that similar concentrations (0.25 - 1.0 μg/mL) of CEMA permeabilized the *P. aeruginosa* H309 outer membrane to lysozyme and NPN. In contrast, the concentrations of polymyxin B which were able to permeabilize cells to NPN, were not sufficient to permeabilize them to lysozyme. These data could be explained if polymyxin B caused more subtle membrane perturbations at low (< 1 μg/mL) concentrations and larger disruptions at higher (> 1 μg/mL) concentrations. The similarities of CEMA activity in both assays could be explained if the peptide caused substantial perturbations at both low and high concentrations. Similar arguments could be made for CEME (cf CEMA), which showed no difference in the two assays and melittin (cf polymyxin B), which showed much lower permeabilization concentrations in the NPN assay. Under the conditions of the NPN assay, little or no cell lysis occurred as measured by OD_{600}.

The results from NPN uptake experiments performed on *E. cloacae* 218R1 showed that the cationic peptides were able to enhance the uptake of the hydrophobic probe at concentrations similar to those in the *P. aeruginosa* H309 experiments. Although conclusions regarding the differences between the outer leaflets of *E. cloacae* 218R1 and *P. aeruginosa* H309 cannot be made, the results do indicate that cationic peptide-induced outer membrane permeabilization is a phenomenon that is not restricted to the *Pseudomonads*.

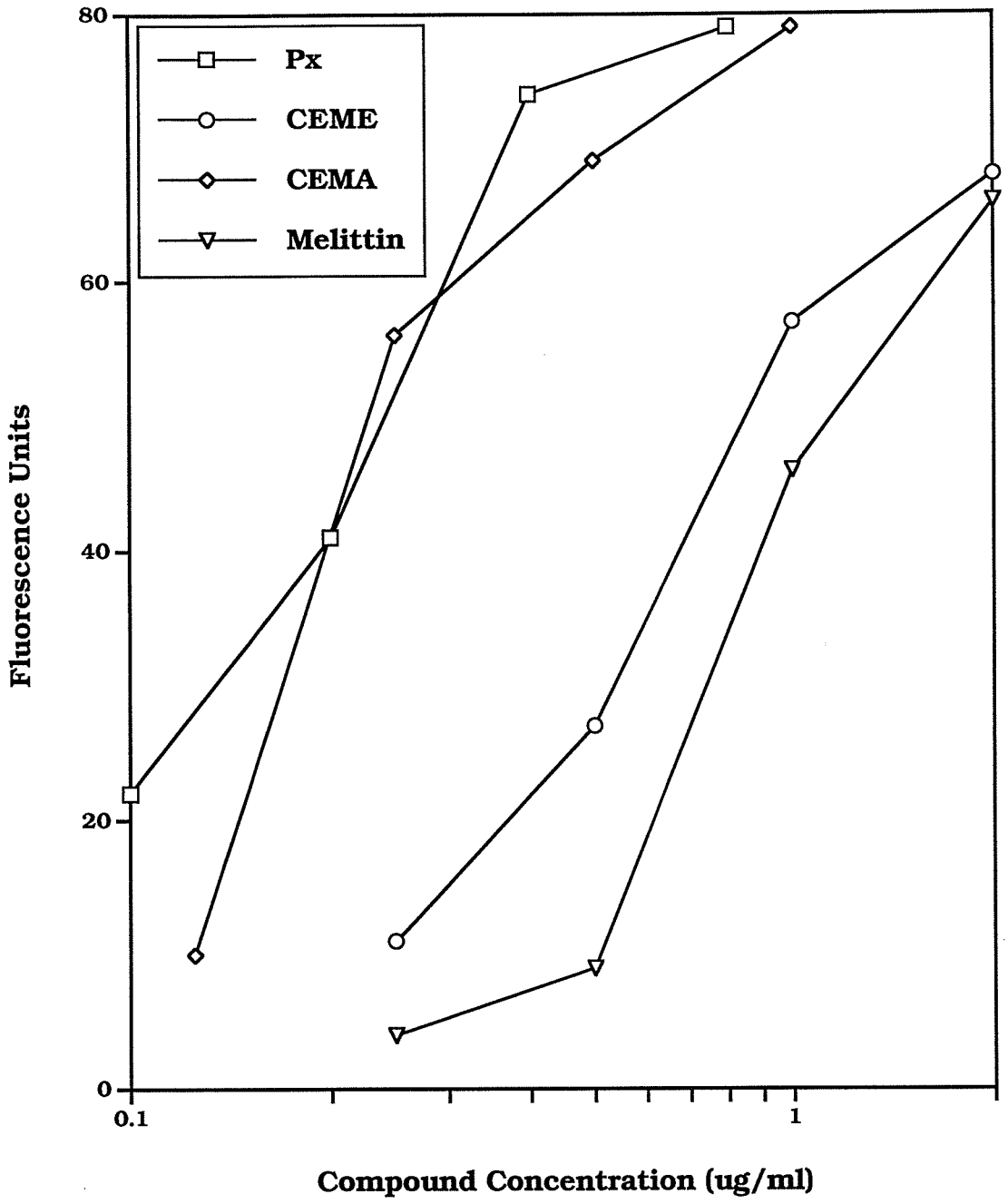

Figure 31: Peptide-mediated NPN uptake in *P. aeruginosa* H309.

P. aeruginosa H309 cells were incubated with NPN in the presence of various concentrations of cationic peptide antibiotics (Materials and Methods, section L.4.). Enhanced uptake of NPN was measured by an increase in fluorescence due to the partitioning of NPN in the hydrophobic membrane. The data was plotted as arbitrary fluorescence units as a function of peptide concentration.

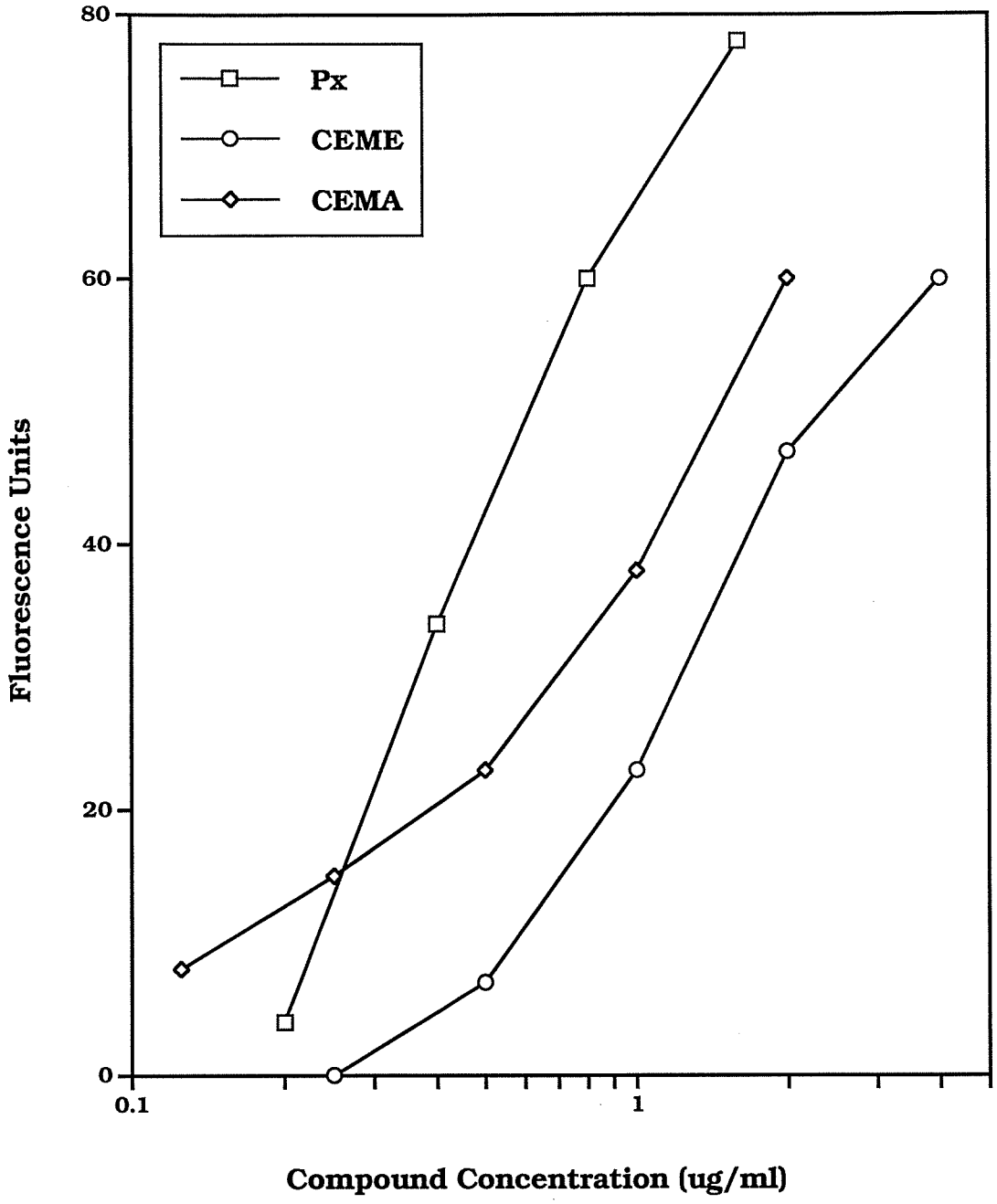

Figure 32: Peptide-mediated NPN uptake in *E. cloacae* 218R1.

E. cloacae 218R1 cells were incubated with NPN in the presence of various concentrations of cationic peptide antibiotics (Materials and Methods, section L.4.). Enhanced uptake of NPN was measured by an increase in fluorescence due to the partitioning of NPN in the hydrophobic membrane. The data was plotted as arbitrary fluorescence units as a function of peptide concentration.

D. Dansyl Polymyxin B Displacement Assays.

Another proposal of the self-promoted uptake model is the ability of the compounds that access this pathway to bind to the divalent cation binding sites of LPS. This is proposed to promote membrane destabilization, leading to the uptake of the molecule. The above experiments in this study provided suggestive evidence that cationic peptides might bind to LPS to initiate their antimicrobial effects. MIC assays on mutants with altered LPS anionicity (Chapter 3.B.), and Mg^{2+} inhibition of the peptide's antibacterial (Chapter 3.C.) and permeabilizing (Chapter 4.B.) activities were consistent with the proposal that the sites at which the cationic peptides bind are also the ones at which Mg^{2+} binds to form cross bridges between adjacent LPS molecules.

To further investigate this question, dansyl polymyxin B displacement assays were performed using purified LPS or whole cells. Dansyl polymyxin B has been shown to bind LPS, resulting in enhanced fluorescence of the dansyl group (Moore et al., 1984). This property led to the development of an assay for determining the LPS-binding affinities of certain antibiotics (Materials and Methods, section L.4.c.; Moore et al., 1986). Dansyl polymyxin B was added to a sample of LPS until approximately 90% of the binding sites were occupied as indicated by 90% of maximal fluorescence enhancement. Cationic peptides or other polycations were then titrated in and the displacement of dansyl polymyxin B monitored by the decrease in fluorescence. The results, which were plotted as the fraction of dansyl polymyxin B bound versus the compound concentration (Figure 33), indicated that all the compounds, with the exception of gentamicin, had high binding affinities for purified LPS. To quantify these affinities, the I_{50} value, which was the concentration of compound that resulted in 50% maximal displacement of the dansyl polymyxin B, was calculated for

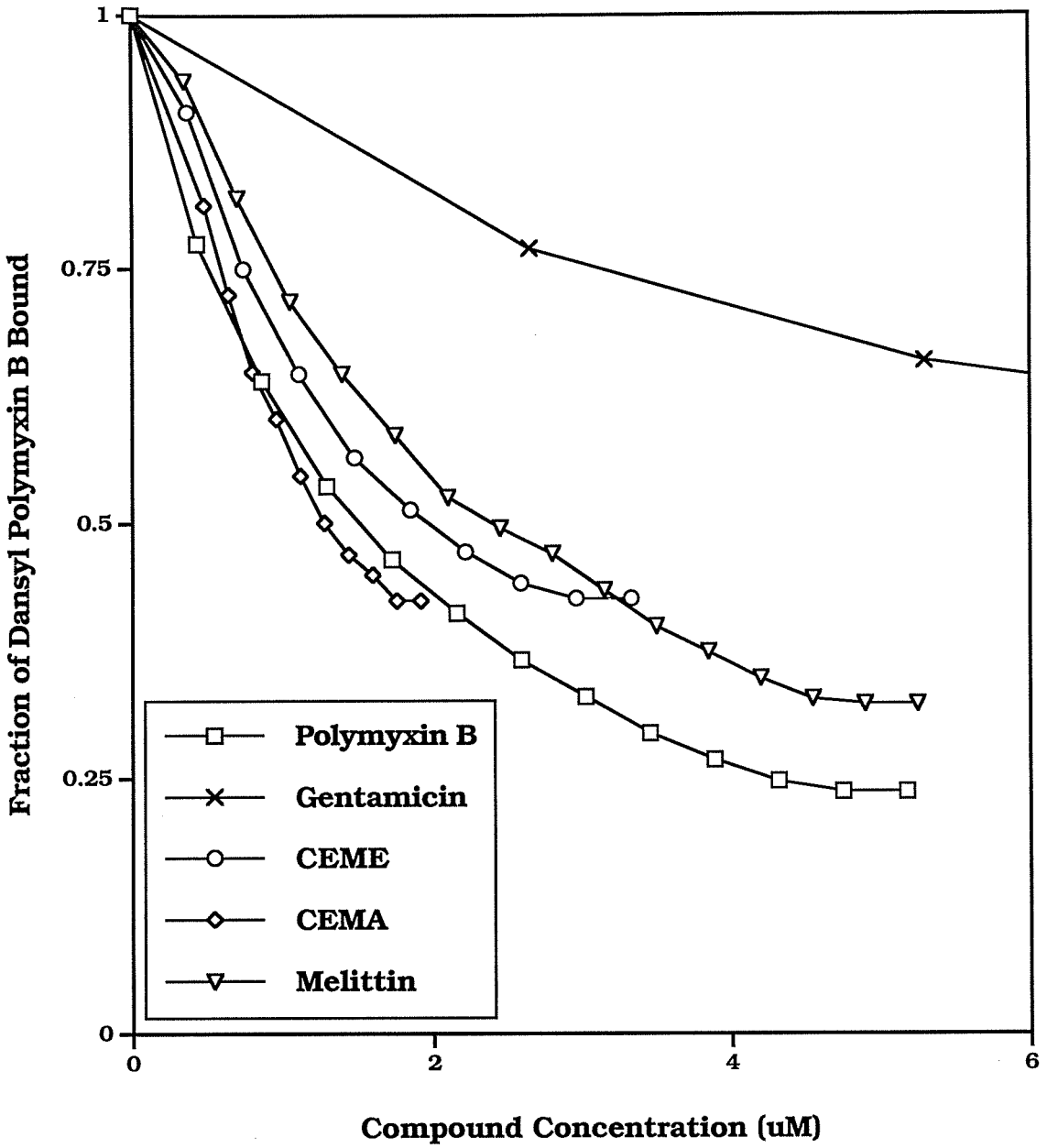

Figure 33: Inhibition of dansyl-polymyxin B binding to *P. aeruginosa* H103 LPS by various compounds.

Cationic antibiotics were titrated into cuvettes containing LPS that had dansyl polymyxin B bound to it (Materials and Methods, section L.5.c.). Displacement of the dansyl polymyxin B from the LPS was measured as a decrease in fluorescence. The fraction of dansyl polymyxin B bound at a given compound concentration was calculated as 1-{(maximum fluorescence – fluorescence at compound concentration)/maximum fluorescence}. The data was plotted as the fraction of dansyl polymyxin B bound as a function of compound concentration.

each compound (Table XII). These values were read directly from graphs such as the one in Figure 33. The values showed that CEMA had the highest affinity for purified LPS, followed closely by polymyxin B, and then CEME and melittin.

To test whether these compounds bind to LPS in native outer membranes, the assay was repeated using *P. aeruginosa* H309 whole cells instead of purified LPS (Figure 34). It was clear from the graph that all three cationic peptides showed a slightly higher affinity for cellular LPS than polymyxin B, which was confirmed by the calculated I_{50} values (Table XII). In contrast only CEMA (and CEME at low concentrations) was shown to be a better permeabilizer than polymyxin B. This indicated that the initial binding affinity of the compound to the LPS and the subsequent ability to permeabilize the outer membrane are not strictly related. Nevertheless, one must be cautious of any conclusions drawn from these whole cell experiments because of the complexity of the system. The levels of competing divalent cations, the possible release of LPS molecules, the influence of dansyl polymyxin B which itself perturbs the permeability barrier, potential conformational changes in the peptides themselves, and the effects of the cationic peptides on membrane structure are all aspects that may affect the results. Nevertheless, the peptides were apparently able to bind LPS in the context of whole cells.

E. Summary.

The membrane permeabilizing activities and LPS binding affinities of CEME, CEMA and melittin were investigated. CEMA was found to be a potent permeabilizer of outer membranes to both lysozyme and NPN in *P. aeruginosa* and *E. cloacae*. CEME also demonstrated strong permeabilizing activity, although not as good as CEMA. Different results obtained in the two assays

Table XII: I_{50} Values (in μM) for Various Compounds Against *P. aeruginosa* LPS and Whole Cells.[a]

Compound	*P. aeruginosa* H103 LPS	*P. aeruginosa* H309 Whole Cells
Polymyxin	0.93 ± 0.03	0.85 ± 0.13
Gentamicin	12.17 ± 0.58	19.0 ± 5.2
$MgCl_2 \cdot 6H_2O$	850 ± 132	127 ± 31
CEME	1.30 ± 0.37	0.41 ± 0.08
CEMA	0.70 ± 0.10	0.33 ± 0.03
Melittin	1.41 ± 0.05	0.43 ± 0.06

[a] Each value is the average of at least three trials ± the standard deviation.

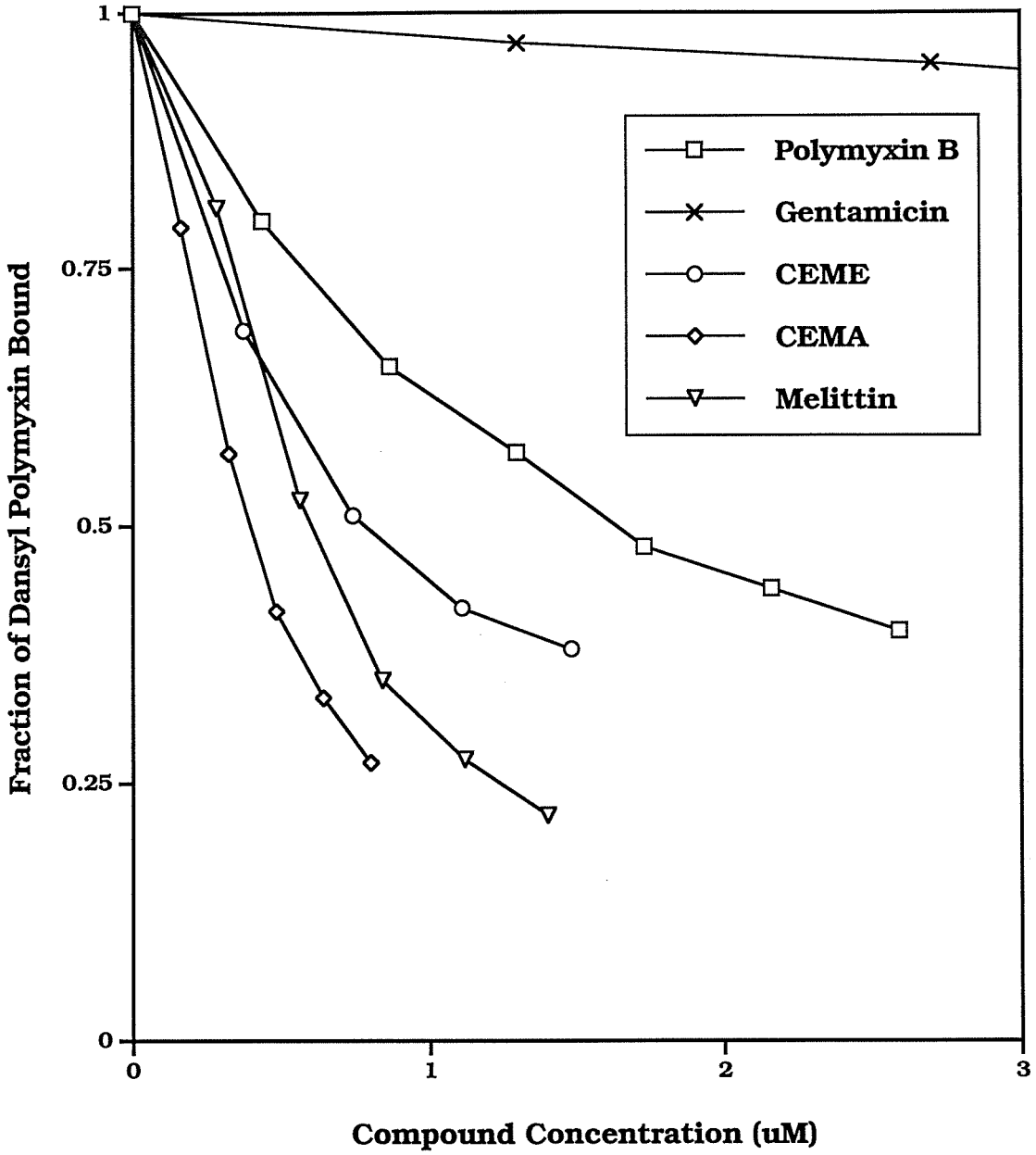

Figure 34: Inhibition of dansyl-polymyxin B binding to *P. aeruginosa* H309 whole cells by various compounds.

Cationic antibiotics were titrated into cuvettes containing whole cells that had dansyl polymyxin B bound to them (Materials and Methods, section L.5.c.). Displacement of the dansyl polymyxin B from the whole cells was measured as a decrease in fluorescence. The fraction of dansyl polymyxin B bound at a given compound concentration was calculated as 1-{(maximum fluorescence – fluorescence at compound concentration)/maximum fluorescence}. The data was plotted as the fraction of dansyl polymyxin B bound as a function of compound concentration.

suggested there may be degrees of permeabilizing activity, with polymyxin B and melittin showing minor perturbations at low concentrations and larger disruptions at high concentrations, while CEME and CEMA showed major disruptions even at low concentrations. Dansyl polymyxin B displacement assays with pure LPS demonstrated that the LPS binding affinities of the compounds (CEMA > polymyxin B > CEME > melittin) correlated well with their membrane permeabilizing activities as determined by the NPN uptake assay. This correlation was not maintained in dansyl polymyxin B displacement assays which used whole cells instead of purified LPS. Nonetheless, all the cationic peptides showed high LPS binding affinities and strong membrane permeabilizing activities, which are both required of compounds proposed to be taken up by the self-promoted uptake pathway.

DISCUSSION

A. General.

This study describes the establishment of a bacterial expression system that allows the production of antimicrobial cationic peptides in bacteria. A number of different systems were investigated, including direct expression and the expression of fusion proteins. In the latter systems, different proteolytic cleavage methods were used to remove the carrier protein. Various fusion proteins required different purification techniques, and during these studies, a novel method for the purification of inclusion bodies was developed. Two α-helical peptides, CEME and CEMA, were produced as fusion proteins to S. *aureus* protein A, and, after being cleaved from the affinity tag, were purified to homogeneity. These peptides were shown to be biologically active, possessing a broad host range of antibacterial activities. The peptides, together with other antibiotic controls, were also tested for their ability to permeabilize the outer membranes of Gram-negative bacteria. Overall, the data from the killing and membrane permeabilization studies indicated that cationic peptides cross the outer membrane by the self-promoted uptake pathway.

This work is the first report of the successful production of an α-helical, antimicrobial peptide in bacteria. During these studies, a unique method of purifying inclusion bodies was developed. Although many previous studies have focused on the interaction of α-helical antimicrobial peptides with the cytoplasmic membrane, none have investigated the effects of these molecules with the outer membrane. The experiments in this thesis, therefore, represent new information regarding the mechanism of action of antibacterial cationic peptides on Gram-negative bacteria.

B. Direct Expression.

My first attempt at producing cationic peptides in bacteria, was to clone the HNP-1 gene into the direct expression vector pT7-5. Intuitively, it did not seem feasible to produce an antibacterial peptide directly in bacteria because of the potential for host toxicity. It was hypothesized that by exporting the peptide to the periplasm, and thus removing the peptide from any potential cytoplasmic targets, this threat could be minimized. Therefore, the signal sequence of *E. coli* alkaline phosphatase was included in the genetic construct to drive this export. This hypothesis was highly speculative since the mechanism of action of defensins was, at the time of these experiments, largely unknown. In fact, exporting the peptide to the periplasm may enhance host toxicity since defensins were subsequently shown to form ion channels in planar lipid membranes (Kagan et al., 1990). Nonetheless, the alkaline phosphatase signal sequence was included, since the export of the peptide to the periplasm had other potential advantages such as proper formation of disulfide bonds (which is necessary for defensin activity; Selsted and Harwig, 1989), an increased chance of stability and solubility, and simplification of the purification procedure. All of these advantages proved to be irrelevant since no HNP-1 was detected in any of the expression attempts using the pT7-5 system.

Since translation in the pT7-5 system was driven by a synthetic ribosome binding site included in the cloned fragment that encoded the alkaline phosphatase signal sequence and the HNP-1 peptide, it was possible that it was not recognized by the *E. coli* translation machinery. Therefore the alkaline phosphatase signal sequence and the HNP-1 gene were transferred to the pT7-7 vector which contained a strong ribosome binding site that was known to function in *E. coli* (Tabor and Richardson, 1985). This, however, did not result

in the production of any HNP-1 peptide, despite the fact that a transcript that hybridized to a portion of the HNP-1 gene was being produced. These results could be explained by one of two scenarios. First, the peptide was not produced at all, perhaps due to a non functional translational start site. Second, and more plausible, the peptide was produced, but rapidly degraded by proteases in the cytoplasm or periplasm. Indeed, only one report of successful direct expression of a disulfide-containing cationic peptide has been documented, but the isolated peptide possessed no biological activity (Pang et al., 1992).

C. Comparison of Different Fusion Protein Systems.

The initial affinity tag fusion protein system that was used in this study was that of GST. It was chosen since it provided a quick method of fusion protein purification by affinity chromatography and since it had a specific proteolytic cleavage site that allowed for the release of the target protein (Smith and Johnson, 1988). As well, many fusion proteins produced by this system are soluble although some do form inclusion bodies (Smith and Johnson, 1988; Hartman et al., 1992). When HNP-1 was produced as a GST fusion protein (Figure 13), two interesting observations were made. First, most of the protein was found in the insoluble pellet as inclusion bodies. Since inclusion bodies are believed to be formed by the aggregation of improper folding pathway intermediates (Mitraki and King, 1989), it is possible that the addition of the 30 amino acids of HNP-1 to the GST carrier protein interfered with the proper folding of the protein, which resulted in aggregation. On the basis of this argument, however, one would expect that most GST fusion proteins would form inclusion bodies, due to interference by the target sequence in proper folding mechanisms. This, however, is not the case. Another explanation for

the formation of these aggregates is that the cysteine residues of HNP-1 and GST formed incorrect disulfide bonds and thus obstructed the proper folding pathway of the fusion protein that normally leads to a soluble product. This latter explanation is more likely since many cysteine-containing eukaryotic peptides form inclusion bodies in *E. coli* (Fischer et al., 1993). The second observation on the expression of GST/HNP-1 was that the small amount of soluble protein detected was degraded. The presence of a protein band in the sample of soluble, purified fusion protein that comigrated with GST, indicated that the GST moiety retained its proteolysis-resistant nature even when produced as a fusion protein. This further implied that the soluble fusion protein consisted of two distinct protein domains that were differentially susceptible to proteolysis. The fact that some intact fusion protein was still present, suggested that the conformation adopted by the HNP-1 moiety was partially resistant to proteolytic degradation, or that more than one conformation was adopted by the fusion protein.

An attempt was made to increase the stability and solubility of the GST/HNP-1 protein by inserting the prepro defensin gene cartridge at the fusion joint. The anionic prepro region is believed to stabilize the cationic defensin peptide during its synthesis and compartmentalization in the eukaryotic host (Michailson et al., 1992). Therefore it was hypothesized that the presence of the prepro region in GST/proHNP-1 might interact with and protect the HNP-1 peptide from proteolytic degradation. There is evidence that the alteration of amino acid sequences in the region linking the carrier and target proteins can abolish the formation of inclusion bodies (Strandberg and Enfors, 1991). It was possible, therefore, that the inclusion of the prepro region would also enhance the solubility of the fusion protein. The production of GST/proHNP-1 (Figure 19) revealed that the prepro region had no effect on the

solubility of the fusion protein since it was still primarily found in the insoluble pellet. This result showed that the structural features of HNP-1 that contributed to inclusion body formation were unaffected by the prepro sequence. This indicated that the positive charge density of HNP-1, which might have been neutralized in GST/proHNP-1, may not have been a contributing factor in the aggregation process. Alternatively, the prepro region may not have neutralized the positive charges on the HNP-1 peptide. In contrast to the solubility of the fusion protein, the stability of the soluble protein was enhanced by the prepro region. This enhancement could reflect the different conformation adopted by the HNP-1 peptide in response to the presence of the prepro region. It must be emphasized that the effects on stability applied only to the soluble protein since it appeared as though the protein contained in inclusion bodies was completely stable.

The GST system was also used to produce the α-helical peptide CEME (Figure 20). GST/CEME had different properties than GST/HNP-1. First, it was found almost exclusively in the soluble fraction of lysed cells. Since CEME and HNP-1 are similar in size and have similar charge densities, this result provided even more evidence that the cysteine residues played a key role in the formation of GST/HNP-1 inclusion bodies. Second, while purified GST/HNP-1 was found to be partially degraded, GST/CEME was found to be completely degraded. This extensive degradation may have been due to the structure of the CEME moiety. Since CEME has a random coil configuration in an aqueous environment (Wade et al., 1990), it may be present as a peptide appendage protruding out from the proteolysis-resistant GST carrier protein, thus making it highly susceptible to proteolytic degradation by *E. coli* proteases. This is in contrast to HNP-1, whose tight, β-sheet structure may contribute to its partial resistance of cellular proteases.

Using the same logic as for GST/proHNP-1, the prepro defensin gene cartridge was inserted into pGEX-CEME in an attempt to stabilize the soluble fusion protein. Unlike GST/proHNP-1, the production of GST/proCEME (Figure 21) revealed that the prepro region had no effect on the stability of the fusion protein. The inability of the prepro region to protect CEME from proteolysis could be a reflection of the ultrasensitivity of the CEME peptide to cellular proteases. Part of the problem may lie in the prepro sequence itself, since it was derived from mammalian defensins. In contrast to the pro region of defensins, the cecropin A pro region is quite short and is charge neutral (Gudmundsson et al., 1991). It is possible, therefore that a cecropin-like peptide such as CEME is unable to interact with a defensin pro region in a way that would protect it from proteolytic degradation. The other interesting observation in the production of GST/proCEME was that it formed inclusion bodies. In an indirect way, this was an advantage since the inclusion bodies provided a source of stable GST/proCEME that could be purified. Nonetheless, since GST/CEME was able to fold properly into a soluble product, the prepro region must have interfered with that process in GST/proCEME. In contrast, the formation of GST/proHNP-1 inclusion bodies could not be attributed directly to the presence of the pro region since the GST/HNP-1 fusion protein already formed inclusion bodies.

An interesting result was obtained when the GST fusion protein system was used to produce the CEMA peptide (data obtained by Dr. M. Brown in our laboratory). The GST/CEMA fusion protein was found to be both soluble and stable when purified on glutathione agarose beads. This is interesting when compared to CEME which differs from CEMA by three amino acids and two positive charges at the C-terminus. Taken by itself, this result suggests that

there are only fine lines that separate insolubility from solubility, which can determine whether a fusion protein is stable or unstable.

The other affinity tag fusion protein system that was used in this study was that of protein A from *S. aureus* (Nilsson et al., 1985a). The vector chosen for this study was pRIT5 since it could be propagated in both *E. coli* and *S. aureus*. When *E. coli* was used as a host to produce PA/CEME, no stable protein was detected (Figure 24). This instability was also observed when the same vector was used to produce human IGF-I in *E. coli* (Nilsson et al., 1985b; Moks et al., 1987a). Recently, shorter protein A fragments and synthetic fragments that maintained IgG binding capabilities were used as affinity tags. Fusion protein produced in these systems have been shown to leak into the external medium when grown in *E. coli* (Abrahmsén et al., 1986; Moks et al., 1987b). These vectors were not used in the present study, but were no more successful when used by Dr. M. Brown in our laboratory.

A number of different cationic peptides, including CEME, CEMA, and HNP-1 were produced in *S. aureus* using the pRIT5 system. The peptides were exported to the culture supernatant in a stable, soluble form (Figure 24). Some degradation products were seen in samples of PA/CEME, but this has been shown to occur with other fusion protein produced by this system in *S. aureus* (Nilsson et al., 1985a; Moks et al., 1987a).

The production of cationic peptides as fusion proteins has a number of advantages. First, purification can be performed in a single step when the carrier protein is an affinity tag (Sassenfeld, 1990). Second, the presence of a fusion molecule can prevent the antibacterial peptide from being active against the host organism (as was shown for PA/CEME, Figure 27). Third, the heterologous protein can be used to elicit an antibody response without prior conjugation to a hapten (Löwenadler et al., 1987). Fourth, the fusion partner

can be manipulated to improve peptide stability, as seen above in certain instances with the insertion of the defensin prepro region. Fifth, the peptide can be fused to the carrier molecule in such a way that it can be released by chemical or enzymatic proteolytic cleavage without leaving any extra amino acids on the N-terminus (see below). This is important with respect to functional studies using the peptide since it has been shown that the addition of one or more amino acids can alter its biological activity (Bessalle et al., 1992; Pang et al., 1992).

The use of specific fusion protein systems provide additional advantages. The GST system had a high expression level of fusion protein, especially when used in different *E. coli* hosts (Table VII). The problem with this system is its unpredictability with respect to the stability and solubility of the produced fusion protein. This is clearly unacceptable when trying to establish a general expression system that can be used for the production of many different cationic peptides. The protein A system overcomes many of these problems, primarily by exporting the fusion protein out of the cytoplasm. It has generally been shown that protein A fusion proteins directed to the external medium in either *E. coli* or S. *aureus* are both stable and soluble (Nilsson et al., 1985b; Abrahmsén et al., 1986; Moks et al., 1987b). This was not true for the cationic peptide fusion proteins in this study, since only when produced in S. *aureus* were the fusion proteins stable. One drawback to the protein A system is its low expression level. This is in part due to the system's promoter which is not very strong and cannot be induced. Nonetheless, extracellular protein accumulations of 75 μg/mL (Moks et al., 1987b) and 100 μg/mL (this study) can be obtained. These expression levels can undoubtedly be improved upon by manipulating the type of promoter and growth conditions.

D. A New Strategy for Solubilizing Inclusion Bodies.

When insoluble inclusion bodies are formed, there are different methods that can be used to solubilize them (Marston, 1986; Fischer et al., 1993). Traditionally, inclusion body purification procedures consist of a series of solubilization steps that will selectively solubilize the inclusion bodies while leaving many of the contaminating proteins in the insoluble pellet. Such extractions often employ a chaotropic agent or detergent to denature the inclusion bodies. There are several disadvantages associated with this procedure. First, it can be difficult to obtain a pure sample of fusion protein since conditions required to solubilize the inclusion bodies will also bring contaminants into solution (Schoner et al., 1985; Cheng et al., 1990). This is not a problem if the solubilized inclusion bodies are to be further purified by conventional techniques (Jayaram et al., 1989; Cheng et al., 1990). It is, however, a problem when the protein is to be immediately refolded and used for functional studies. In addition, if the solubilized fusion protein is to be cleaved by site-specific proteases, the denaturant must be removed carefully to avoid re-precipitation of the protein (Stein, 1990; Claassen et al., 1991). Second, the solubilization procedure is protein specific and can even vary for a single protein depending on the growth conditions under which the inclusion bodies are formed (Stein, 1989). Therefore, the solubilization conditions for each protein must be empirically determined. Third, due to the heterogeneity of the inclusion body composition, a single solubilization condition will not necessarily dissolve all of the inclusion bodies (Schoner et al., 1985). This was the case for the solubilization procedure of GST/HNP-1 inclusion bodies which resulted in the loss of significant amounts of the protein (Figure 14). Fourth, due to the

harsh conditions necessary to solubilize the inclusion bodies, the fusion protein may be irreversibly modified (Marston and Hartley, 1990).

Theoretically, another method of purifying inclusion bodies would be to solubilize preferentially all the contaminating proteins in the insoluble pellet prior to solubilizing the inclusion bodies. To date, there has been no report of such an approach, possibly because the washing conditions used in the past either solubilize the inclusion bodies, or do not selectively remove the contaminating proteins. This problem was observed with urea in the solubilization of GST/HNP-1. In this study, a novel solubilization scheme was developed for GST/proCEME inclusion bodies based on the observation that the major contaminating proteins in inclusion bodies are outer membrane proteins (Veeraragavan, 1989). The initial washes with Triton X-100, Tris-HCl pH 8.0, and EDTA remained the same as those described for the GST/HNP-1 inclusion bodies. The novel extraction used 3% O-POE, a detergent known to solubilize outer membrane proteins (Siehnel et al., 1992). Analysis of the supernatants and pellets after these extractions revealed that not only were the contaminating proteins removed completely, but the 3% O-POE did not solubilize any of the fusion protein (determined by Western immunoblotting, Figure 21). This procedure resulted in a preparation of GST/proCEME protein that had only minor amounts of contaminating proteins. This method could be applied to any inclusion body preparation, regardless of how they are eventually to be solubilized. For those preparations of fusion proteins that are to be proteolytically cleaved by chemical methods such as CNBr, the inclusion bodies can be solubilized directly in 70% formic acid since the preparation is relatively pure. One of the key advantages of this solubilization protocol is that it avoids the loss of fusion protein through premature solubilization.

E. Comparison of Fusion Protein Cleavage Methods.

In many instances, a purified fusion protein must be specifically cleaved to release the target protein of interest. Two different cleavage methods were used in the study, one enzymatic (factor X_a) and one chemical (CNBr).

Solubilized GST/HNP-1 inclusion bodies were cleaved with factor X_a to release HNP-1 without any extra N-terminal amino acids (Figure 15). The release of HNP-1 by factor X_a at 4°C did not occur, even though factor X_a has been shown to be active at this temperature (Ellinger et al., 1989). Even at temperatures as high as 37°C, the reaction required 60 h and an enzyme to substrate ratio of 1:25 to cleave the protein. Conditions similar to these have been shown to be necessary for other fusion proteins (Nambiar et al., 1987; Baldwin and Schultz, 1989), but in other cases, the conditions were much milder (Nagai and Thøgersen, 1984; Ellinger et al., 1989). This suggests that the accessibility of the cleavage site varies between fusion proteins, and that this may have contributed to the inefficient cleavage of GST/HNP-1. Furthermore, it may have been the aggregation of the fusion partner and the peptide that was preventing the factor X_a enzyme from reaching its target sequence. In addition to the inefficiency of the reaction, some non-specific cleavage of the fusion protein occurred (Figure 16). Although the factor X_a cleavage site has a consensus sequence of Ile-Glu-Gly-Arg, there have been reports of other sequences at which cleavage can occur (Carter, 1990; Greenwood, 1993). In all of these examples, the cleavage site was on the C-terminal side of an arginine residue. The proteolytic cleavage of GST/HNP-1 resulted in three distinct peptide products. Upon further analysis, it was discovered that these peptide fragments corresponded to internal fragments of GST. Based on N-terminal amino acid sequence analysis, it was determined

that the cleavages giving rise to these fragments occurred after specific arginine residues. The four amino acid residue sequences preceding these cleavage sites, Asp-Lys-Trp-Arg and Ala-Ile-Ile-Arg, had not previously been reported as factor X_a recognition sites (Carter, 1990; Greenwood, 1993). In GST that had been previously denatured and renatured, these same two sites were susceptible to factor X_a cleavage (Figure 18), suggesting that these internal sites were made accessible to factor X_a as a consequence of the inability of GST to refold properly under these conditions. It is interesting to note that there are many other arginine residues in the GST amino acid sequence that were not cleaved to any large extent by factor X_a, indicating that even the "non-specific" cleavage was not random. Due to the inefficient and the expensive nature of the factor X_a cleavage reaction, this was not the method of choice for the production of large quantities of cationic proteins. Furthermore, the biggest advantage of enzymatic cleavage, namely its high specificity, could not be utilized in the purification of HNP-1 from GST/HNP-1.

The gene encoding CEME was preceded directly by a methionine codon which enabled the release of the peptide from a fusion protein by CNBr cleavage. Biologically active CEME was released from both GST/proCEME and PA/CEME fusion proteins (Figures 22 and 27). The major disadvantage of using CNBr on large fusion proteins is the generation of other peptide fragments due to the presence of methionine residues in the carrier molecule. Unlike the recognition site for factor X_a, the methionine residues are rarely inaccessible since CNBr is a small chemical molecule and the reaction is carried out in conditions under which most proteins are denatured. This problem was evident in both fusion protein systems since GST (Smith et al., 1986) and protein A (Uhlén et al., 1984; Nilsson et al., 1985a) both contain several methionine residues. The difficulty of purifying the target protein from a CNBr

digest depends on the size and pI of the various peptide fragments produced by the reaction. In the GST/proCEME digest, such peptide fragments made further purification of the cationic peptide impractical. In contrast, all the contaminating peptide fragments in the PA/CEME digest could be separated from CEME by gel exclusion and reverse phase chromatographies. Since this cleavage method is both inexpensive and efficient, it can be used in a large scale process, as described for PA/CEME.

F. Do Cationic Peptides Cross the Outer Membrane via the Self-Promoted Uptake Pathway?

The self-promoted uptake model was originally proposed as a mechanism of uptake for polycationic antibiotics such as polymyxin B and gentamicin (Hancock, 1984). It has been shown that peptides of the defensin family were able to bind LPS (Sawyer et al., 1988) and permeabilize outer membranes of various bacteria (Sawyer et al., 1988; Lehrer et al., 1989), two activities that are observed for compounds proposed to cross the outer membrane via this pathway. The hypothesis that cationic peptides are taken up via the self-promoted uptake pathway was strengthened by the fact that several different cationic peptides such as defensins (Sawyer et al., 1988), magainins (Rana et al., 1990) and melittin (David et al., 1992) are able to interact directly with LPS. Furthermore, the ability to permeabilize outer membranes has been demonstrated for various defensins (Sawyer et al., 1988; Lehrer et al., 1989) as well as bactenicins (Skerlavaj et al., 1990). There have been, however, no studies to elucidate the interaction of cecropins and melittin with the outer membrane, despite their documented Gram-negative bactericidal activity (Wade et al., 1990). Therefore, this study set out to obtain evidence to support the

hypothesis that these cationic peptide antibiotics cross the outer membrane permeability barrier by promoting their own uptake.

The self-promoted uptake of a compound is proposed to be initiated by its interaction with the negatively charged sites on LPS molecules that are normally occupied by Mg^{2+} ions which form stabilizing cross bridges between adjacent LPS molecules. Using dansyl polymyxin B as a probe, the LPS binding capabilities of CEME, CEMA and melittin were examined and compared to those of other cations including polymyxin B, which has been shown to bind very tightly to LPS (Moore et al., 1986). Indeed the cationic peptides all showed high binding affinities for pure LPS (Figure 33) or LPS in the context of the whole cell environment (Figure 34). The calculated I_{50} values even showed that with whole cells, all the cationic peptides could displace dansyl polymyxin B more readily than polymyxin B. This, however, may not reflect the affinities of the compounds for LPS per se, but rather the accessibility of the sites to the different compounds. For instance, since the α-helical peptides consist mainly of random coil in an aqueous environment, they may more easily penetrate a slightly perturbed membrane (due to the bound dansyl polymyxin B) than polymyxin B, which has a constrained, cyclic structure. Regardless, these experiments clearly demonstrated the ability of these peptides to bind LPS.

These studies also provided evidence that the peptides were binding to the divalent cation binding sites on the LPS. First, dansyl polymyxin B that was displaced from LPS by the cationic peptides could also be displaced by Mg^{2+}, which was consistent with a common binding site for all of these compounds. Second, the presence of Mg^{2+} in MIC assays rendered the bacteria more resistant to the action of cationic peptides (Table X), presumably due to its saturation of divalent cation binding sites and the resulting stabilization of the outer membrane. Third, the ability of the cationic peptides to permeabilize the

outer membrane to compounds such as lysozyme was inhibited by Mg^{2+}. Taken together, these data suggest that the initial interaction of cationic peptides with Gram-negative bacteria occurs at the divalent cation binding sites on the LPS.

In the self-promoted uptake model, the binding of cationic compounds to the LPS is proposed to accompany a localized destabilization of the outer membrane. It has been shown in this study that CEME, CEMA and melittin all have the ability to permeabilize the outer membrane of *P. aeruginosa* and *E. cloacae* to lysozyme and NPN (Figures 29-32). In fact, under some conditions CEMA and CEME are better permeabilizers that polymyxin B, whose potent outer membrane disrupting ability has been well documented (Vaara, 1992). This permeabilization of the outer membrane, although necessary for self-promoted uptake, is not sufficient. The molecule must be able to dissociate from the LPS to which it is bound and move through the disrupted outer membrane into the periplasm. This may occur as a result of changes in the structure of LPS or the peptide itself, once the outer membrane has been destabilized. It is very difficult to examine the nature of such post-destabilization events, but some indirect evidence is available which supports the hypothesis that these peptides are, in fact, taken up. First, studies have shown that defensins (Lehrer et al., 1989) and bactenicins (Skerlavaj et al., 1990) sequentially permeabilize outer and inner membranes. Second, many peptides have been shown to form ion channels in planar lipid membranes (Hanke et al., 1983; Christensen et al., 1988; Kagan et al., 1990) which led to the hypothesis that the disruption of cytoplasmic membrane integrity was the cause of cell death (Christensen et al., 1988; Lehrer et al., 1989). Third, studies on the interaction of melittin with lipid A revealed that when bound to LPS, melittin had no hemolytic activity (David et al., 1992). This suggested that

melittin must be released from the LPS before it can exert its bactericidal activity, and therefore it must be taken up into the periplasm. These data, together with the findings that CEME, CEMA and melittin caused outer membrane permeabilization at concentrations that are not bacteriolytic, suggest that the disruption of the outer membrane is not sufficient to kill Gram-negative bacteria, and that killing requires both uptake and further action of the peptides. The evidence provided in this thesis strongly implies that these cationic peptides are taken up by self-promoted uptake.

Self-promoted uptake involves certain basic steps that all compounds taken up by this pathway are thought to go through. This thesis provides evidence that different compounds proceed through these steps in various ways. For example, CEME and CEMA showed different permeabilizing properties when compared to polymyxin B and melittin. It seems reasonable that a more profound disruption of the outer membrane would be required to permeabilize it to lysozyme (a 14 kDa protein) than to NPN (a small hydrophobic chemical). While all four compounds, at low concentrations, enabled NPN to enter the membrane environment (Figure 31), only CEME and CEMA could promote the uptake of lysozyme at those same concentrations (Figure 29). Polymyxin B and melittin, on the other hand, required higher concentrations for this, suggesting that they could only induce major outer membrane disruptions at high concentrations. While this may not be surprising for polymyxin B, given its different secondary structure, it is for melittin, since it has a similar secondary structure to CEME and CEMA. The only difference in secondary structure that the latter two peptides may have compared to melittin, is an additional hinge region, but this has not been confirmed and therefore remains speculative. It is also interesting to note that the concentrations of melittin required to permeabilize the outer membrane to lysozyme, were only slightly

lower than those that lysed cells (Figure 29). This implies that the rate-limiting step in the bactericidal mechanism of melittin was its ability to cross the outer membrane permeability barrier. This has also been suggested for polymyxin B (Vaara and Vaara, 1983) as well as human (Viljanen et al., 1988) and rabbit (Sawyer et al., 1988) defensins. In contrast, CEMA was able to permeabilize the membrane to lysozyme at concentrations >10-fold lower than its MIC, demonstrating that it could breach the permeability barrier relatively easily, but could not kill cells unless a sufficient concentration accumulated in the periplasm. Therefore, while all these cationic compounds are proposed to be taken up by the self-promoted uptake pathway, differences exist in their interactions with the outer membrane.

G. Cationic Peptides As Therapeutic Agents.

Cationic peptides have been referred to as "natural peptide antibiotics", reflecting their broad range of antimicrobial activity. Work in the last few decades has focused on isolating and characterizing dozens of different peptides in an effort to understand how they function. As this understanding increases, a logical extension of this work is to begin to develop them as therapeutic agents that can be used to combat various infectious diseases.

One property common to many of the cationic peptides that prevents them from being used as antibiotics, is their cytolytic activity against mammalian cells. Melittin has been shown to have potent hemolytic activity (Habermann and Jentsch, 1967; Wade et al., 1990), and defensins demonstrate a broad range of cytotoxicity against mammalian cells (Lehrer et al., 1993). In an attempt to identify structural regions within proteins that are responsible for cytolytic activity, Kini and Evans (1989) compared the sequences of over 30

cytolysins. They concluded that a cationic site flanked by a hydrophobic region was a common feature to all these proteins. This conclusion, however, remains too general to aid in the design of antibiotic peptides without cytolytic activity. More helpful have been the studies in which analogues of various peptides have been synthesized that maintain high antibacterial activity but do not possess hemolytic activity (Boman et al., 1989a; Wade et al., 1990; Andreu et al., 1992; Bessalle et al., 1992; Wade et al., 1992).

Another problem that these peptides may encounter is proteolytic degradation inside the host. One potential solution to this problem is to make D-enantiomers of the peptides which would increase their resistance to host proteases resulting in a longer *in vivo* half-life. It has already been shown that D-forms of certain antibacterial peptides are as biologically active as their naturally occurring L-form counterparts (Wade et al., 1990). One drawback to this solution is that the D-forms of the peptides could not be produced in a bacterial expression system. Other solutions could include the alteration of flanking amino acids, which can influence proteolysis, or the encapsultion of the peptide in either liposomes or polymers to protect it during delivery. An alternative solution to the problem of peptide degradation would be to concentrate on the β-sheet, disulfide bond-containing peptides, since their tightly packed tertiary structure renders them quite resistant to proteolytic degradation (Fujii et al., 1993).

Another aspect that must be addressed before the peptides can be used as therapeutic agents is their size. There are a number of reasons why it is of interest to develop peptides that are smaller. First, the marketing of the peptides would be enhanced if their molecular weight was in the range of other conventional antibiotics. For example, CEMA, with a molecular weight of 2800 daltons, is almost double that of polymyxin B. Second, a smaller peptide may

have improved solubility properties, which could have ramifications for delivery and bioavailability in clinical trials. Third, when creating analogues of a certain peptide in order to improve it, the task is significantly simplified by starting with a shorter parent peptide. Therefore, one of the challenges of designing these compounds would be to decrease the size of the peptides without compromising antimicrobial activity. Andreu et al. (1992) investigated how many C-terminal amino acids could be deleted from CEME without affecting the bactericidal activity. They managed to bring the length of the peptide down to 18 amino acids (from 26) with no significant loss of antibacterial activity or increase in hemolytic activity and suggested that this was possible because the flexible hinge region between the two α-helices was retained. When the deletions brought the peptide down to 15 amino acids, there was a marked increase in the lethal concentration against certain bacteria. It was suggested that this hinge containing pentadecapeptide no longer had the ability to span the apolar portion of the membrane and form ion channels (Andreu et al., 1992). The antibacterial activity of other 15 mers that did not have the flexible hinge region was almost as good as the 18 and 20 mers. This suggested that either, (1) the peptide formed a 3_{10} α-helix that was able to span the membrane, (2) the peptide associated as multimers that were able to span the membrane, or (3) these short peptides have a mechanism of action that has not yet been adequately described (Andreu et al., 1992; Wade et al., 1992). It remains abundantly clear that reducing these peptides to a therapeutically useful size cannot be done by indiscriminately deleting residues from an active peptide without considering the structural ramifications.

Another focus of future research may be to design peptides with improved specific activity (LPS binding, membrane permeabilization, channel formation), which could be used in conjunction with conventional antibiotics.

An example of this is PMBN, which is not antibacterial (MIC >100μg/mL), but possesses potent membrane permeabilizing activity that can increase the sensitivity of various Gram-negative bacteria to different antibiotics (Vaara, 1992). This thesis has described a CEME variant, CEMA, which has a 2-5-fold increase in permeabilizing activity as compared to CEME. Synergy studies with CEMA and hydrophobic antibiotics showed that the peptide was unable to enhance bacterial susceptibility to these antibiotics. This could be because the MIC of CEMA is relatively low and since concentrations required to open the membrane to hydrophobic antibiotics would kill the cell, the permeabilizing effect would be masked. This phenomenon has previously been documented for polymyxin B (Vaara and Vaara, 1983). It would be interesting to create shortened CEMA analogues and examine whether they can retain the membrane permeabilization activity of CEMA regardless of the effects on antibacterial activity. By doing this, it may be possible to derive a peptide that is short enough to use as a therapeutic, and yet still be useful in conjunction with other antibiotics. Indeed, there is a precedent for *in vitro* synergy between cationic peptides and conventional antibiotics. Darveau et al. (1991) found that sub-inhibitory concentrations of β-lactam antibiotics such as cefepime were able to potentiate the activity of magainin-2 (or vica versa) against *E. coli in vitro* and in an *in vivo* mouse model. Magainins have also been shown to have a synergistic effect with erythromycin on *P. aeruginosa* (MacDonald et al., 1991). In light of such studies it seems very possible that the next few decades will see the emergence of cationic peptides as boni fide therapeutic agents.

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