Title
The membrane insertion and transmembrane topography of Colicin Ia

Author
Mel, Stephanie F.

Publication Date
1992

Peer reviewed|Thesis/dissertation

The Membrane Insertion and Transmembrane Topography of Colicin Ia

by

Stephanie F. Mel

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pathology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

To the members of my family who were with me all the way -- my Mother and Father, Amelie, Bartlett, Maria, and Partho

ACKNOWLEDGEMENTS

Graduate school would have been infinitely more difficult than it was without the help, friendship and encouragement of a number of people. A most important source of support and camaraderie came from the members of the Stroud lab, with whom I shared lab space, scientific discussions, much laughter, tales of woe, and the occasional glass of wine. It never ceases to amaze me that 20 or so people could co-habitate in close quarters such as we did, week after week, month after month, yet continue to get along so well, linked by a common sense of humor and of the absurd. I will truly miss the current members of the lab who have made my day to day existence, especially at the end, not only bearable but fun - Ramu, Alok Mitra, Diana Cherbavaz, Raj, Chris Schafmeister, Anton Krukowski, George Turner, Mary Betlach, Janet Finer-Moore, Earl Rutenber, Louise Chang, Bob Keenan (thanks for making me feel like my opinion really counted for something you moop you), BobRose (I promise I won't yell at you ever again), Eric Fauman (Eric, please turn the music down), Paul Foster (50! without cheating...you are the best big lug that I know); and Michael Wiener (2-3 seconds of milk, but please, err on the side of 3). Special thanks goes to Kathy Perry (KP) for being a great inspiration to all women juggling families and careers - (and here's to the good old days, when WE used to be the livewires around here). Special thanks also to Eric Fauman who answered COUNTLESS computer questions. Rich Fallon has vastly

improved the quality of all of our lives, with his world-class sense of humor that was frequently translated into masterful skits and songs. I will especially miss Mel Jones and Doug Freymann for all sorts of things, but mostly for just being Mel and Doug. Finally, the members of the Agard and Fletterick labs as well as the other 10th floor residents have greatly enlivened my graduate experience.

Larry Miercke gets his own paragraph - he probably should get his own chapter. I cannot thank Larry enough for his tremendous assistance with both the theoretical as well as practical aspects of column chromatography, and most importantly, for always being willing and ready to help with a giant smile on his face. Every lab in the world needs a Larry Miercke.

Several people who have left the lab in recent years deserve special mention. The lab was a very special place when Michael Shuster was there, largely due to his warm personality and his unbelievable tenacity that could not help but inspire us all. He has been greatly missed by me, and by others. Thomas and Julie Earnest (no relation) were also mainstays in the lab a few years ago, and I am grateful that my friendship with both of them has continued. When Sasha Kamb arrived in the lab, both my scientific and personal life became greatly enriched, (and not just because I like cowboys and the wild west). Sasha has the wonderful ability to engage just about anyone in conversation, on just about any subject. As I am fond of conversation myself, it was a perfect match. Many of my best memories of graduate school are of our afternoons together in

Moffitt cafeteria, contemplating such things as the transmembrane topography of colicin Ia, presidential politics, bicycle routes, life, love, and the pursuit of happiness (not necessarily in that order).

In addition to the Stroud lab, several other people at UCSF made my life as a graduate student much more pleasant indeed. In particular, the weekly Breakfast Club gatherings, which usually included Caroline Goutte, Dana Smith, Evi Strauss, Debbie Zimmerman, Sigrid Reinsch, Jill Heemskirk and Delia Lakich were something I always looked forward to, despite the hour at which I had to awaken to get there on time. Knowing that the ups and downs of graduate life that I was experiencing were more often than not the same ups and downs that others were experiencing was of great solace to me. Plus, it was always fun. The offshoot group of Research Club - Evi, Debbie, Sigrid, and myself - was one of the most valuable experiences of graduate school to me, both scientifically as well as personally. Not only did I learn a tremendous amount about fields other than my own, but I learned it all in an environment in which I could ask stupid questions to my heart's content. I am SO glad that Debbie decided to have Valentina, and to take a few months off so that we could continue to see each other on a regular basis while I was finishing up. I especially appreciate the fact that Debbie too worked on membrane proteins -- she helped me to put my graduate work, and all of life for that matter, into the appropriate context and focus in her infinite and calm wisdom. Also, Cindy Keleher Kruger deserves very special

thanks, for without her, I would not have survived medical school Physiology. If you ever need a tutor at the last minute with the patience of a saint, Cindy is the one to go find.

Evi Strauss was my true kindred spirit in graduate school. Not only did she understand each and every one of my many and varied moods in gory detail, but usually, she would relive every one of them herself at one time or another. I so appreciated being able to run downstairs, tell Evi the latest calamity in my life and have her break into the world's greatest smile and say "I just had the same thing happen to me!" Life around here is not the same with Evi off in France -- thank god for Fax machines and telephones.

Other UCSF friends played an important role in my life here. Mark Grimes and Gretchen McCaffrey (and now Lindsey and Shannon!) have been great friends and stabilizing influences. Our earlier years together with Henrik Semb and Judy White and Sam Green and monday night dinner club were wonderful times, and welcome breaks from the lab. I do miss the days of my runs with Judy, though now my knees and her baby leave us both doing other forms of exercise.

My pre-graduate school years at UCSF must not go unmentioned. If my time as a technician in the Cancer Research Institute had not been so fruitful, I surely would not have continued down this path. I learned many things from many people - Narla Mohandas, Tim Heath, Larry Corash, Mary Rossi, Steve Shohet, Dimitri Papahadjopoulos, Paul Meers, Nejat Duzgunes, Bob Straubinger. I would especially like to acknowledge my friend

Margaret Clark, who continues to be a great friend and constant inspiration, and who ALWAYS seems to understand me.

Very special thanks to Arnie Falick who taught me all about chromatography, mass spectrometry, and who provided the perfect balance to my otherwise less-than-balanced personality. I will miss all of the hours that we worked together.

Thanks also to Dan Friend, who has always been a Friend and a supporter.

While it is hard to recall at this stage, there were times during graduate school when I had a life, with friends on the outside. Several of them deserve special mention -- firstly, members of the Rumsey family -- GEORGE, I DID IT! -- I know you always believed that I would before the age of 40, and only really had a problem with my shoes. Mary and Jim have been wonderful friends through all of this -- I truly appreciate that they really CARE that a molecule is smaller than a bread box. I would also like to thank Sandy Still for always being there for me and for getting me hooked on bike riding, which contributed greatly to my sanity on a number of occasions. All of the members of my family also deserve special thanks for their understanding of my many and varied mood swings. Last but not least, graduate school would have been impossible were it not for the constant help and support of Partho. He has been my true best friend and I couldn't have done it without him.

I would finally like to thank my advisor, Bob Stroud. He has taught me the value of critical, quantitive thinking, as well as how

to have a great time. I feel completely prepared to tackle new scientific problems, no matter how challenging they might be. I will remember my days in the Stroud lab with great fondness for years to come.

THE MEMBRANE INSERTION AND TRANSMEMBRANE TOPOGRAPHY OF COLICIN Ia

Stephanie F. Mel

ABSTRACT

The bacterial toxin colicin Ia is a member of the family of ion-channel forming colicins. These proteins kill by inserting into the inner plasma membrane of a target *E. coli* and subsequently depleting the cell of its electrochemical gradient. In the following work, the membrane insertion and transmembrane topography of colicin Ia have been investigated. Colicin Ia inserts only into negatively charged membranes and only at acidic pH, as determined by fluorophore leakage from liposomes. This insertion is not accompanied by a secondary structural change, but does require an alteration of protein tertiary structure, as measured by circular dichroism, proteolysis, and tryptophan fluorescence. Furthermore, membrane insertion follows single-hit kinetics. Specific regions of colicin Ia become protected from proteolysis after protein association with negatively charged membranes. Protected regions include the C-terminal channel-forming domain, as well as a portion of the putative N-terminal translocation domain. These protected regions thus define those portions of colicin Ia that are closely membrane associated, and are likely involved in ion channel-formation. Coupled with the recently solved x-ray structure of the

soluble form of colicin Ia (Partho Ghosh, Ph.D. thesis UCSF, 1992), these data provide new insight into how proteins are able to exist in both water soluble as well as a transmembrane environments.

TABLE OF CONTENTS

LIST OF TABLES

exclusive of Chapter 3, Part 1

LIST OF FIGURES

exclusive of Chapter 3, Part 1

CHAPTER 1

INTRODUCTION

Toxins are defined as proteinaceous poisonous substances that are secreted by a microbe and are capable of causing a pathological condition. Many toxins are directly pathogenic to humans, wreaking havoc first at the cellular and ultimately at the organismal level. In the 1870's and 1880's, it was first predicted that pathogenic bacteria might secrete toxic, soluble substances. The first evidence for this (albeit indirect) came in 1884, when Loeffler discovered that lesions from fatal cases of diphtheria, while widely spread throughout the body, were always sterile. The bacillus itself could only be isolated from the primary lesion in the throat (Loeffler, 1884). Four years later, Roux and Yersin demonstrated that by injecting sterile filtrates from diphtheria bacillus cultures into animals, they could mimic both the symptoms as well as the manner of death that normally arose from infection with live bacteria (Roux and Yersin, 1888). This prompted researchers to look for additional soluble toxins that were released from other pathogenic bacteria, and in 1890, two groups simultaneously discovered the toxin from the organism *Clostridium tetani,* which causes tetanus (Brieger and Frankel, 1890; Faber, 1890). The final proof that some toxin molecules were indeed the causative agents of disease came in 1890, when von Behring and Kitasato demonstrated that diphtheria and tetanus antitoxins were protective against the lethal effects of the parent toxin (von Behring and Kitasato, 1890).

Several of the toxins which cause human disease have been well characterized at both a biochemical as well as a structural

level: diphtheria toxin from *Corynebacterium diphtheriae*, the causative agent of diphtheria; cholera toxin from *Vibrio cholerae*, which leads to the debilitating disease of cholera; heat labile enterotoxin from enteropathogenic strains of *E. coli*, which causes severe diarrhea, and others. In addition to toxins which act on mammalian cells, there exist toxins whose targets are instead other bacteria. Among these are a family of colicins (kill *E. coli*), proteins which are secreted from one species of *E. coli* and kill members of other species of *E. coli*. The killing action of these bacterial toxins was observed as early as 1952 (Jacob, et al., 1952).

One of the most interesting and unusual features of the toxin molecules is their ability to exist as both water-soluble as well as transmembrane proteins. In effecting their lethal hit on a target cell, toxins will insert into cellular membranes from an aqueous environment. In the case of diphtheria toxin, for example, the low pH environment of the endosomal compartment triggers conformational changes in both the A and B subunits of this protein. This leads to the insertion of the B subunit into the endosomal membrane, as well as the translocation of the A subunit across the membrane and into the cytosol. Within the cytosol, the A-chain ADP-ribosylates and thereby inactivates elongation factor 2, which ultimately leads to the inhibition of protein synthesis (Neville and Hudson, 1986). In another case, the toxin secreted by the bacteria *Vibrio cholerae* first binds to the ganglioside G_{M1} receptor and subsequently inserts into human gut epithelial cell membranes. Like

diphtheria toxin, the A-subunit then enters the cytosol where it is a potent adenylate cyclase activator -- the increased levels of cAMP lead to debilitating, and frequently deadly diarrhea (Holmgren, 1981) Not all toxins act enzymatically in the cell. Instead, several of these human toxins form ion-conducting channels within their target cell membrane. Both tetanus and botulinum toxins form channels in cell membranes (Bouquet and Duflot, 1982)(Donovan and Middlebrook, 1986) and indeed, it has been found that the B-subunit of diphtheria toxin forms an ion-conducting pore (Donovan, et al., 1981).

Similarly, certain colicins act enzymatically, and others act by forming ion-conducting channels in their target membranes. Colicins E2 and E3 have RNase and DNA endonuclease activity respectively, while colicins A, E1, Ia, Ib, K, and N form ion channels. These channel-forming colicins first bind to cell surface receptors on the outer membrane of sensitive *E. coli.* (A sensitive strain is defined as one which does not harbor an immunity protein to the attacking colicin molecule.) After traversing the periplasmic space, they insert into the bacterial cytoplasmic membrane, ultimately depleting the cell of its electrochemical gradient and causing cell death (reviewed in (Cramer, et al., 1990) (Pattus, et al., 1990)). One of three linearly arranged domains is responsible for each of the three functions: receptor binding, translocation, and channel-formation. As the colicins are plasmid encoded, easily purified, and available in large amounts, they are therefore excellent tools with

which to study both membrane insertion as well as ion-channel function.

The study of channel-forming colicins has focused primarily on colicins A and E1 in recent years, and more specifically on their proteolytically derived C-terminal channel-forming domains. A large body of data has accumulated on the voltage-dependence, gating characteristics, selectivity, and molecularity of these channels, largely from studies in planar lipid bilayers (reviewed in (Slatin, 1988). More recently, focus has shifted to studying the mechanism of membrane insertion, guided by insights gained from the recently solved x-ray structure of the colicin A channel-forming fragment (Parker, et al., 1989). While the channel-forming domain of the related protein colicin Ia is clearly of interest, the Stroud lab chose to focus instead on the structure and function of the entire molecule.

Previously, Jordon Konisky and colleagues had worked out a purification scheme for colicin Ia and had determined that it killed sensitive strains of *E. coli* by forming an ion channel in the inner plasma membrane of the target cell (Konisky, 1982). While on sabbatical in Herb Boyer's lab at UCSF, Konisky cloned colicin Ia and it's corresponding immunity protein into pBR322, with the resultant plasmid pJK5 (Weaver, et al., 1981). We were fortunate that this plasmid remained at UCSF, and that we were able to use it for the ensuing studies.

Crystals of colicin Ia were grown by Nancy Helmers and Susan Hershenson, with protein that had been purified from pJK5. Seunghyon Choe, a graduate student in the Stroud lab, determined that these crystals were in a space group of $C222_1$, that they diffracted to 3.4 Å resolution, and that they were approximately 79% water (Choe, 1987). In the electron microscope, crushed crystals of colicin Ia appeared to grow in a honeycomb-like pattern (Choe, 1987). Based on this pattern, coupled with the known number of 8 colicin Ia molecules/unit cell, Choe and Stroud proposed that colicin Ia was a Y-shaped molecule, with each arm of the Y representing one of the three functional domains.

It was at this stage that I joined the lab. I was poised to begin asking questions about the function of colicin Ia, and how we could correlate function to the structural information available at that time. I discovered colicin Ia as a frozen pellet in the deep freeze, and thus this story begins...

Over the years, I studied many aspects of colicin Ia, beginning with defining the conditions under which it would insert into a membrane. I found that colicin Ia inserts only into membranes made from negatively charged lipids and only at acidic pH values (see Chapter 2). At the same time, Partho Ghosh and I were able to measure single-channel recordings of colicin Ia , showing that in planar bilayers it exhibited voltage dependence and multiple conductance states that ranged between 10-30 pS (see Appendix 1). When Cynthia Wolberger arrived in the lab to study colicin Ia,

together we worked out a new scheme that vastly simplified the purification, shortening it to one centrifugation, one ion-exchange column, and one size-exclusion HPLC column (see Chapter 2). With large quantities of purified protein in hand, I began a series of circular dichroism experiments aimed at determining any secondary structural changes which might accompany the membrane insertion of colicin Ia (see Chapter 2).

At the same time, I began a fruitful collaboration with Arnie Falick and Al Burlingame of the Mass Spectrometry Facility at UCSF, wherein we sought to define the transmembrane topography of colicin Ia, using a combination of biochemistry and mass spectrometry. Arnie and I spent many long hours at the HPLC, separating proteolytic fragments of colicin Ia for future mass spectrometric analysis, in an effort to "map" the entire molecule (see Chapter 3, Part 1). Our goal was to then label, with a radioactive probe, those portions of the protein which traversed the membrane , and to determine where these labelled portions mapped to the colicin sequence. While the radioactive probe 3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) did successfully label colicin Ia in it's membrane bound configuration (Chapter 3, Part 2), the labelling was not of great enough efficiency to define the transmembrane sequences. As an alternate approach to identifying the transmembrane sequences, I then began using the protease pepsin, added to the outside of lipid vesicles, to define those regions of colicin Ia that were protected by the membrane.

These experiments resulted in the definition of several discrete "protected" fragments of colicin Ia, which together begin to define the topography of the membrane-associated conformation of colicin Ia in asolectin membranes (see Chapter 4).

In addition to mapping the topography in asolectin liposomes, I embarked on a series of experiments aimed at mapping the topography of colicin Ia in liposomes made from *E. coli* lipids as well (Chapter 5). Due to problems experienced with sample heterogeneity in the experiments described in Chapter 4, I attempted to separate peptides protected in *E. coli* membranes using column chromatography rather than gel electrophoresis. This ongoing work is described in Chapter 5.

Concomitant with this work, Partho Ghosh solved the x-ray crystal structure of colicin Ia to 3.4 Å resolution. Although the electron density is not of sufficiently high resolution to allow the placement of amino acid side chains, the structure is found to be Y-shaped, and composed of three separate domains. With the eventual assignment of amino acids to the protein density, we will be able to interpret results from the topography mapping experiments in the context of this structure. For the first time, structural information will exist for both the soluble as well as the transmembrane forms of a channel-forming colicin. This should greatly enhance our understanding of how these molecules carry out a dual existence as both water soluble as well as transmembrane proteins.

REFERENCES

Bouquet, P. & Duflot, E. (1982) *Proc. Natl. Acad. Sci. USA 79*, 7614-7618.

Brieger & Frankel (1890) *Berl. Klin. Wochschr. 11.*

Choe, S., Ph.D., University of California, San Francisco, 1987.

Cramer, W. A., Cohen, F. S., Merrill, A. R. & Song, H. Y. (1990) *Molec. Microbiol. 4*, 519-526.

Donovan, J., J. & Middlebrook, J., L. (1986) *Biochem. 25*, 2872-2876.

Donovan, J. J., Simon, M. I., Draper, R. & Montal, M. (1981) *Proc. Natl. Acad. Sci. USA 78*, 172-176.

Faber, K. (1890) *Berl. Klin. Wochschr. 11*, 717.

Holmgren, J. (1981) *Nature 292*, 413-417.

Jacob, F., Siminovitch, L. & Wollman, L. (1952) *Ann. Inst. Pasteur 83*, 295-315.

Konisky, J. (1982) *Ann. Rev. Microbiol. 36*, 125-144.

Loeffler, F. (1884) *Mitt. Klin. Gesundheitsamte 2*, 421.

Neville, D. M. J. & Hudson, T. H. (1986) *Ann. Rev. Biochem. 55*, 195-224.

Parker, M. W., Pattus, F., Tucker, A. D. & Tsernoglou, D. (1989) *Nature 337*, 93-96.

Pattus, F., Massotte, D., Wilmsen, H. U., Lakey, J., Tsernoglou, D., Tucker, A. & Parker, M. W. (1990) *Experientia 46*, 180-192.

Roux, E. & Yersin, A. (1888) *Ann. Inst. Pasteur 2*, 629.

Slatin, S. L. (1988) *Int. J. Biochem. 20*, 737-744.

von Behring & Kitasato (1890) *Deut. Med. Woch. 16*, 1145, 1245.

Weaver, C. A., Redborg, H. & Konisky, J. (1981) *J. Bacteriol. 148*, 817-828.

CHAPTER 2

COLICIN Ia INSERTS INTO NEGATIVELY CHARGED MEMBRANES AT LOW pH WITH A TERTIARY BUT LITTLE SECONDARY STRUCTURAL CHANGE

Stephanie F. Mel and Robert M. Stroud

submitted to *Biochemistry*

ABBREVIATIONS:

ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; β-OG, octyl-β-D-glucopyranoside; BME, 2-mercaptoethanol; CD, circular dichroism; CL, cardiolipin; DPX, p-xylylenebis[pyridinium bromide]; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; O.D., optical density; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Tris, [Tris(hydroxymethyl)aminomethane].

ABSTRACT

Colicin Ia, a member of the channel-forming family of colicins, inserts into model membranes in a pH- and lipid-dependent fashion. This insertion occurs with single-hit kinetics, requires negatively charged lipids in the target membrane, and increases in rate as the pH is reduced below 5.2. The low pH requirement does not act by inducing a secondary structural change in colicin Ia, which remains 66% +/- 4% α-helical between pH's 7.3 and 3.1 as determined by circular dichroism. The secondary structure also remains unchanged between pH's 7.3 and 4.2 in the hydrophobic environment provided by the detergent octyl-β-D-glucopyranoside (β-OG). However, at pH 3.1 in the presence of β-OG, an 11% +/- 3% decrease in the α–helical content is observed. Further, β-OG induces a change in tryptophan fluorescence and an altered pattern of proteolytic digestion, indicative of a tertiary structural change. This suggests that colicin Ia undergoes a tertiary but little or no secondary structural change in its transition from a soluble to a transmembrane protein.

INTRODUCTION

The spontaneous insertion of proteins from aqueous environments into membranes is widely seen in biology. Examples include perforin (Podack et al., 1991), the C-9 component of the complement cascade (Stanley, 1989), several toxins which are pathogenic to humans such as diphtheria and cholera toxins (Holmgren, 1981; Kagan et al., 1981), as well as members of the ion channel-forming family of colicins (Parker et al., 1990). The mechanism by which such molecules are able to insert into the hydrophobic environment of a lipid bilayer from aqueous solution is not fully understood at the molecular level. To understand both the biochemical and structural basis of this insertion process, we have focused on one member of this class of proteins, the bacterial toxin colicin Ia.

Colicin Ia is a member of the ion channel-forming family of colicins, proteins that undergo a transition from a soluble to a transmembrane configuration. The members of this family, which includes colicins A, B, E1, Ia, Ib, K, and N, are synthesized in and released from *E. coli* as soluble proteins. These colicins kill sensitive, target *E. coli* by first binding to a specific receptor on the outer membrane, translocating across the periplasmic space, and inserting into and forming ion-conducting channels within the inner membrane. The channels are relatively non-specific, voltage-

dependent, and kill by depleting the target cell of its electrochemical potential (reviewed in Konisky, 1982; Lazdunski et al., 1988; Cramer et al., 1990; Pattus et al., 1990).

Many of the proteins which spontaneously insert into membranes require low pH (reviewed in Parker et al., 1990) and several also require negatively charged lipids (Shone et al., 1987; Cramer et al., 1990). Two of the channel-forming colicins, A and E1, require both low pH and negatively charged lipids (Bullock et al., 1983; Pattus et al., 1983; Davidson et al., 1984; Davidson et al., 1985; Massotte et al., 1989) for membrane insertion. This insertion appears to be accompanied by a tertiary but little or no secondary structural change for both of these colicins (Brunden et al., 1984; Merrill et al., 1990a; Lakey et al., 1991a; van der Goot et al., 1991).

We sought to determine whether colicin Ia, which shares less than 30% amino acid sequence identity with colicins A and E1, has specific pH and lipid requirements for membrane insertion and channel formation. We further examine the degree and type of structural change accompanying its transition from a hydrophilic to a hydrophobic environment. Our results are discussed in the context of the membrane insertion model proposed from the x-ray crystal structure of the soluble, channel-forming domain of colicin A (Parker et al., 1989).

MATERIALS AND METHODS

PURIFICATION OF COLICIN Ia: Cell growth and lysis . Cells harboring the colicin Ia-producing plasmid pJK5 (Weaver et al., 1981) were grown to mid-log phase in LB culture medium (Miller, 1972) at 37° C and induced by the addition of mitomycin C (Boehringer Mannheim) (stock solution of 2 mg/ml in 150 mM NaCl) to a final concentration of 0.2 μg/ml. Four to 12 hours after induction, the cell pellet was harvested by centrifugation at 5000 rpm for 10 min in a GSA or GSA3 rotor. The cell pellet was then solubilized in 1.5 ml of lysis buffer (50 mM Tris, pH 8; 2 mM EDTA, 0.1 mM DTT, 1 mM BME, 5% glycerol, 100 mM NaCl) per gram of cells, and lysed using a probe sonicator, while the temperature was maintained below 10° C. The lysate was diluted 4-fold in buffer C (20 mM Tris, pH 8; 2 mM EDTA, 0.1 mM DTT, 5% glycerol) containing 25 mM NaCl, and centrifuged in an SS-34 rotor at 18,000 rpm for 20 minutes.

Ion exchange and size exclusion chromatography . After centrifugation, the supernatant was applied to a CM-Sephadex C-50 column (Pharmacia) equilibrated with buffer C + 25 mM NaCl, at 4° C. Colicin Ia adheres to the column matrix and is eluted as the major peak at O.D. 280, using a salt gradient which was 6X the column volume and increased linearly from buffer C + 25 mM NaCl to buffer C + 400 mM NaCl. This peak was concentrated at 4° C (Amicon, YM30 filter) to 5-7 mg/ml, then applied to a Biosil TSK 250 HPLC column equilibrated with 50 mM NaCl and 20 mM citrate, pH 5.1 (buffer D).

Colicin Ia elutes as a large, single peak after approximately 66 minutes, at a flow rate of 2 ml/min, and is sufficiently pure (Figure 1) to grow crystals which diffract to at least 2.8 Å (Ghosh, unpublished observation). Typically, 30 mg of purified protein per liter of cells were obtained. An extinction coefficient of 0.83 cm^2/mg was determined by measuring the O.D. 280 (using a Shimadzu UV 160 Spectrophotometer) of duplicate samples of colicin Ia. The mass of these samples was quantitated by amino acid content using a Beckman 121MB amino acid analyzer after gas phase hydrolysis in 6N HCl for 24 hours at 108° C.

Secondary structure determination using circular dichroism. To assess secondary structure, circular dichroism spectra of colicin Ia were recorded on a Jasco 500 spectrophotometer, which was calibrated with ammonium-d-camphor-10-sulfonate. As colicin Ia adheres to glass, all protein samples were handled using plastic tubes and plastic pipette tips. Colicin Ia (6.1 mg/ml stock solution in buffer D) was diluted to 0.1 mg/ml either with or without 1.5% β-OG (Pfanstiehl Lab, IL), in citrate/phosphate buffer, for which the pH was adjusted by titrating 5 mM citric acid with 10 mM dibasic sodium phosphate. Samples for circular dichroism were scanned from 240 to 190 nm in a 0.1 cm cell. To minimize colicin adherence to the quartz CD cell, it was washed with nitric acid and then rinsed extensively with water between each sample run. Duplicates of each sample were scanned 4 times and averaged, and a pH and β-OG-

adjusted baseline was subtracted for each. Data were digitized on a Jasco DP-J500/PC Data Processor, with a sampling interval of 0.1 nm.

Protein concentration was measured by O.D. 280 and a modified Lowry assay (Markwell et al., 1978) for all samples. The CD spectra were normalized using the concentration that was determined by O.D. 280, with the exception of colicin Ia at pH 7.3 in the presence of 1.5% β-OG. In this sample, some aggregation was observed as determined by light scattering, so a modified Lowry's assay alone was used to measure this protein concentration.

Secondary structure content was estimated by least squares analysis, using a data base of 15 solved protein structures (Chang et al., 1978; Yang et al., 1986). Standard deviations in secondary structure estimation were determined by propagation of the standard deviation of CD measurements of duplicate samples with the standard deviation of protein concentration measurements.

Liposome preparation. Vesicles were prepared following the Reverse Phase Evaporation (REV) method (Szoka & Papahadjopoulos, 1978). Approximately 10 μmole of either egg PC (Avanti Polar Lipids, AL) or purified (Kagawa & Racker, 1971) asolectin (Associated Concentrates, NY) were dried to a thin film, then rehydrated in 1 ml of diethyl ether and 1 ml of the following buffer: 12.5 mM ANTS (Molecular Probes; Eugene, OR), 45 mM DPX (Molecular Probes; Eugene, OR) 45 mM NaCl, 10 mM TES, 0.1 mM EDTA. After 3

minutes of bath sonication, the emulsion was evaporated at room temperature for approximately 15 minutes to remove residual ether. To obtain a uniformly sized population, vesicles were extruded first through a 0.2 μm and then through a 0.1 μm polycarbonate membrane (Nucleopore) with a stream of argon at 40-50 psi. Unencapsulated ANTS and DPX were separated from the liposomes by applying the vesicles to a Sephadex G-75 column (1 x 20 cm) (Pharmacia) equilibrated with 10 mM TES, 0.1 mM EDTA, 140 mM NaCl, and 10 mM citrate (G-75 buffer). The osmolarity of this buffer was adjusted to that of the ANTS/DPX buffer with NaCl. The phospholipid content of the vesicles was measured by an inorganic phosphate analysis (McClare, 1971).

Monitoring membrane insertion by fluorescence. Colicin Ia insertion into membranes was measured using an ANTS/DPX fluorescence leakage assay (Ellens et al., 1984). When co-encapsulated inside liposomes, DPX quenches the fluorophore ANTS, resulting in no measurable fluorescence. The leakage of ANTS and DPX from vesicles into the surrounding medium leads to an increase in fluorescence, which results from the dequenching of ANTS. A solution of either asolectin or egg PC liposomes (50 μM final phospholipid concentration in G-75 buffer) adjusted to the desired pH with concentrated HCl, was stirred in a quartz cuvette at room temperature. The baseline fluorescence leakage (defined as 0% fluorescence leakage) from the vesicles was monitored for 100

seconds, at which time colicin Ia was added to a final molar lipid:protein ratio of 850:1 for the pH experiments, and at varying ratios for concentration dependence experiments. The final volume of each reaction mixture was 2 ml. Fluorescence release was recorded for 400 seconds; at 500 seconds, 50 µl of 10% Triton X-100 was added to each 2 ml sample, and samples were monitored for an additional 100 seconds. The addition of detergent leads to liposome lysis and defines 100% fluorescence leakage. All experiments were performed using a Spex Fluorolog-2 Spectrofluorometer (Edison, NJ), with an excitation wavelength of 360 nm and an emission wavelength of 520 nm.

Proteolytic digestion. Colicin Ia, both in the absence and in the presence of 1.5% β-OG, was digested with pepsin (Sigma). For all samples, a 6.1 mg/ml stock solution of colicin Ia in buffer D was diluted to 0.4 mg/ml in 50 mM ammonium acetate buffer (pH 4.1), either with or without 1.5% β-OG. Immediately after the addition of pepsin (final concentration of 0.4 µM) to the solution of colicin Ia, 12 µl (from a total volume of 60 µl) was removed for the zero time point, added to sample buffer (Laemmli, 1970), and immediately frozen on dry ice to inhibit further proteolysis. At 5, 15 and 30 minutes after the addition of pepsin, additional 12 µl aliquots were removed and similarly treated. The digests were carried out both at room temperature and at 37° C. Samples were examined by 16% SDS-PAGE (Laemmli, 1970).

Tryptophan fluorescence. Tryptophan fluorescence of colicin Ia was measured both in the presence and absence of 1.5% β-OG, in citrate/phosphate buffer pH 4.1 (buffer as described for circular dichroism experiments). Final sample concentrations were all 0.1 mg/ml, diluted from a 6.1 mg/ml stock solution in buffer D. All samples were prepared in plastic tubes, and plastic pipette tips were used for the transfer of any material due to problems of colicin Ia sticking to glass. Duplicate samples were excited at 278 nm, then scanned once from 300-400 nm with data recorded every 0.1 nm; data were corrected using the emission correction data file provided by Spex (MCORRECT). All experiments were performed on a Spex fluorolog 1680 0.22m double spectrometer (0.25mm excitation slit width and 2.5mm emission slit width), and the data were analyzed using Spex DM3000 software. A pH and β-OG-adjusted baseline spectra was subtracted from each sample.

RESULTS

Colicin Ia inserts into negatively charged lipids, but only at acidic pH. Colicin Ia spontaneously inserts into negatively charged asolectin membranes, with increasing efficiency as the pH is decreased below 5.2. Both the rate of fluorophore release as well as the percent of total fluorophore leaked increase as the pH is lowered (Figures 2a and 2b). At pH's greater than 5.2, the addition of colicin

Ia to negatively charged membranes results in no fluorophore release, indicating that membrane insertion does not occurr at these pH values. Decreasing the pH is not sufficient for membrane insertion, however, as the addition of colicin Ia to vesicles made from the neutral lipid egg PC results in no fluorophore leakage over a pH range of 7.3-3.1. Thus, colicin Ia will insert into negatively charged phosopholipids and not into neutral lipids, and will do so only at acidic pH.

The maximum rate of fluorophore leakage increases as the pH is decreased, with a midpoint ≤ pH 4.5 (Figure 2b). The midpoint of the titration cannot be accurately defined, however, as below pH 4, the rate of fluorophore release increases dramatically upon colicin Ia addition to vesicles, but less than 50% of encapsulated fluorophore is released. This points to an altered behavior of colicin Ia or vesicles in this assay at these lower pH values, so these data were not included.

At pH 4.1, the rate of fluorophore leakage from asolectin vesicles increases linearly with increasing colicin Ia concentration, within the range (12-177nM) evaluated (Figures 3a and 3b). This supports a single-hit kinetic model of channel insertion (Wendt, 1970; Bruggemann & Kayalar, 1986; Levinthal et al., 1991), in which one molecule, or a rapidly associating molecular complex inserts into the membrane to form a channel.

Colicin Ia releases 100% of encapsulated fluorophore at protein concentrations ≥ 89 nM (Figure 3a: a and b). At 89 nM colicin

Ia, the protein to liposome ratio is estimated to be 100:1. This estimate assumes an average liposome diameter of 1000 Å (based on extrusion through a 1000 Å filter, see Materials and Methods) and a surface area of 70 $Å^2$/lipid (Small, 1986). Assuming colicin Ia acts as a monomer, a Poisson distribution shows that at 89 nM colicin Ia, the probability of finding a liposome with no protein molecules attached is diminishingly small, in essence zero. Since all colicin Ia is associated with liposomes as determined by ultracentrifugation (data not shown), multiple colicin Ia molecules will be attached to each vesicle at this protein concentration. Thus, as 100% of fluorophore is released only at an estimated colicin:liposome ratio of 100:1, on the order of 1-10 % of colicin Ia molecules are functionally inserting under these experimental conditions.

The transition from hydrophilic to hydrophobic environment requires little or no change in secondary structure. To determine whether the acidic pH required for membrane insertion induces a structural change in colicin Ia, the secondary structure composition of the soluble form of colicin Ia was evaluated at pH's between 7.3 and 3.1, using circular dichroism (CD). A comparison of spectra at four pH values reveals that the overall shapes and zero crossings (wavelength at which molar ellipticity $\varepsilon = 0$) are essentially identical, but that the amplitudes of these spectra are somewhat different (Figure 4a). As the shape and zero crossing of a CD spectrum directly reflect the proportional contribution of each

secondary structure element in a protein (α-helix, β-sheet, turn, random coil), any genuine change in secondary structure would lead to a change in either the shape, zero crossing, or both, of that spectrum. The overall amplitude of a raw spectrum, however, is directly proportional to protein concentration.

To determine the source of the differences among the spectra of colicin Ia at the different pH values, the CD spectra were linearly scaled to optimize overlap (Figure 4b). After scaling, the calculated secondary structure proportions were identical to within +/-1%, indicating that the proportion of α-helix to random coil in colicin Ia does not change over the pH range 7.3 - 3.1. Within this pH range, the percent α-helix remained constant at 66% +/- 4%, and the remaining secondary structure was comprised of random coil (34% +/- 4%) (Table I). Scaling the curves supposes that there is variation in concentration between samples, possibly due to colicin Ia sticking to glass.

To test whether a hydrophobic environment alone or in combination with pH induces a change in secondary structure content, CD spectra of colicin Ia solubilized in the non-ionic detergent β-OG were evaluated across a pH range of 7.3 - 3.1 (Figure 4c). β-OG was used to mimic the hydrophobic nature of the membrane in order to minimize problems due to light scattering from lipids (Wallace & Mao, 1984). The secondary structure content of colicin Ia at pH's of 7.3 and 4.1 is constant at 68 +/- 1% α-helix and 32 +/-1% random coil, in the presence of 1.5% β-OG (critical

micelle concentration = 0.8%) (Table I). At pH 3.1 in 1.5% β-OG, however, the amount of α-helix drops to 59.8 +/- 1%, with a compensatory increase in the percent of random coil. Notably, the zero crossing is shifted to a lower wavelength by 0.8 nm (Figure 4c, inset, as compared to Figures 4a and 4b insets). This reflects a decrease of 11 +/- 3% in the amount of α-helix, as reflected in both the overall shape and zero crossing of this spectrum, when compared with the spectra of colicin Ia in β-OG at pH's 4.2 and 7.3.

Therefore, at pH 3.1, the structure of colicin Ia is slightly altered by the presence of a hydrophobic environment. Between pH 7.3 and pH 4.2, however, neither a hydrophobic environment nor an acidic pH significantly alter the amount of secondary structure of this highly α-helical protein. As colicin Ia will release the contents of liposomes below pH 5.2, membrane insertion does not require a significant change in secondary structure content.

The transition from hydrophilic to hydrophobic environment is accompanied by a tertiary structural change To assess tertiary structural changes as a function of the hydrophobicity of the environment, proteolytic digestion of colicin Ia was carried out in the presence and absence of β-OG at pH 4.1. The susceptibility of colicin Ia to proteolysis is altered in the presence of 1.5% β–OG. After a 30 minute digestion at 37° C in the absence of 1.5% β–OG, a strong proteolytic product of ~25 kD remains uncleaved (Figure 5a, lanes 2-4). This 25 kD product is not visible in a parallel digest in

the presence of 1.5% β–OG, indicating an increased susceptibility to proteolysis in the presence of this detergent (Figure 5a, lanes 6-8). The different digestion pattern does not result from a temperature-induced secondary structural change of colicin Ia in the presence of detergent, as the α-helical content of colicin Ia in 1.5% β-OG at 25° C is identical to that at 37° C (data not shown). A similar digestion carried out at 25° C reveals that the reaction products from colicin Ia digested in the presence of 1.5% β–OG are different from those of colicin Ia digested in the absence of detergent (Figure 5b, lanes 1-8).

The appearance of different proteolytic digestion products in the presence of detergent can be explained in one of two ways. Either colicin Ia has undergone a tertiary structural change resulting in the appearance of new proteolytic cut sites and the disappearance of others, or the protein structure remains unaltered in detergent but the presence of β-OG limits the access of pepsin to existing cut sites. That the digestion of colicin Ia proceeds more rapidly in the presence of detergent (Figure 5a) argues in favor of a structural change in the protein, rather than a masking of available cut sites by β-OG.

Further evidence supporting a tertiary structural change in the presence of a hydrophobic environment comes from tryptophan fluorescence of colicin Ia. The addition of 1.5% β-OG to colicin Ia leads to a 15% increase in fluorescence intensity at both pH 4.2 and pH 7.3, as well as a small shift of 1 nm in wavelength maximum

(from 321 to 322 nm at pH 4.2 and from 323 to 324 nm at pH 7.3) (Figure 6), indicating that the detergent alters the environment of tryptophan residues. When scaled to correct for protein absorption to glass, these tryptophan fluorescence spectra (Figure 6) were not superimposable, indicating a genuine change in tryptophan environment in the presence of detergent, further consistent with a tertiary structural change in colicin Ia.

In contrast, pH alone has a minor effect on the environment surrounding tryptophan residues. In the absence of β-OG, the tryptophan fluorescence spectrum of colicin Ia at pH 7.3 exhibits a maximum near 323 nm (Figure 6). Lowering the pH to 4.2 results in a very slight blue shift of 1-2 nm to 321 nm, with a 2% change in fluorescence intensity. These small changes in both wavelength maximum and fluorescence intensity indicate little or no significant change in protein tertiary structure in response to pH changes alone.

DISCUSSION

Colicin Ia is a member of a class of proteins which spontaneously insert into membranes from aqueous environments. For colicin Ia, insertion will occur only in the presence of negatively charged membranes and only at low pH. The dependence on negatively charged lipids may be indicative of an electrostatic attraction between colicin Ia and its target membrane surface. The

inner *E. coli* membrane, into which colicin Ia inserts and forms a channel *in vivo*, has a phospholipid composition of PE:PG:CL in a ratio of 74:19:3 (Gennis, 1989), which results in an overall net negative charge of 0.25 electrons per lipid. The requirement for negatively charged lipids suggests an electrostatic attraction between the membrane and positively charged colicin Ia, which has a calculated isoelectric point of 9.72 (Stroud, PREDICT program). The mechanism of electrostatic attraction is probably conserved across the channel-forming colicin family, as both colicins A and E1 are also basic proteins (isoelectric points of 8.86 and 10.08 respectively) and require negatively charged phospholipids for membrane insertion (Bullock et al., 1983; Massotte et al., 1989).

Specific residues that might be involved in this electrostatic interaction have not yet been directly identified for colicin Ia. However, it has been proposed based on the x-ray crystal structure of the colicin A channel-forming fragment that 8 positively charged residues are responsible for the interaction of that protein with a negatively charged target membrane (Parker et al., 1989). Of these 8 residues, only 2 positive charges are conserved between colicin A and colicin Ia. The overall number of positive charges within the channel forming domains, however, is similar between these two colicins: there are 28 positively charged residues in the colicin A channel-forming domain, and 30 in the same region of colicin Ia. Therefore, although the general mechanism of electrostatic

attraction is likely to be similar between colicin A and colicin Ia, the details of this mechanism may differ.

In common with the other channel-forming colicins, colicin Ia also requires a low pH for membrane insertion (Bullock et al., 1983; Pattus et al., 1983; Davidson et al., 1984; Davidson et al., 1985), a condition which these proteins may encounter in the periplasmic space. It has been suggested that this space is acidified by the proton translocation machinery (Cramer et al., 1983). This requirement for low pH probably reflects the necessity to protonate colicin Ia, phospholipid head groups, or both. Since the midpoint of the maximum rate of fluorophore leakage is $\leq$ pH 4.5 (Figure 2b), it is likely that the titration of groups with pK's near this value is responsible for this effect. The secondary phosphate groups in asolectin, which are strongly acidic with a pK < 2, would not be significantly titrated between pH 4-5. While the phosphatidic acid groups in asolectin have a pK near pH 4 and thus would be titrated between pH 4-5, they constitute only 8% of the asolectin mixture. It is more likely that low pH is required for protonation of negatively charged carboxyl groups on aspartic and glutamic acids, whose pK values are near pH 4.5. The protonation of the carboxyls may serve to reduce the repulsion between the negatively charged membrane and these negatively charged groups on colicin Ia. The protonation might further serve to neutralize carboxyls that are to cross the membrane. Site-directed mutagenesis experiments on the channel-forming domain of colicin E1 indicate that more than one carboxylic

acid residue must be protonated to account for the pH-dependent increase in channel-forming activity (Davidson et al., 1985; Shiver et al., 1987; Shiver et al., 1988).

As the insertion of colicin Ia into asolectin vesicles is pH-dependent, we sought to understand whether lowering the pH leads to a conformational change in the protein. CD spectra indicate that soluble colicin Ia remains highly α-helical (66%) as the pH is decreased from 7.3 to 4.2, reflecting no change in secondary structure within this range. Furthermore, lowering the pH from 7.3 to 4.2 induces little or no tertiary structural change, as determined by tryptophan fluorescence. Similarly, the tertiary structure of the colicin A channel-forming domain does not change as the pH is varied between 7 and 4, and the channel-forming regions of both colicins A and E1 exhibit little or no change in secondary structure as the pH is lowered to 4 (Brunden et al., 1984; van der Goot et al., 1991).

Below pH 4, however, a pH-induced structural change is seen in both colicins A and E1. Spectroscopic analysis of colicin A demonstrates the appearance of a "molten-globule" form of the protein, in which there is a compact, water-excluding protein core, significant secondary structure, but no native tertiary structure (van der Goot et al., 1991). A tertiary structural change has also been identified for colicin E1, as determined by altered proteolytic digestion patterns of the protein and increased accessibility below pH 4 to a fluorescent probe on a previously buried cysteine (Merrill

et al., 1990a). For colicin Ia, the secondary structure of the soluble form below pH 4 is not altered.

In contrast to the effect of pH alone, the presence of detergent at pH 3.1 leads to an 11% decrease in the α-helical content of colicin Ia. In addition to a loss of α-helix, the presence of detergent may also lead to a rearrangement of tertiary structure. At pH 4.1, proteolytic digestions of colicin Ia in the presence and absence of β-OG reveal different products, consistent with the appearance of new proteolytic cut sites and loss of others, indicating a possible change in tertiary structure. Furthermore, at both pH's 7.3 and 4.2, the addition of 1.5% β-OG leads to an increase in tryptophan fluorescence intensity, indicating a change in the environment surrounding tryptophan residues, also consistent with a tertiary structural change in the protein. This type of structural rearrangement appears to be conserved within the colicin family, as colicin A also exhibits a change in tertiary structure upon exposure to a membrane (Lakey et al., 1991a).

Exposure to a hydrophobic environment evokes no change in the secondary structure of colicin Ia at either pH 7.3 or 4.2. Similarly, colicins A and E1 undergo little or no secondary structural change within this pH range in the presence of either lipids or detergent (Brunden et al., 1984; Goormaghtigh et al., 1991; Rath et al., 1991). It therefore appears that a tertiary but not secondary structural change accompanies the membrane insertion of members of this colicin family.

Such a change is probably necessary for this family of colicins to insert into membranes and form ion channels. Colicins A, E1, and Ia are all highly α-helical proteins (Brunden et al., 1984; Pattus et al., 1985; Parker et al., 1989; Wormald et al., 1990) and although they have very little sequence identity, they share strong amphipathic α-helical characteristic within their carboxy-terminal one-third which comprises the channel-forming region (Dankert et al., 1982; Ohno-Iwashita & Imahori, 1982; Martinez et al., 1983; Cavard et al., 1986; Shiver et al., 1989; and reviewed in Baty et al., 1988). The tertiary structural changes probably reflect an alteration in these amphipathic α-helices from a soluble conformation in which the apolar faces are packed within the protein interior, to a membrane configuration in which the apolar faces are exposed to the lipid (Parker et al., 1989; Lakey et al., 1991b) A two-step model explaining this membrane-insertion process has recently been proposed in which colicins, after first binding to a membrane in an "umbrella" conformation, are then driven into the bilayer by a transmembrane voltage (Parker et al., 1989).

The "umbrella" model provides an explanation for the finding that not all fluorophore is released from vesicles in the ANTS/DPX leakage assay, even in the presence of multiple copies of colicin Ia per vesicle. In this model, colicin molecules first become anchored within a target membrane via a hydrophobic helical hairpin, but insert fully only after the application of a transmembrane voltage. We have found that in the absence of a transmembrane voltage, only

~1 in approximately 100 colicin Ia molecules will spontaneously insert into the membrane and form channels. For colicin E1, the presence of a transmembrane voltage leads to increased channel formation in liposomes (Merrill & Cramer, 1990b). The high colicin to liposome ratios necessary for fluorophore release in our case could also be explained by the possibility that colicin Ia forms a channel as an oligomer. It seems more likely, however, that the lack of a transmembrane voltage is responsible for this effect, since most recent evidence argues in favor of the pore forming colicins acting as monomers (Bruggemann & Kayalar, 1986; Peterson & Cramer, 1987; Slatin, 1988; Levinthal et al., 1991).

The membrane insertion of colicin Ia appears to require a change in tertiary structure without a significant change in secondary structure. A similar structural alteration has been observed for filamentous bacteriophage Pf1 coat protein. It also undergoes a tertiary structural change with no corresponding change in secondary structure during membrane-mediated viral assembly, as determined by neutron diffraction and nuclear magnetic resonance (NMR) studies (Nambudripad et al., 1991). The tertiary alteration of pre-existing secondary structures may represent a generalized mechanism of action for proteins which exist in both hydrophobic and hydrophilic environments. The study of the channel-forming family of colicins will greatly aid in understanding the mechanism of insertion as well as the structural basis for the transition from hydrophilic to a hydrophobic environment.

FIGURE LEGENDS

1. Purification of colicin Ia. Lane A, cell lysate; B, flow-through from CM-Sephadex column, material that did not stick to the column upon loading; C, column wash, material that eluted from CM-Sephadex column with a buffer wash; D, major O.D. 280 peak eluted from CM-Sephadex column with NaCl gradient; Lane E, 13 µg purified colicin Ia eluted from HPLC column.

2. (A) Effect of pH on fluorophore leakage from asolectin vesicles. Colicin Ia (final concentration of 59 nM) was added to asolectin vesicles after 100 seconds of mixing (indicated with an arrow). Samples were stirred while the fluorescence leakage was monitored for an additional 5 minutes. a) pH 4.1; b) pH 4.5; c) pH 4.6; d) pH 4.8; e) pH 5.2; f) pH 5.7. 0 and 100% leakage values are defined in Materials and Methods. (B) Maximum rate of fluorophore leakage from asolectin (open symbols) and egg PC (closed symbols) vesicles as a function of pH. Rates were determined by measuring the slope at the steepest part of each leakage curve (units are %leakage/second).

3. (A) Rate of fluorescence leakage from asolectin vesicles at pH 4.1 as a function of colicin Ia concentration. Colicin Ia was added after 100 seconds of mixing (indicated with an arrow) at the following

concentrations: a) 177 nM; b) 89 nM; c) 30 nM; d) 12 nM; e) 0 nM. (B) Maximum rate of fluorophore leakage from asolectin vesicles as function of colicin Ia concentration. Leakage rates determined as in legend for figure 1B.

4. Circular dichroism of colicin Ia as a function of pH and 1.5% β-OG. Boxed areas on spectra are displayed as insets. (A) CD spectra in the absence of β-OG, data not scaled; (B) CD spectra in the absence of β-OG, data scaled; (C) CD spectra in the presence of β-OG, data not scaled. Units of molar ellipticity are degree cm^2 $dmole^{-1}$; pH 3.1 solid line, pH 4.2 dotted line, pH 5.1 shorter dashed line, pH 7.3 longer dashed line.

5. Pepsin digest of colicin Ia +/- 1.5% β-OG. (A) Digest at 37^o; (B) Digest at room temperature. 0.1*,5,15, and 30 refer to minutes of digestion. (One large and 3 smaller bubbles are visible between lanes 7-8, panel A). *0.1 time point was taken immediately after pepsin addition.

6. Tryptophan fluorescence spectra of colicin Ia in the presence and absence of 1.5% β-OG. Solid line, pH 4.1; dashed line, pH 7.3.

TABLE I: % α-helix of colicin Ia +/- β-OG[1]

	-β-OG		+β-OG	
	calculated	scaled	calculated	scaled
pH 3.1	66.5	66.5	59.8	NA
pH 4.2	64.5	66.5	68.5	NA
pH 5.1	70.2	67.5	N.D.	NA
pH 7.3	61.3	65	68.3	NA

[1]In all cases, the non-α-helical secondary structure is random coil, with no calculated β-sheet. Standard deviations for all reported values are less than +/- 2%. Scaling of values is desribed in the Results section.

Figure 1

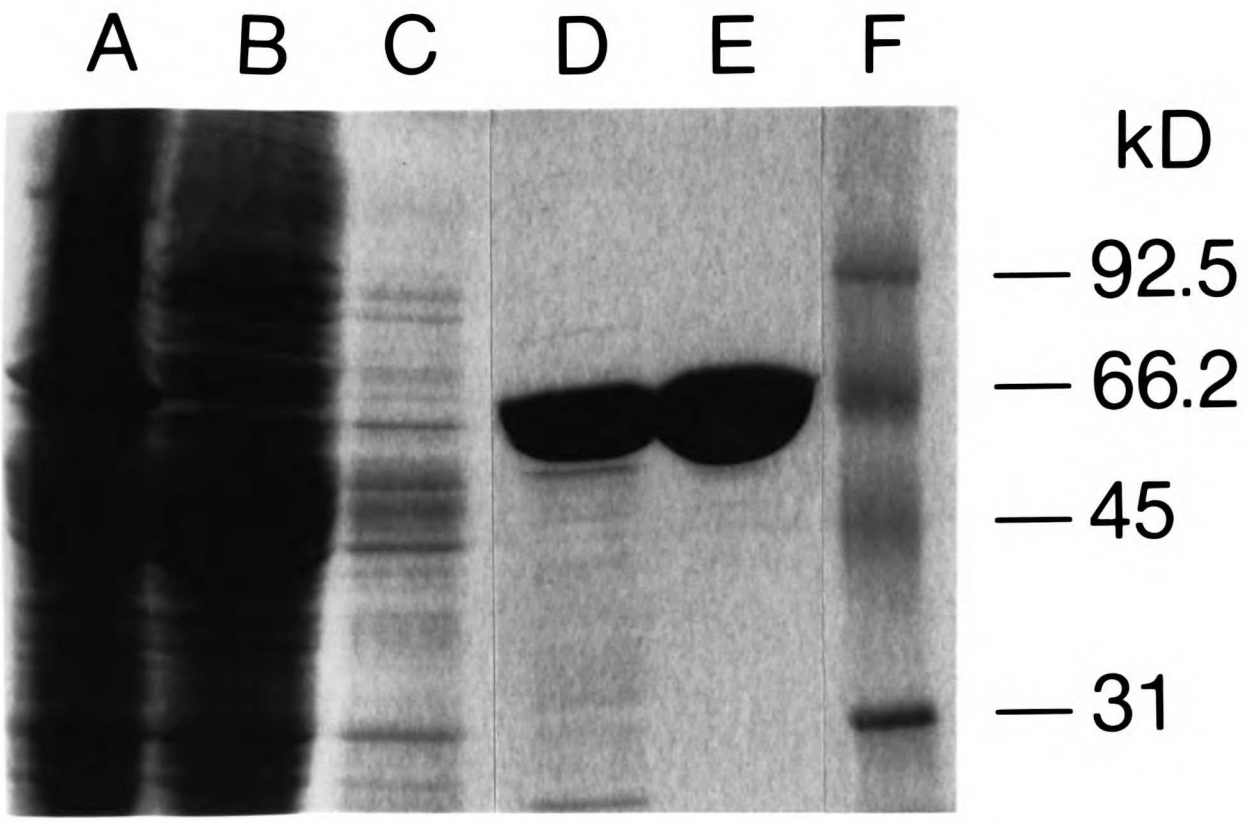


Figure 2A

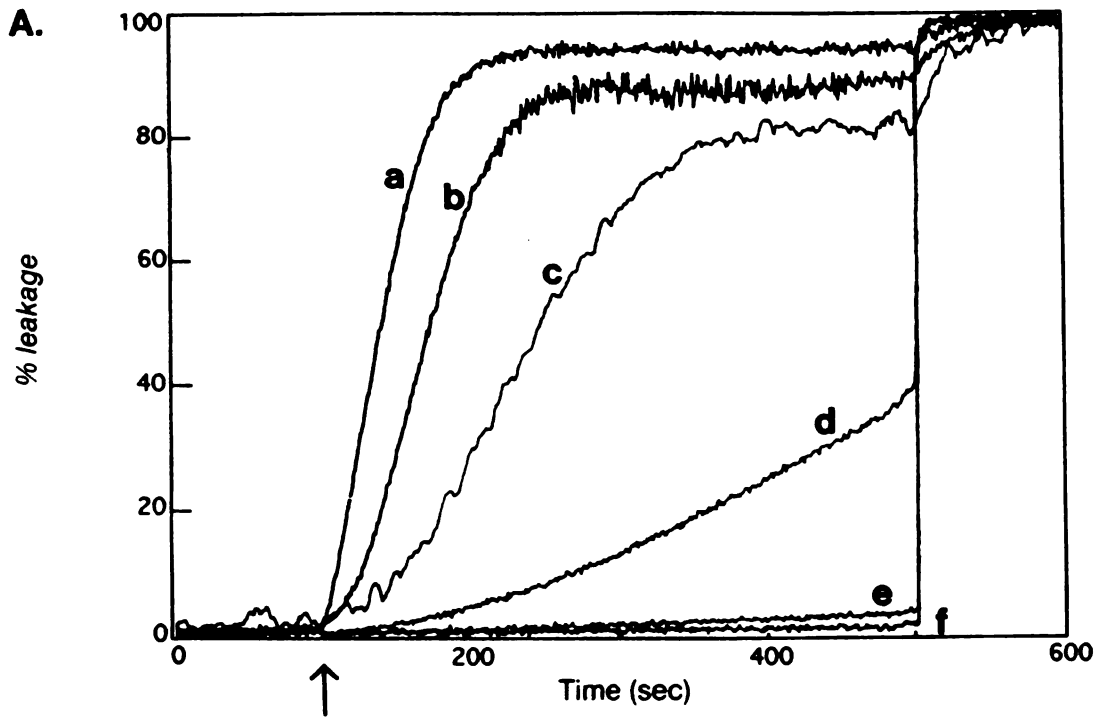


Figure 2B

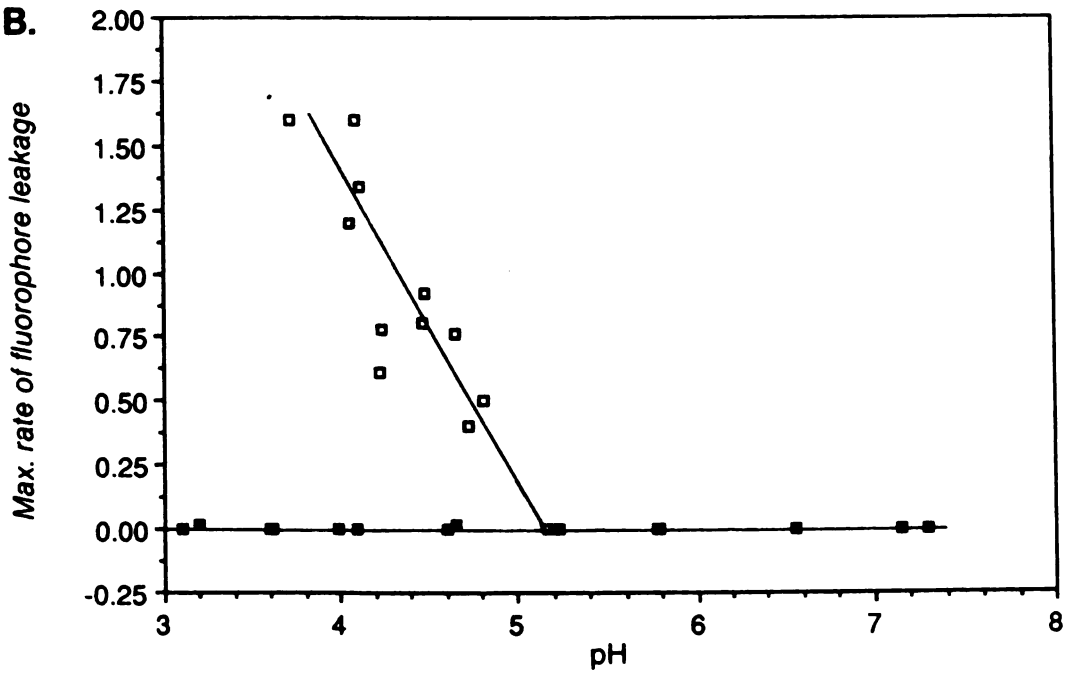

B.
Max. rate of fluorophore leakage
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
-0.25
3
4
5
6
7
8
pH

Figure 3A

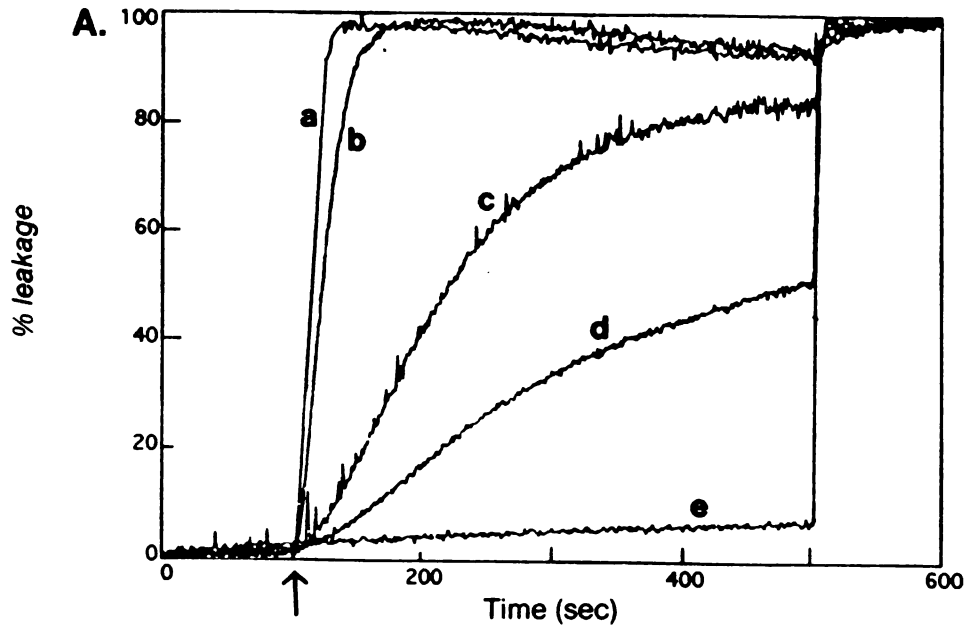


Figure 3B

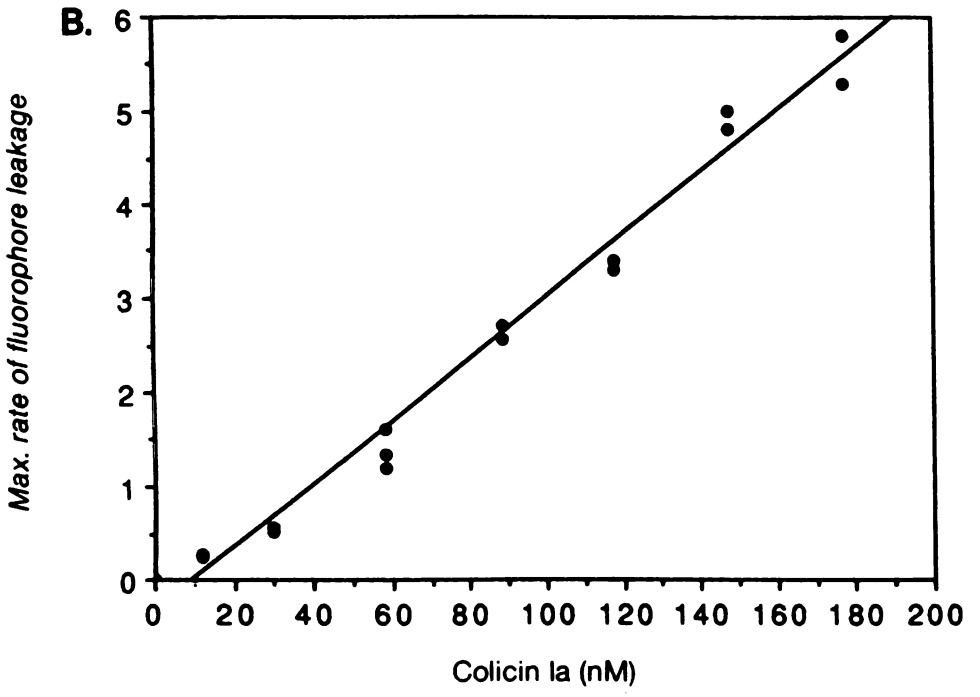

B.
Max. rate of fluorophore leakage
0
1
2
3
4
5
6
0
20
40
60
80
100
120
140
160
180
200
Colicin Ia (nM)

Figure 4A

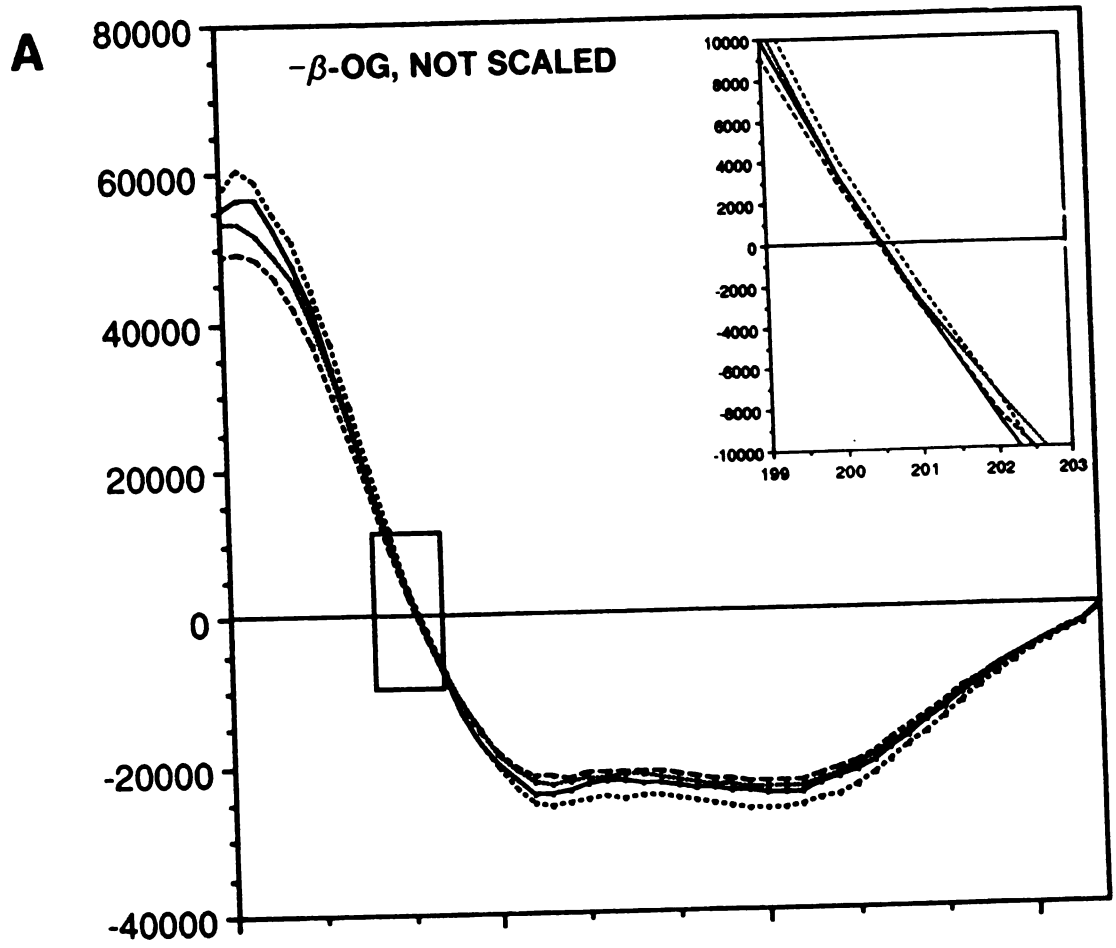

A
80000
60000
40000
20000
0
-20000
-40000
-β-OG, NOT SCALED
10000
8000
6000
4000
2000
0
-2000
-4000
-6000
-8000
-10000
199
200
201
202
203

Figure 4B

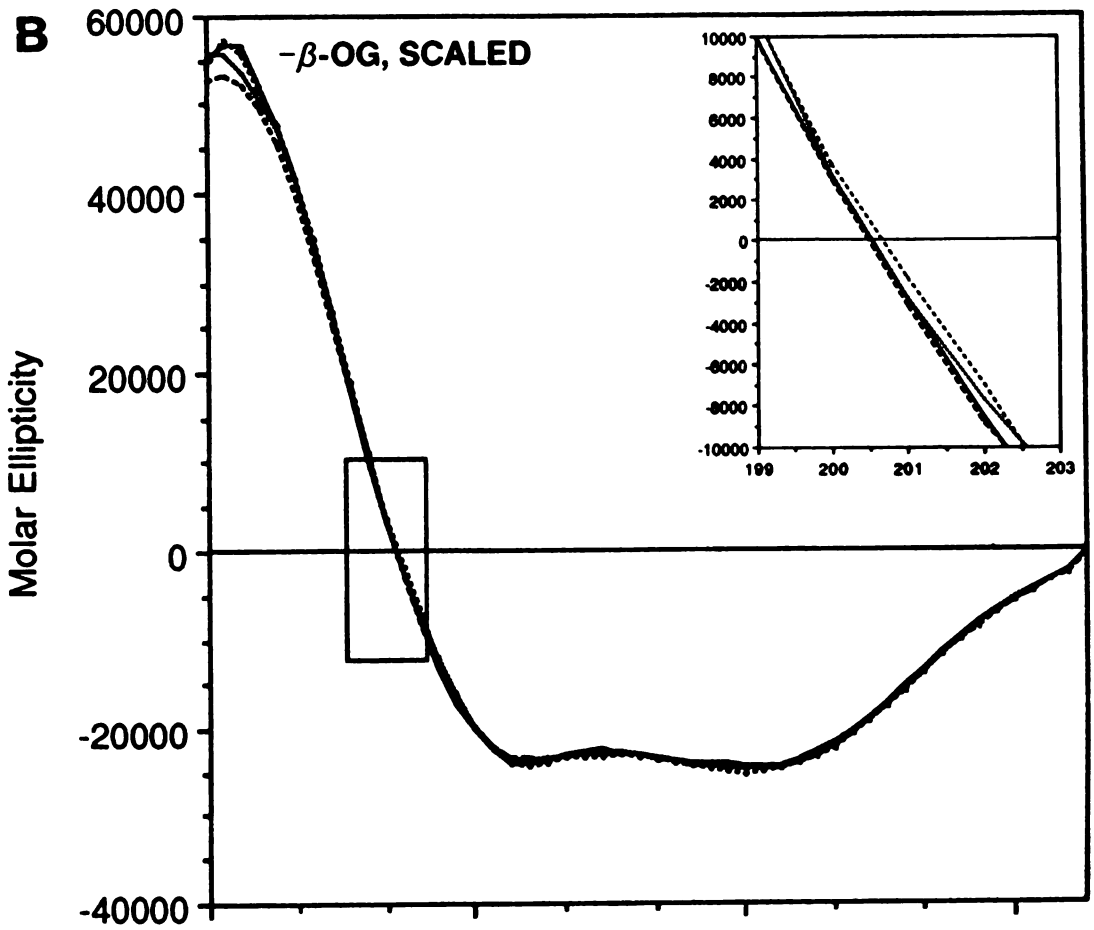

B
60000
40000
20000
0
-20000
-40000
Molar Ellipticity
-β-OG, SCALED
10000
8000
6000
4000
2000
0
-2000
-4000
-6000
-8000
-10000
199
200
201
202
203

Figure 4C

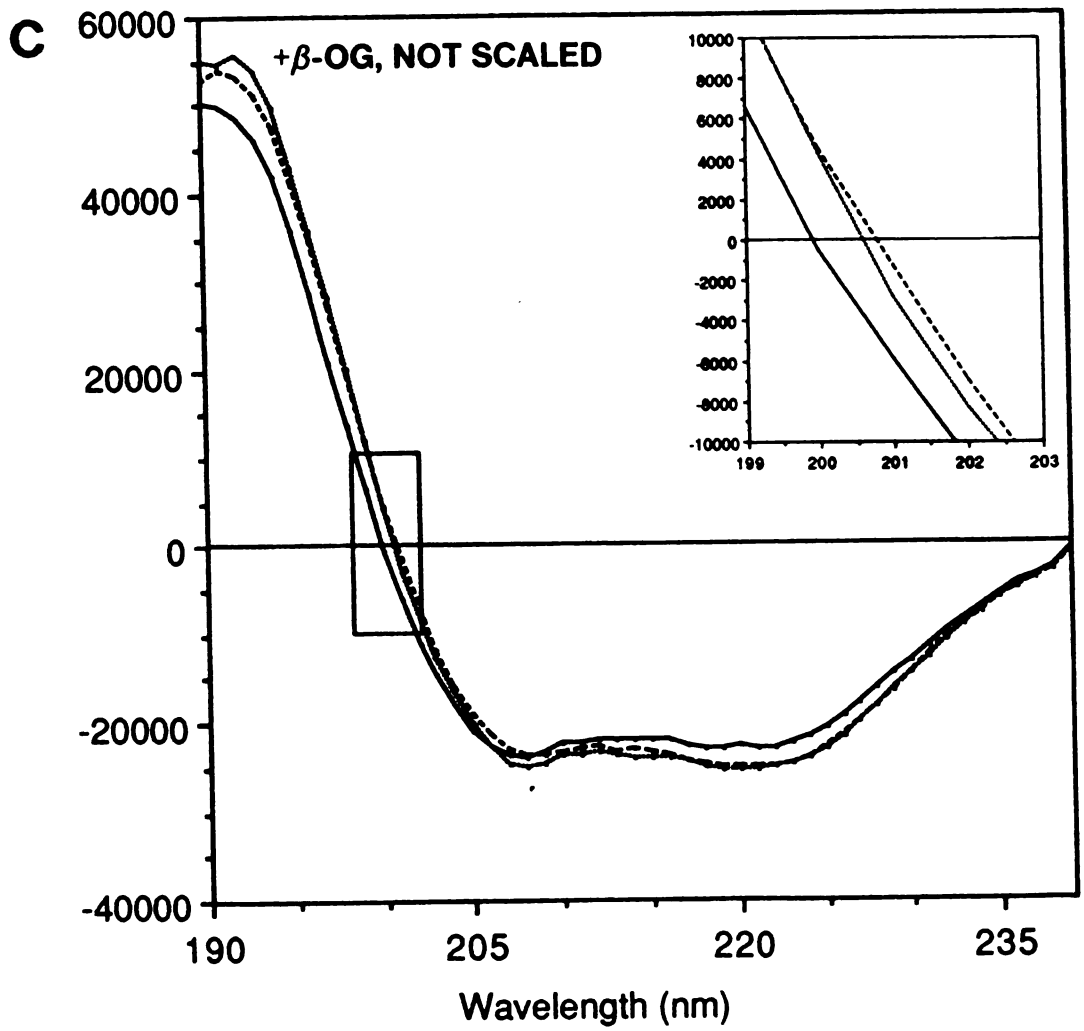

C
+β-OG, NOT SCALED
60000
40000
20000
0
-20000
-40000
190
205
220
235
Wavelength (nm)
10000
8000
6000
4000
2000
0
-2000
-4000
-6000
-8000
-10000
199
200
201
202
203

Figure 5A

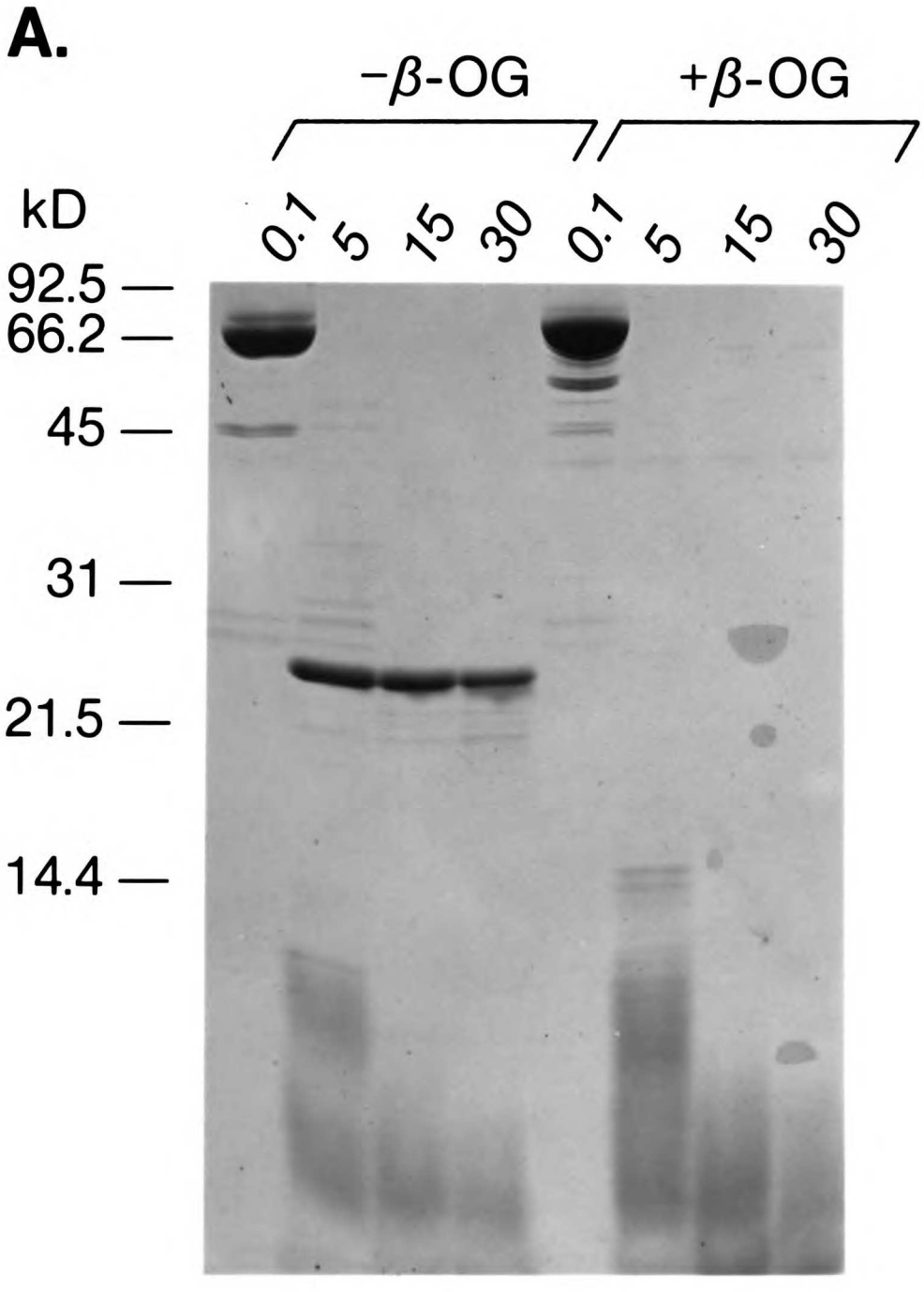


Figure 5B

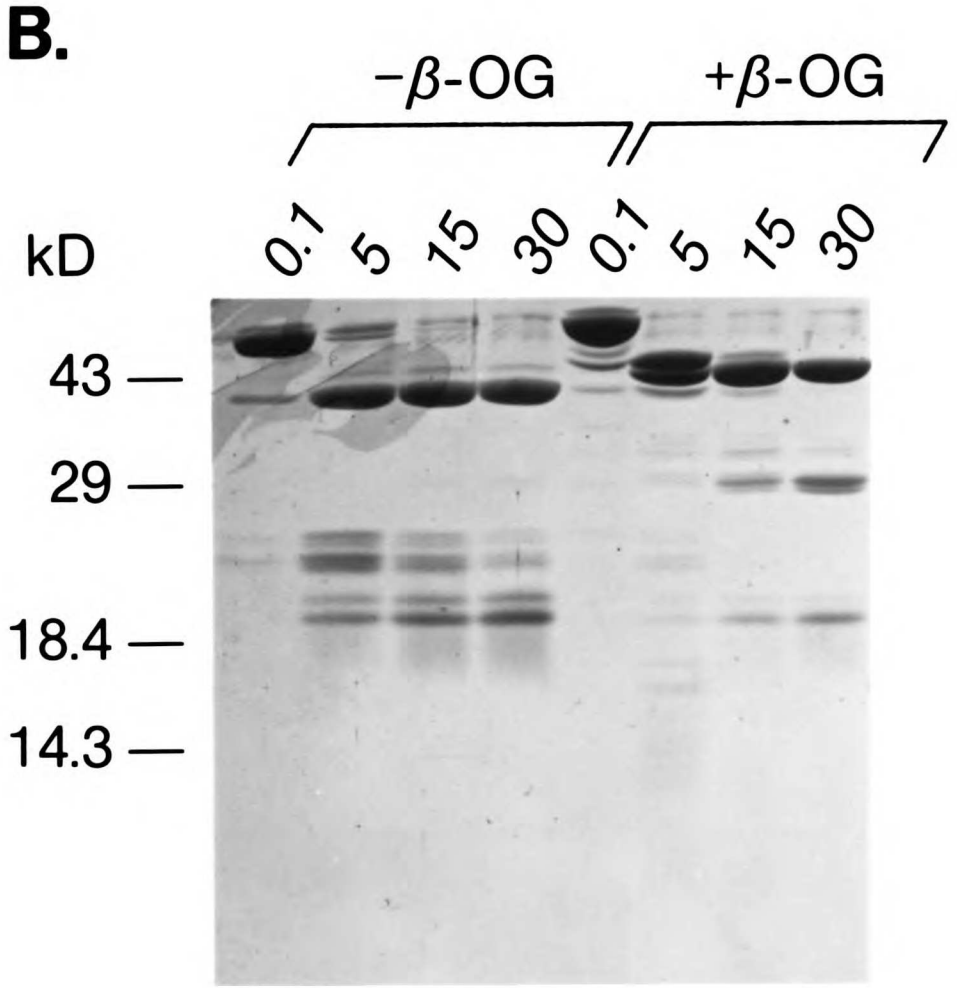
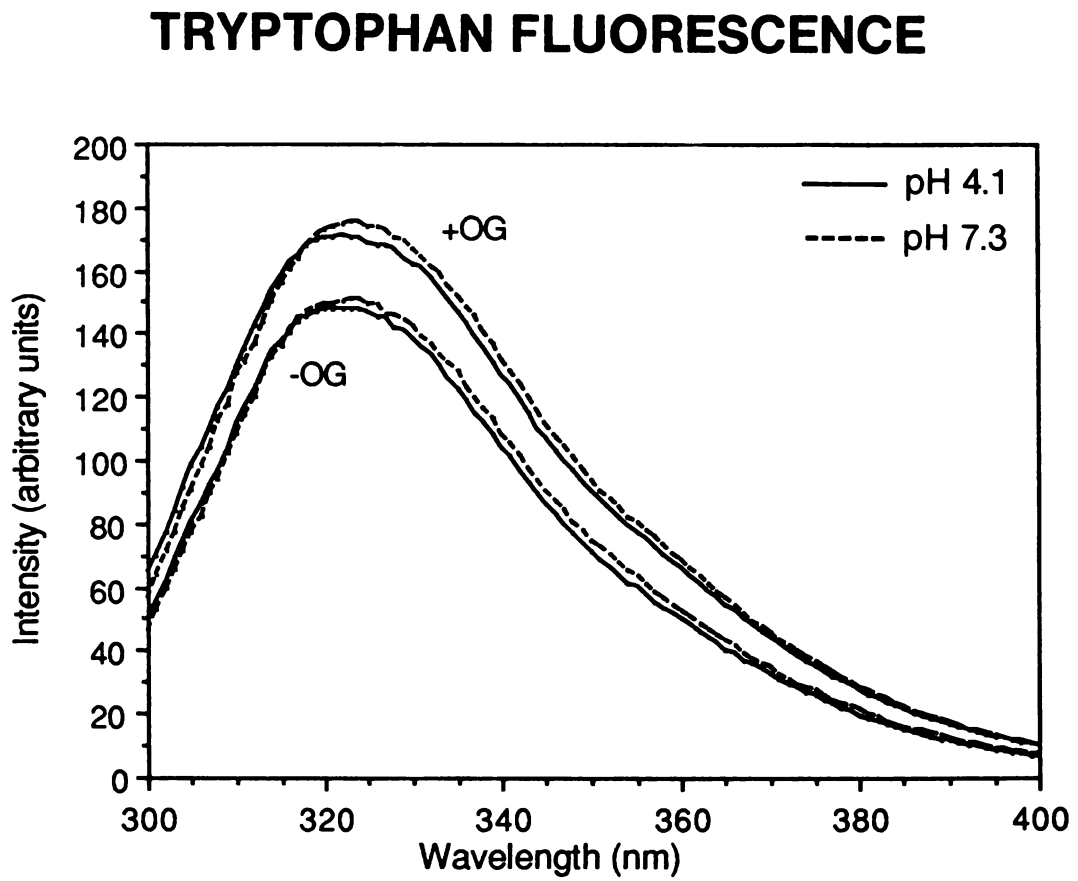


Figure 6


TRYPTOPHAN FLUORESCENCE
Intensity (arbitrary units)
200
180
160
140
120
100
80
60
40
20
0
300
320
340
360
380
400
Wavelength (nm)
pH 4.1
pH 7.3
+OG
-OG

REFERENCES

Baty, D., Frenette, M., Lloubes, R., Geli, V., Howard, S. P., Pattus, F. & Lazdunski, C. (1988) *Mol. Micro. 2*, 807-811.

Bruggemann, E. P. & Kayalar, C. (1986) *Proc. Natl. Acad. Sci. USA 83*, 4273-4276.

Brunden, K. R., Uratani, Y. & Cramer, W. A. (1984) *J. Biol. Chem. 259*, 7682-7687.

Bullock, J. O., Cohen, F. S., Dankert, J. R. & Cramer, W. A. (1983) *J. Biol. Chem. 258*, 9908-9912.

Cavard, D., Crozel, V., Gorvel, J.-P., Pattus, F., Baty, D. & Lazdunski, C. (1986) *J. Mol. Biol. 187*, 449-459.

Chang, C. T., Wu, C.-S. C. & Yang, J. T. (1978) *Anal. Biochem. 91*, 13-31.

Cramer, W. A., Dankert, J. R. & Uratani, Y. (1983) *Biochim. Biophys. Acta 737*, 173-193.

Cramer, W. A., Cohen, F. S., Merrill, A. R. & Song, H. Y. (1990) *Molec. Microbiol. 4*, 519-526.

Dankert, J. R., Uratani, Y., Grabau, C., Cramer, W. A. & Hermodson, M. (1982) *J. Biol. Chem. 257*, 3857-3863.

Davidson, V. L., Cramer, W. A., Bishop, L. J. & Brunden, K. R. (1984) *J. Biol. Chem. 259*, 594-600.

Davidson, V. L., Brunden, K. R. & Cramer, W. A. (1985) *PNAS 82*, 1386-1390.

Ellens, H., Bentz, J., & Szoka, F. C. (1984) *Biochem. 23,* 1532-1538.

Gennis, R. B. (1989) in *Biomembranes: Molecular Structure and Function* (Cantor, C. R., Ed.) Springer-Verlag, NY.

Goormaghtigh, E., Vigneron, L., Knibiehler, M., Lazdunski, C. & Ruysschaert, J.-M. (1991) *Eur. J. Biochem. 202*, 1299-1305.

Holmgren, J. (1981) *Nature 292*, 413-417.

Kagan, B. L., Finkelstein, A., & Colombini, M. (1981) *Proc. Natl. Acad. Sci. USA 78*, 4950-4954.

Kagawa, Y. & Racker, E. (1971) *J. Biol. Chem. 246*, 5477-5487.

Konisky, J. (1982) *Ann. Rev. Microbiol. 36*, 125-144.

Laemmli, U. K. (1970) *Nature 227*, 680-685.

Lakey, J. H., Massotte, D., Heitz, F., Dasseux, J.-L., Faucon, J.-F., Parker, M. W. & Pattus, F. (1991) *Eur. J. Biochem. 196*, 599-607.

Lakey, J. H., Baty, D. & Pattus, F. (1991) *J. Mol. Biol. 218*, 639-653.

Lazdunski, C. J., Baty, D., Geli, V., Cavard, D., Morlon, J., Lloubes, R., Howard, S. P., Knibiehler, M., Chartier, M., Varenne, S., Frenette, M., Dasseux, J.-L. & Pattus, F. (1988) *Biochem. Biophys. Acta 947*, 445-464.

Levinthal, F., Todd, A. P., Hubbell, W. L. & Levinthal, C. (1991) *Proteins: Struct. Func. Genet. 11*, 254-262.

Markwell, M. K., Haas, S. M., Bieber, L. L. & Tolbert, N. E. (1978) *Anal. Biochem. 87*, 206-210.

Martinez, M. C., Lazdunski, C. & Pattus, F. (1983) *EMBO J. 2*, 1501-1507.

Massotte, D., Dasseux, J.-L., Sauve, P., Cyrklaff, M., Leonard, K. & Pattus, F. (1989) *Biochem. 28*, 7713-7719.

McClare (1971) *Anal. Biochem. 39*, 527-530.

Merrill, A. R., Cohen, F. S. & Cramer, W. A. (1990) *Biochem. 29*, 5829-5836.

Merrill, A. R. & Cramer, W. A. (1990) *Biochem. 29*, 8529-8534.

Miller, J. H. (1972) in *Experiments in Molecular Genetics* Cold Spring Harbor Laboratory, NY.

Nambudripad, R., Stark, W., Opella, S. J. & Makowski, L. (1991) *Science 252*, 1305-1308.

Ohno-Iwashita, Y. & Imahori, K. (1982) *J. Biol. Chem. 257*, 6446-6451.

Parker, M. W., Pattus, F., Tucker, A. D. & Tsernoglou, D. (1989) *Nature 337*, 93-96.

Parker, M. W., Tucker, A. D., Tsernoglou, D. & Pattus, F. (1990) *TIBS 15*, 126-129.

Pattus, F., Martinez, M. C., Dargent, B., Cavard, D., Verger, R. & Lazdunski, C. (1983) *Biochem. 22*, 5698-5703.

Pattus, F., Heitz, F., Martinez, C., Provencher, S. W. & Lazdunski, C. (1985) *Eur. J. Biochem. 152*, 681-689.

Pattus, F., Massotte, D., Wilmsen, H. U., Lakey, J., Tsernoglou, D., Tucker, A. & Parker, M. W. (1990) *Experientia 46*, 180-192.

Peterson, A. A. & Cramer, W. (1987) *J. Memb. Biol. 99*, 197-204.

Podack, E. R., Hengartner, H. & Lichtenheld, M. G. (1991) *Annu. Rev. Immunol. 9*, 129-157.

Rath, P., Bousche, O., Merrill, A. R., Cramer, W. A. & Rothschild, K. J. (1991) *Biophys. J. 59*, 516-522.

Shiver, J. W., Cramer, W. A., Cohen, F. A., Bishop, L. J. & de Jong, P. J. (1987) *J. Biol. Chem. 262*, 14273-14281.

Shiver, J. W., Cohen, F. S., Merrill, A. R. & Cramer, W. A. (1988) *Biochem. 27*, 8421-8428.

Shiver, J. W., Peterson, A. A., Widger, W. R., Furbacher, P. N. & Cramer, W. A. (1989) *Meth. Enz. 172*, 439-461.

Shone, C. C., Hambleton, P. & Melling, J. (1987) *Eur. J. Biochem. 167*, 175-180.

Slatin, S. L. (1988) *Int. J. Biochem. 20*, 737-744.

Small, D. M. (1986) in *Handbook of Lipid Research: The Physical Chemistry of Lipids: From Alkanes to Phospholipids,* (Hanahan, D. J., Ed.) Plenum Press, New York.

Stanley, K. (1989) *Current Topics in Microbiology and Immunology 140*, 49-65.

Szoka, F. & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. USA 75*, 4194-4198.

van der Goot, F. G., Gonzalez-Manas, J. M., Lakey, J. H. & Pattus, F. (1991) *Nature 354*, 408-410.

Wallace, B. A., & Mao, D. (1984) *Anal. Biochem. 142,* 317-328.

Weaver, C. A., Redborg, H. & Konisky, J. (1981) *J. Bacteriol. 148*, 817-828.

Wendt, L. (1970) *J. Bact. 104*, 1236-1241.

Wormald, M. R., Merrill, A. R., Cramer, W. A. & Williams, R. J. P. (1990) *Eur. J. Biochem. 191*, 155-161.

Yang, J. T., Wu, C.-S. C. & Martinez, H. M. (1986) *Methods in Enzymology 130*, 208-269.

Acknowledgements:

We would like to thank Jordon Konisky for the plasmid pJK5, Keelung Hong for technical assistance with the fluorometer and Larry Miercke for assistance with HPLC. We would also like to thank Cynthia Wolberger for her contributions to the purification protocol. We further thank Sasha Kamb, Michael Wiener and Partho Ghosh for helpful discussions and critical reading of the manuscript.

CHAPTER 3
PART 1

A NEW STRATEGY FOR MAPPING THE TOPOGRAPHY OF A TRANSMEMBRANE PROTEIN USING MASS SPECTROMETRY

Arnold M. Falick, Stephanie F. Mel, Robert M. Stroud, and A. L. Burlingame

reprinted with permission from *Techniques in Protein Chemistry*

A NEW STRATEGY FOR MAPPING THE TOPOGRAPHY OF A TRANSMEMBRANE PROTEIN USING MASS SPECTROMETRY[1]

Arnold M. Falick[2], Stephanie F. Mel[3], Robert M. Stroud[4], and A. L. Burlingame[2]

University of California, San Francisco, California 94143.

I. INTRODUCTION

In order to completely understand the mechanism of signal transduction across cellular membranes, one must first define the structure of the signal transducing molecule. To this end, we have undertaken to map the transmembrane topography of a bacterial ion channel, colicin Ia, using a combination of proteolytic digestion, HPLC, and mass spectrometric techniques. In this paper, we aim to demonstrate the power of mass spectrometry (MS), in particular tandem mass spectrometry (MS/MS), as applied to a specific problem in structural biology.

Colicin Ia is a 626 amino acid protein which forms an ion channel in the inner membrane of an *E. coli* and fatally depletes that cell of its electrical gradient (Konisky, 1978). Colicin is an unusual protein, leading a dual existence as both a water soluble and a transmembrane protein, a property that is required for its mode of action. It is made and released as a soluble protein during the SOS response in *E. coli.* It then binds to an iron receptor on the outer membrane of a sensitive strain, and subsequently inserts a channel forming region into the inner plasma membrane (Bowles, et al., 1983). Members of the colicin-producing strain of bacteria are immune to this killing

---

[1]This work was supported by the National Institutes of Health (Grant RR01614 to ALB and Grant GM24485 to RMS), the National Science Foundation Biological Instrumentation Program and the University of California, San Francisco (Claypole Fellowship and Graduate Division Mentorship to SFM).

[2] Mass Spectrometry Facility, Department of Pharmaceutical Chemistry

[3]Department of Pathology

[4]Department of Biochemistry and Biophysics

as they have an immunity protein that resides in the inner plasma membrane and protects the cell (Weaver, et al., 1981).

Our overall strategy is to proteolytically digest colicin Ia in its soluble form, to separate the peptide fragments using HPLC, and to identify the individual fragments using liquid SIMS (Secondary Ion Mass Spectrometry) and MS/MS. Subsequently, the protein will be chemically modified in its membrane bound conformation. Analysis by MS/MS will allow us to determine the exact location of modified residues, which in turn will give precise structural information about the transmembrane conformation of the protein.

II. MATERIALS AND METHODS

Colicin Ia was purified from *E. coli* strain JK365, according to the method of Mel, et al. (1988).

Each proteolytic digestion was carried out on 2 nmoles of colicin Ia, with Endoproteinase Glu-C (EC-3.4.21.19) from Boehringer Mannheim (referred to below as *S. aureus* V8 protease). Digestions were done at 37°C for 2 hours in 0.05M phosphate buffer, pH 7.8, and either 2.4M urea or 0.1% SDS at a substrate to enzyme ratio of 5:1.

For sequencing, protein was digested for 2 hours under the above conditions, and run on 16% polyacrylamide SDS gels. The bands were cut out of the gels and electroeluted for 21 hours at 4°C, at 200V. Edman sequencing was performed on an Applied Biosystems 470A Protein Sequencer.

HPLC separations were done on a Vydac C-18 reverse phase column (4.6 mm ID x 25 cm) using 0.1% trifluoroacetic acid in water (A) and 0.08% trifluoroacetic acid in acetonitrile (B). A linear gradient was used, 0-35% B in 105 min at a flow rate of 1 mL/min. The eluent absorbance was monitored at 215 nm.

Mass spectrometry was carried out on a Kratos MS-50S double focusing mass spectrometer equipped with a high-field magnet (mass range 3000 daltons at 8kV), a cesium ion liquid SIMS ion source (Aberth, et al., 1982; Falick, et al., 1986a), and a coolable sample introduction probe (Falick, et al., 1986b). Tandem mass spectrometry experiments were performed on a Kratos "Concept" 4-sector instrument fitted with a cesium liquid SIMS ion source, coolable probe and an electro-optical array detector (Cottrell and Evans, 1987; Elliott, et al., 1988).

III. RESULTS AND DISCUSSION

Our first goal was to proteolytically digest colicin Ia into component peptides that were of a convenient size to be identified by mass spectrometry. Figure 1 shows the time course of a 2 hour digestion of colicin Ia with *S. aureus* V8 protease in the presence of urea. Within 2 minutes, native colicin

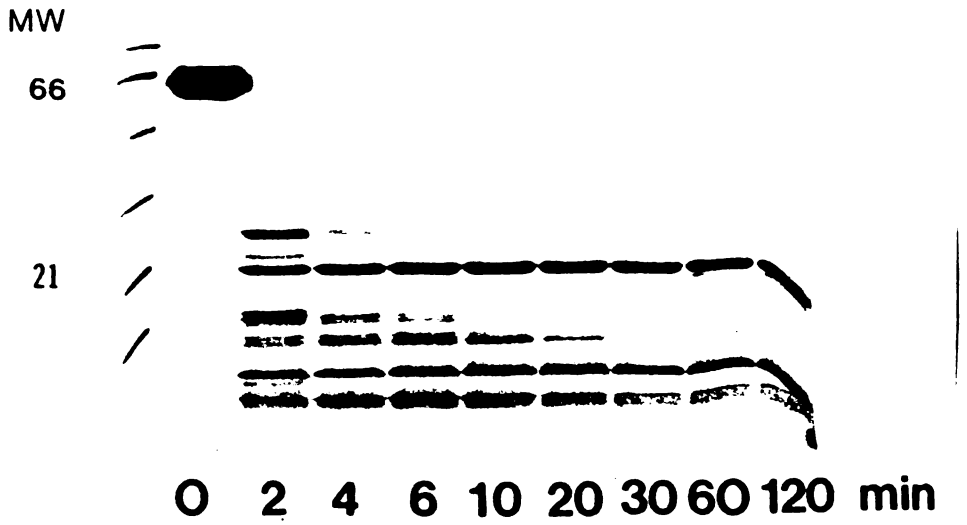


Fig. 1: Polyacrylamide gel (16%) of a colicin digestion. Lane 1 has low molecular weight standards, with 21kD and 66kD marked. Remaining lanes are colicin before the addition of *S. aureus* V8 protease (lane marked 0), and after 2, 4, 6,10,20,30,60, and 120 minutes of digestion. Note that colicin (MW 69kD), runs slightly aberrantly on a 16% gel.

completely disappeared from the gel, and a number of bands representing proteolytic fragments became apparent. At 2 hours, two major fragments that are more resistant to the action of the protease remain, one at approximately 20kD and the other at less than 14kD. After 19 hours at 37°, the strong 20kD band was still present on a gel (data not shown).

Following digestion, the fragments were separated on HPLC, as shown in Figure 2. Following separation by HPLC, fractions were analyzed by liquid SIMS. Figure 3 shows a portion of a typical mass spectrum of a single HPLC fraction. Multiple peptides are often present in a single fraction, but this generally presents no difficulty when using mass spectrometry, as the presence of each individual peptide is revealed by a peak in the mass spectrum at its specific mass. (For a recent comprehensive review of current developments in mass spectrometry, see Burlingame, et al., 1988). In the case of a known primary sequence, most of the peptides predicted from specific proteolytic enzyme cleavages have unique masses and are therefore easily identified. It does occur, however, that a small number of peptide pairs will, by chance, have very similar or identical masses and will therefore not be distinguishable on the basis of molecular mass measurements. Such cases occur with moderate frequency in relatively small proteins such as colicin, and with correspondingly greater frequency in digests of larger proteins. Some examples of such "isobaric peptides" from colicin Ia are shown in Table I. Any of these pairs is easily distinguishable by MS/MS (see below), which gives sequence information directly.

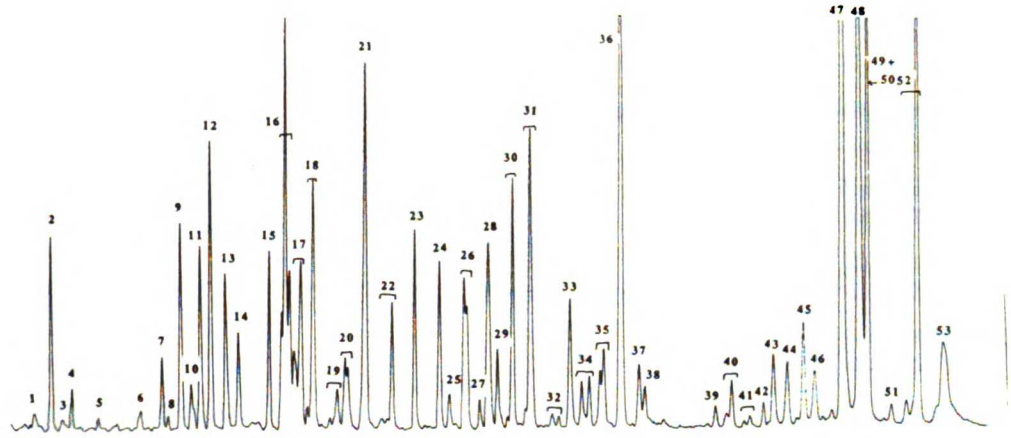


Fig. 2. HPLC chromatogram of *S. aureus* V8 digest of colicin Ia.

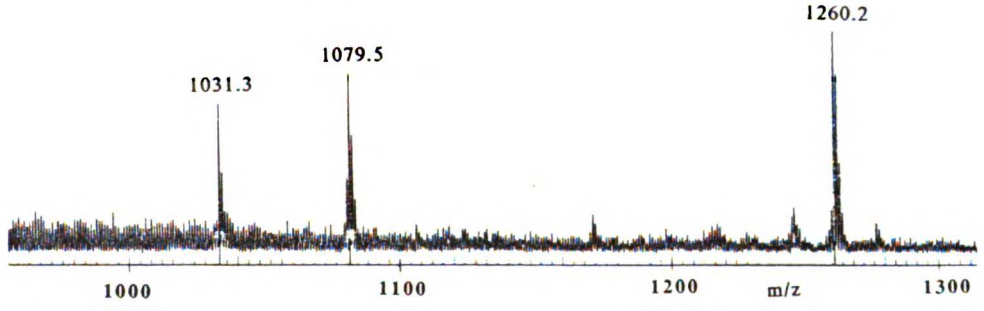


Fig. 3. A portion of the mass spectrum of Fraction 11. Three peptides were present in this fraction: AQNADKKAAD, m/z (calc. for MH^+) = 1031.5; SLGYDSDGHE, m/z (calc.) = 1079.4; and VVKPATMSNNAE, m/z (calc.) = 1260.6. The experimentally measured masses are noted above the peaks. The first two of these are predicted colicin peptides, while the third is an *S. aureus* V8 autodigestion product.

Tandem mass spectrometry was carried out on several representative peptides from the digest. In a tandem instrument, ions representing a variety of molecular species present in the sample are generated in the ion source of the first mass spectrometer (MS-1). A particular molecular species of interest is selected from this spectrum by means of the mass analyzer of MS-1, while ions representing other species are rejected (i.e., one selects only a single peak from the spectrum shown in Figure 3, for example). The selected ions are then focused into a collision cell for the purpose of generating structurally informative fragment ions via collision-induced unimolecular decomposition. Ions collected from the collision cell are refocused and mass-analyzed in a second mass spectrometer (MS-2). One thus obtains a clean parent plus daughter ion spectrum of one particular "pure" chemical species, with the secure knowledge that observed peaks are in fact logically related to the selected ions, with no interference from various background ions or other species present in the sample.

TABLE I. Examples of isobaric peptides in *S. aureus* digest of colicin Ia.

Sequence	Position/Length	Mass (MH^+)
WITE	551/4	548.2720
IMAVD	24/5	548.2754
KRQKD	188/5	674.3949
KRKQD	440/5	674.3949
SRMLFAD	162/7	839.4085
ALRLHTE	155/7	839.4739
FLKSVSEKYGAKAE	459/14	1556.8324
AGKRLSAAIAAREKD	98/15	1556.8872
RRKGILDTRLSELE	209/14	1685.9662
AGKRLSAAIAAREKDE	98/16	1685.9298

Figures 4 and 5 are MS/MS spectra of two peptides from the *S. aureus* V8 digest of colicin Ia. It has been established that high-energy gas collisions generate reproducible and interpretable fragmentation patterns from which the primary sequence of a peptide can be deduced (Biemann and Scoble, 1987; Johnson and Biemann, 1987; Johnson, et al., 1987; Crabb, et al., 1986; Anderegg, et al., 1988). In general, ion series are present that begin from both the C- and N-termini. Nomenclature for the various ion series observed has been summarized by Anderegg, et al.(1988). Some of the fragment ion mass differences that indicate the specific sequences present in Figures 4 and 5 are shown on the figures. Other ion series (not labeled in these figures) are also present, giving confirmatory evidence. It is clear from these spectra that covalent modification of any residue is easily detectable and that those residues that have been modified can be directly identified from the MS/MS spectrum. Furthermore, an MS/MS spectrum is rapidly obtainable on sub-nanomole quantities of material, even in the presence of other digestion fragments or other contaminants, and without regard to the presence of N-terminal blocking groups or other post-translational modifications of the peptide.

Figure 6 is a linear diagram of colicin Ia, showing those portions of the molecule that have been successfully mapped. Thus far, we have mapped approximately 67% of colicin Ia, using a combination of *S. aureus* V8 proteolytic digestion, HPLC separation, liquid SIMS, and MS/MS. Much of the remaining one third of the molecule that has not been mapped lies in the carboxy-terminal, channel-forming region. One explanation for the difficulty in mapping this region is that there are fewer appropriate cleavage sites in the C-terminal portion of the molecule. Alternatively, the results may indicate something of biological significance about colicin Ia. An analysis of the gel in Figure 1 shows several strong bands remaining after a 2-hour digestion in the

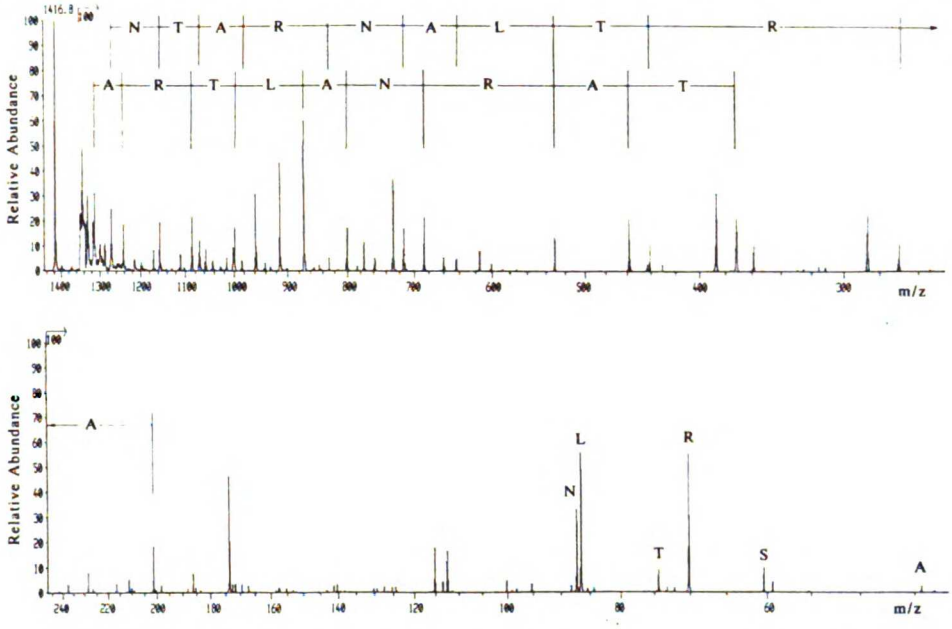


Fig. 4. MS/MS spectrum of the peptide SLNTARNALTRAE from Fraction 22, m/z (MH^+) = 1416.8. The spectrum contains several series of peaks from which the sequence can be confirmed or deduced. The series indicated in this figure are the "w" (upper) and "d" series (lower). The labeled low mass peaks provide confirmatory evidence of the presence of the residues noted.

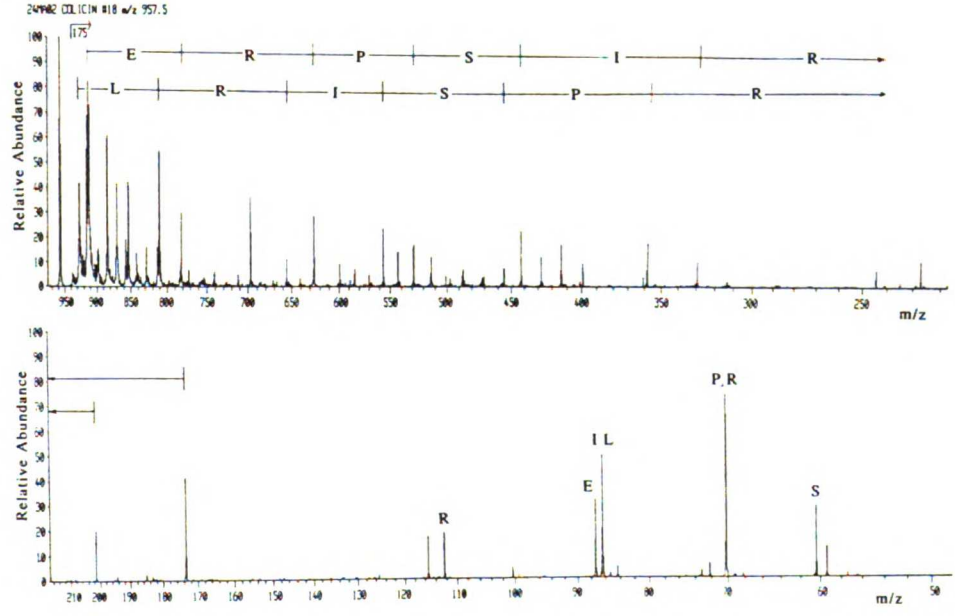


Fig. 5. MS/MS spectrum of the peptide SLRISPRE from Fraction 18, m/z (MH^+) = 957.5. The fragment ion series shown are the "a" series (upper) and the "w" series(lower).

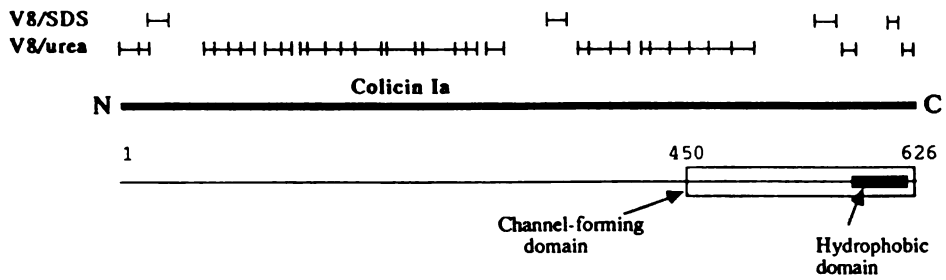


Fig. 6. Mapped portions of colicin Ia using enzymatic digestion and mass spectrometry. The *S. aureus* V8 digest with SDS produced all of the fragments that were found with *S. aureus* V8 protease in urea. The fragments labeled V8/SDS above represent additional pieces not found in the V8/urea digest.

presence of urea. Indeed, sequence analysis of the major band (20kD) reveals that, in fact, it corresponds to the channel-forming fragment of colicin Ia (Ghosh and Stroud, 1988). When SDS is used as a denaturant in the digestion, the 20 kD band is nearly absent from the gel, and addtional fragments are detected by HPLC and mass spectrometry. The resistance to digestion of this band suggests that this portion of the protein is folded very tightly in a domain-like structure.

Further support for this idea comes from electron microscopic studies of a 3-dimensional crystal lattice of colicin Ia (Choe and Stroud, 1988). An analysis of this crystal lattice has led these authors to propose a model for the structure of colicin in which three clearly visible sections of the molecule, as seen by EM, correspond to three functional domains (receptor-binding, translocation, and channel-forming), as described by biochemical studies (Konisky, 1982). Our present results support the idea that colicin Ia does have three functionally and structurally separate domains.

In this paper, we describe a strategy by which one can specifically define stuctural features of a molecule using standard biochemical analysis coupled with mass spectrometric techniques. We have proteolytically digested a bacterial ion channel, colicin Ia, and have identified the peptide fragments using liquid SIMS and MS/MS. Thus far, we have precisely mapped approximately two thirds of the molecule. Chemical modification of colicin Ia in its membrane-bound conformation, followed by MS/MS analysis to locate the modified residues will give us precise information about the structure of this ion channel. Mass spectrometry coupled with biochemistry promises to be an extremely powerful and important combination of techniques, allowing new insights into problems in structural biology.

Acknowledgement

The authors are indebted to F. C. Walls and B. L. Gillece-Castro for running the MS/MS spectra shown in Figures 4 and 5.

References

Aberth, W., Straub, K.M. and Burlingame, A.L. (1982) *Anal. Chem.* **54**, 2029-2034.
Anderegg, R. J., Carr, S.A., Huang, I. Y., Hiipakka, R.A., Chang, C. and Liao, S. (1988) *Biochem.* **27**, 4214-4221.
Biemann, K. and Scoble, H.A. (1987) *Science* **237**, 992-998.
Bowles, L.K., Miguel, A.G., and Konisky, J. (1983) *J. Biol. Chem.* **258**, 1215-1220.
Burlingame, A.L., Maltby, D., Russell, D.H., and Holland, P.T. (1988) *Anal. Chem.* **60**, 294R-342R.
Choe, S.Y. and Stroud, R. (1988) manuscript in preparation.
Cottrell, J.S. and Evans, S. (1987) *Anal. Chem.* **59**, 1990-1995.
Crabb, J.W., Armes, L.G., Carr, S.A., Johnson, C.M., Roberts, G.D. Bordoli,R.S.,and McKeehan, W.L. (1986) *Biochem.* **25**, 4988-4993.
Elliott, G., Gallagher, R., Evans, S., Ashcroft, A., Gooch, G., Wright, A.D., Walls, F. and Burlingame, A.L. (1988) 36th ASMS Conf. on Mass Spectrom. & Allied Topics, June 1988, San Francisco, CA, paper #WPB 38.
Falick, A.M., Wang, G.H. and Walls, F.C. (1986a) *Anal. Chem.* **58**, 1308-1311.
Falick, A.M., Walls, F.C., and Laine, R.A. (1986b) *Anal. Biochem.* **159**, 132-137.
Ghosh, P. and Stroud, R.M. (1988) manuscript in preparation.
Johnson, R.S. and Biemann, K. (1987) *Biochem.* **26**, 1209-1214.
Johnson, R.S., Martin, S.A., Biemann, K., Stults, J. T., and Watson, J.T. (1987) *Anal. Chem.* **59**, 2621-2625.
Konisky, J. (1978) in *The Bacteria* (Ornston, L., and Sokatch, J.R., Eds.) Vol. 6 Academic Press, New York.
Konisky, J. (1982) *Ann. Rev. Microbiol.* **36**, 125-144.
Mel, S., Wolberger, C., and Stroud, R. (1988) manuscript in preparation.
Weaver, C.A., Redborg, A.H., and Konisky, J. (1981). *J. Bact.* **148**, 817-828.

CHAPTER 3
PART 2

[^{125}I]TID LABELING OF COLICIN Ia

INTRODUCTION

As stated in Part 1 of this chapter, the overall strategy that we chose to map the transmembrane topography of colicin Ia was to proteolytically digest the soluble protein, to separate the peptide fragments using HPLC, and to determine the mass of each of these soluble peptides. Subsequently, we planned to chemically modify colicin Ia in its transmembrane form. Mass spectrometric analysis should localize the modifying agent to discrete peptides, based on an increase in the mass. The localization of these peptides in the sequence will give us precise structural information about the transmembrane structure of colicin Ia. Part 1 describes the mapping of the soluble form. A majority of the soluble peptides were identified (Part 1, Figure 6), though certain regions were not amenable to HPLC and/or mass spectrometry, despite a number of attempts at separation and solubilization using different solvents.

We decided to use a lipophilic probe to modify colicin Ia in its membrane bound conformation. A variety of lipophilic probes have been used with varying degrees of success to label regions of transmembrane proteins that insert into the membrane. One such probe, 3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) readily partitions into membranes and labels intrinsic membrane proteins in a highly selective manner (Brunner and Semenza, 1981). Many proteins have been labelled successfully, including influenza hemagglutinin (Harter, et al., 1988), rat brain

sodium channel (Reber and Catterall, 1987), and diphtheria toxin (Dumont and RIchards, 1988). Presented here are the results of experiments aimed at defining the transmembrane regions of colicin Ia using [^{125}I]TID.

[^{125}I]TID labels colicin Ia in membranes

METHODS

To determine if colicin Ia is in close contact with liposome membranes, 20 µl of asolectin liposomes (total of 30 nmoles phospholipid) and 2 µl of [^{125}I]TID (total of 2.5 µCi in EtOH) were incubated in a borosilicate glass tube for 10 minutes with 45 µl of 50 mM ammonium acetate buffer, pH 4. Two µl of [^{125}I]TID and 65 µl of the same buffer were incubated in parallel as a soluble control. After 10 minutes at room temperature, colicin Ia was added to each solution, to a final concentration of 0.09 mg/ml. Both solutions were then exposed to UV light at a distance of 4 cm, for 10 minutes. After UV activation, the protein was precipitated with trichloroacetic acid (TCA), centrifuged, washed, redisolved, and electrophoresed on a 10% SDS-PAGE gel, which was subsequently autoradiographed using XOMAT-AR film.

RESULTS

In the presence of negatively charged liposomes, colicin Ia becomes significantly labeled with [^{125}I]TID, indicating that regions

of the protein are closely associated with or inserted into the lipid bilayer (Figure 1). In contrast, in the absence of these liposomes, the amount of [^{125}I]TID labelling of soluble colicin Ia is significantly less. That some radioactivity is associated with soluble colicin Ia in the absence of lipid may be due to partitioning of [^{125}I]TID into hydrophobic pockets of the protein. Thus, at least some portions of colicin Ia are closely assoicated with the lipid bilayer, and these regions are the ones we seek to identify.

[^{125}I]TID labeling of colicin Ia is pH-dependent.

METHODS/RESULTS

To determine if colicin Ia is labeled to the same extent at different pH values, colicin Ia was incubated, as described above, in the presence of liposomes at pH 4 and at pH 8. After lipids were extracted from the samples, each pellet was solubilized in SDS and run on an SDS-PAGE gel. Individual protein bands were cut out and counted. The results of this experiment show that colicin Ia is labeled more efficiently at pH 4. Duplicate samples contained 1650 counts each, while the samples incubated with liposomes at pH 8 contained 400 counts each. The background level of radioactivity in the gel was approximately 130. Therefore, colicin Ia preferentially associates with liposomes and becomes labeled with this lipophilic probe, at low pH.

Optimization of labeling conditions.

METHODS/RESULTS

To optimize the labeling conditions, two further experiments were performed to determine if 1) increasing the incubation time pre-UV activation would increase protein labeling and 2) increasing the time of UV exposure would increase [^{125}I]TID labeling of colicin Ia.

Pre-UV incubation times were varied between 10 and 40 minutes. 80 µl (12 nmole of phospholipid/µl) of asolectin vesicles were incubated with 720 µl of buffer (as above) and 12 µl of [^{125}I]TID (1.25 mCi/ml) at room temperature for 10 minutes, at which time 8 µl of colicin Ia (stock solution of 6 mg/ml in NaCl/citrate buffer mentioned above) was added. 205 µl was aliquoted into each of four borosilicate glass tubes. Tubes 1-4 were allowed to sit 10, 20, 30, and 40 minutes respectively before the UV lamp was turned on. UV activation proceeded for 15 minutes. Lipids were extracted from each sample with a chloroform/methanol procedure and each of the protein pellets was run on a gel. Protein bands were cut out and counted.

Increasing the amount of pre-UV incubation time does not increase the amount of [^{125}I]TID associated with colicin Ia and we concluded that 10 minutes pre-UV incubation time was sufficient for detectable labeling of protein.

We further investigated the effect of increased times of UV exposure on [^{125}I]TID incorporation into colicin Ia. The experimental reagents were identical to those described in the previous paragraph. After a 10 minute pre-UV incubation, each of tubes 1-4 were exposed to UV light for 10, 20, 30, and 40 minutes respectively. The 10, 20, and 30 minute samples were frozen after UV exposure. At 40 minutes, lipids were extracted from all samples and the remaining protein pellets electrophoresed on a 16% gel, the protein bands cut out and the radioactivity quantitated. Increasing the time of UV exposure appears to increase [^{125}I]TID incorporation into colicin Ia up to 30 minutes, at which time a plateau is reached. Therefore, in subsequent experiments with [^{125}I]TID, we used a pre-UV incubation time of 10 minutes and a UV exposure time of 30 minutes.

Efficiency of [^{125}I]TID labeling; importance of lipid extraction.

Having established that colicin Ia does become labeled with [^{125}I]TID, it was of further importance to measure the ratio of [^{125}I]TID:colicin Ia molecules, to determine the feasability of using this probe to determine membrane topography.

METHODS

These experiments were performed by adding colicin Ia to liposomes that had been first incubated with [^{125}I]TID, then separating the lipid pellet from the supernatant by centrifugation to eliminate any soluble, non-membrane associated radioactivity, and subsequently running the protein on an SDS-PAGE gel and counting the radioactivity in the protein bands. 40 µl of asolectin vesicles (60 nmoles total phospholipid) were incubated for 7 minutes with 354 µl of 50 mM ammonium acetate buffer pH 4, and 6 µl of [^{125}I]TID (from same stock solution referred to in previous paragraph). A parallel sample with no [^{125}I]TID was included, to determine background levels of radioactivity. After 7 minutes, 4 µl of a 6 mg/ml solution of colicin Ia was added to each tube. Tubes were exposed to UV light for 30 minutes, with the light source at a distance of 4 cm. After UV exposure, each tube was vortexed, divided into 3 airfuge tubes, and spun for 15 minutes at 30 psi. The supernatants of each of the 6 tubes were discarded, the lipid pellets resuspended in sample buffer, then run on 16% SDS-PAGE gels. The protein bands were cut out and counted for radioactivity.

RESULTS

The original experiments to determine the [^{125}I]TID:colicin Ia stoichiometry were performed as described above, with both lipid and protein loaded together onto the gel. On the gels, the radioactive lipid runs close to the dye front while the colicin Ia protein band runs near the top. Under these conditions, we determined a

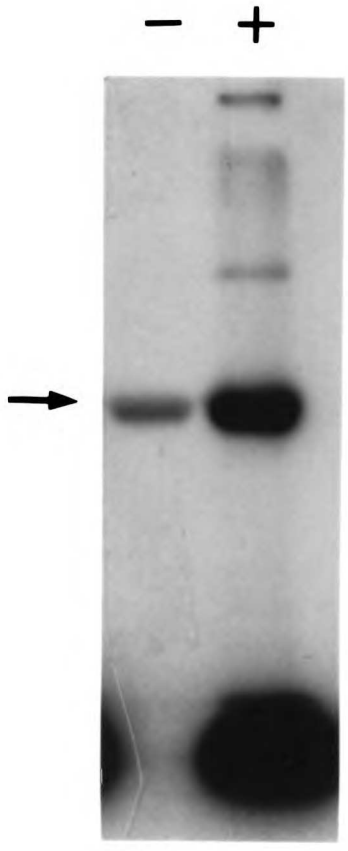
[^{125}I]TID:colicin ratio of approximately 0.1 [^{125}I]TID/colicin. While this ratio was similar to ratios that had been measured for other transmembrane proteins, we questioned whether or not the presence of some residual radioactive lipid attached to the protein might be giving erroneously high stoichiometries. Thus, the experiments were repeated, with a chloroform/methanol lipid extraction step (Wessel and Flugge, 1984) included after the 30 minute UV activation. This extraction step appears to be critical for correct assessment of [^{125}I]TID:colicin Ia stoichiometry, as the ratio dropped to 0.01 [^{125}I]TID/colicin, for a comparable amount of protein. Repeated experiments corroborated this ratio of approximately 0.01/1. We therefore concluded that in our experimental system, one out of approximately 100 colicin Ia molecules was becoming labeled with one molecule of [^{125}I]TID.

CONCLUSIONS

Several experiments were carried out in which colicin Ia was incubated with liposomes and [^{125}I]TID, proteolyzed, and the peptide fragments were separated by HPLC. HPLC separation of peptides did not yield any radioactive peaks that could be correlated to modified colicin Ia peptides. This is very likely due to the low efficiency of [^{125}I]TID labelling, as described above. While low resolution information, such as whether or not a protein is in the membrane, or whether a particular subunit of a protein is transmembranous can be

readily determined using this probe, the very low efficiency of [^{125}I]TID labelling in these experiments makes it impossible to gain high resolution structural information of colicin Ia using this method. Thus, we turned to proteolysis as a probe for membrane topography, as described in the following chapter.

Figure 1



1. [^{125}I]TID labeling of colicin Ia in the absence (lane 1) and presence (lane 2) of asolectin liposomes. Colicin Ia is indicated with an arrow. The radioactivity at the bottom of the autoradiogram is free lipid and the other radioactive bands in the presence of lipid are likely aggregated colicin Ia.

REFERENCES

Brunner, J. & Semenza, G. (1981) *Biochem. 20*, 7174-7182.

Dumont, M. E. & RIchards, F. M. (1988) *J. Biol. Chem. 263*, 2087-2097.

Harter, C., Bachi, T., Semenza, G. & Brunner, J. (1988) *Biochem. 27*, 1856-1864.

Reber, B. F. X. & Catterall, W. A. (1987) *262*, 11369-11374.

Wessel, D. & Flugge, U. I. (1984) *Anal. Biochem. 138*, 141-143.

CHAPTER 4

MAPPING THE MEMBRANE ASSOCIATED CONFORMATION OF COLICIN Ia

Stephanie F. Mel, Arnold M. Falick, Alma S. Burlingame, and Robert M. Stroud

Abbreviations:

CD, circular dichroism; Da, dalton; mA, milliamp; PA, phosphatidic acid; PC, egg phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis;

ABSTRACT

Channel-forming colicins exist in at least 2 different membrane-associated conformations, a voltage-independent closed-channel state and a voltage-dependent open-channel state. In a voltage-independent membrane-associated conformation, we find that two major regions of colicin Ia are protected from pepsin proteolysis, in the presence of negatively charged membranes. In contrast, colicin Ia is rapidly and completely proteolyzed in the absence of membranes. The major protected region includes an electrophysiologically defined C-terminal channel-forming domain as well as 96 residues upstream of this region. Approximately 100 residues spanning Ala 79 -~ Arg 189 within the N-terminal domain are protected as well. In addition to the first N-terminal 76 residues, a large region which includes much of the putative central receptor-binding domain is not protected from proteolysis. Both N- and C- termini of protected peptides have been identified using a combination of gel electrophoresis, N-terminal sequencing and mass spectrometry, thereby defining specific residues that are located outside of the lipid bilayer. These data, combined with the recent solution of the x-ray structure of the soluble form of colicin Ia (Ghosh, 1992), lend new understanding to the question of how this water soluble protein inserts into the hydrophobic interior of a lipid bilayer.

INTRODUCTION

A variety of soluble toxin molecules spontaneously inserts into the lipid bilayer of their target cell. Examples include toxins from the human pathogens *Corynebacterium diphtheriae*, *Clostridium tetani,* and *Vibrio cholerae*, (Donovan, et al., 1981; Holmgren, 1981; Bouquet and Duflot, 1982) as well as the channel-forming colicins, bacterial toxins which are synthesized in and secreted by *E. coli,* and are lethal to other bacteria (Konisky, 1982). These colicins have been widely used in studies aimed at understanding how water soluble proteins spontaneously insert into membranes.

The family of channel forming colicins, which includes colicins A, E1, Ia, Ib, K, and N, form lethal ion channels in a target bacterium (Konisky, 1982). After binding to specific bacterial outer membrane receptors, these colicins translocate across the outer membrane and periplasmic space, then insert into and form channels within the inner plasma membrane. The channels deplete the target cells of their electrochemical gradient and cause cell death.

In vitro, colicins A, E1, and Ia form relatively non-selective, voltage-gated ion channels in planar lipid bilayers upon application of a trans-negative voltage (cis is defined as the side of the bilayer to which protein is added, while trans is defined as the opposite side) (Schein, et al., 1978). These colicins also associate with membranes in the absence of a transmembrane voltage, binding to and releasing the contents of negatively charged liposomes at acidic

pH (Pattus, et al., 1983; Davidson, et al., 1985; Kayalar and Duzgunes, 1986; Mel, 1992). Studies with colicins A and E1 indicate that the structures of the voltage-dependent and voltage independent membrane-associated forms differ, however (Raymond, et al., 1986; Merrill and Cramer, 1990; Slatin, et al., 1986; Lakey, et al., 1991a; Lakey, et al., 1991b).

Proteolysis and deletion analysis studies on colicins A and E1 reveal that separate linearly arranged domains are responsible for each of the three functions, receptor binding, translocation across the outer membrane, and channel-formation (Ohno-Iwashita and Imahori, 1982; Martinez, et al., 1983; Brunden, et al., 1984; Cavard, et al., 1986; Liu, et al., 1986; Baty, et al., 1988). By analogy, colicin Ia likely contains 3 linearly arranged domains as well, due to shared function and 25-30% sequence identity between colicins A, E1, and Ia. Indeed, it has recently been shown that a C-terminal domain of colicin Ia beginning at Asp 451 forms voltage-dependent ion-conducting channels in planar lipid bilayers (Ghosh, 1992).

The majority of studies aimed at defining the transmembrane topography of colicins has been carried out on the 20 kD C-terminal channel-forming domains of colicins A and E1. The experiments described below have instead focused on identifying all regions of the 70 kD protein that associate with and are protected by a target membrane during membrane insertion, using a less well studied member of this colicin family, colicin Ia. The strategy employed

was to proteolytically digest all accessible regions of membrane-associated colicin Ia, then to identify the protected peptides using a combination of gel chromatography, N-terminal sequencing and mass spectrometric analysis. Proteolysis was carried out with pepsin, both because it functions at acidic pH and because it cleaves relatively non-specifically (Sachdev and Fruton, 1970), thereby allowing cleavage close to the membrane surface.

Proteases have been successfully used to identify transmembranous regions of a variety of proteins (reviewed in (Jennings, 1989)). In this study, electrospray mass spectrometry has been used to rapidly and precisely analyze the relatively large membrane-protected peptides generated by proteolysis of colicin Ia in the presence of membranes. We have identified 2 major regions of colicin Ia that are protected by the negatively charged membranes at low pH and in the absence of a transmembrane voltage. Within these regions, residues lying at the N- and C-termini of protected peptides necessarily map to the cis side of the lipid bilayer, thus placing constraints on structure of colicin Ia in this membrane-associated conformation. These results are discussed in the context of the recently solved structure of the soluble form of colicin Ia (Ghosh, 1992).

MATERIALS AND METHODS

Liposome Preparation. Reverse Phase Evaporation vesicles (REV's) were prepared by the method of Szoka & Papahadjopoulos (Szoka and Papahadjopoulos, 1978) using 10 µmole of either purified (Kagawa and Racker, 1971) asolectin (Associated Concentrates, NY), egg PC[1] (Avanti Polar Lipids, AL), or *E. coli* lipid extract (Avanti Polar Lipids, AL), 1 ml of diethyl ether and 1 ml of 50 mM ammonium acetate buffer, pH 4.1.

Pepsin digestion and peptide separation. Colicin Ia was expressed and purified from *E. coli* JK365 (Mel, 1992). For identification of peptide fragments that are protected after 2 hours of digestion, typically 7 nmoles of colicin Ia was added to liposomes to a final concentration of 5.4 µM at a lipid:protein molar ratio of 1500:1, in 50 mM ammonium acetate, pH 4.1. After a 5 minute incubation at room temperature, pepsin was added to a final pepsin:colicin molar ratio of 3:1, and samples were incubated at 37° C. After 2 hours, aliquots of 70 µl-110 µl of the digestion mixture were immediately added to 400 µl of methanol for subsequent lipid extraction (Wessel and Flugge, 1984). After extraction of lipids, peptides were dried in a hood, re-solubilized in sample buffer (Laemmli, 1970), and separated on 16% Tricine-SDS-PAGE gels (Schagger and von Jagow, 1987) which offer improved separation of components of M_r less than 14 kD. Pepsin was added at a final concentration of 19 µM. after 2 hours in each of the time courses.

Identification of N-terminal sequences and masses of protected fragments. For N-terminal sequence analysis of protected fragments, gels were blotted onto Immobilon-P Transfer Membranes (Millipore, Bedford, MA) for 90 minutes at 100 mA, using a TE 70 SemiPhor Semi-Dry Transfer Unit (Hoefer Scientific Instruments, CA) and sequenced directly from Immobilon using Edman degradation. The number of residues sequenced for each gel band is indicated in Table 1. For mass spectrometric analysis, bands were electroeluted from gels using a Model 422 Electro-Eluter (Bio-Rad Laboratories, CA.) in 50 mM ammonium bicarbonate, 0.1% SDS buffer, at 60 mA for 90 minutes. Samples were lyophilized after electroelution and sonicated in 5 µl of formic acid and 3 µl of hexafluoroisopropanol, then immediately analyzed by electrospray mass spectrometry.

Mass Spectrometry. Electrospray mass spectrometry was carried out on a Fisons/VG Bio-Q mass spectrometer, using a 50:50 (v:v) mixture of acetonitrile and water, in 1% acetic acid. Samples were injected in the formic acid/hexafluoroisopropanol solution described above.

RESULTS

Regions of colicin Ia are protected from proteolysis upon association with membranes. Using proteolysis as a probe of

membrane-associated structure, specific regions of colicin Ia that are resistant to pepsin digestion after association with negatively charged membranes at low pH were identified. After vigorous proteolysis (pepsin:colicin Ia molar ratio of 3:1 for 1 hour at 37° C), several protein fragments remain protected in the presence of asolectin liposomes (Figure 1, lanes 1-5). Three major digestion products, at approximate M_r of 23 kD, 15 kD, and 13 kD, as well as a smear of lower molecular weight components remain protected for at least 60 minutes (Figure 1, Lane 5). In contrast, despite a decreased pepsin:colicin ratio, no protected regions are detectable after 10 minutes of digestion in the absence of liposomes, indicating that colicin Ia is rapidly proteolyzed (Figure 1, lanes 6-9). The differences between digestions in the presence and absence of lipid are not due to inhibition of pepsin activity by lipid. This was shown in a separate experiment where the extent of cleavage of a pepsin substrate (sequence ATLNFPISPW) in the presence and absence of asolectin lipids yielded identical results (data not shown). The protective effect appears to be specific to negatively charged lipids, as colicin Ia digested in the presence of vesicles made from neutral egg PC results in no detectable protected regions (data not shown), identical to the results observed with soluble colicin Ia in the absence of membranes (Figure 1, lanes 6-9). Since colicin Ia will release the contents of negatively charged asolectin liposomes but not those of liposomes made from the neutral lipid egg PC (Mel, 1992), the fragments that are protected after

associating with negatively charged membranes are likely to be those portions of the molecule that are functionally important in membrane attachment, membrane insertion, or ion-channel formation.

Identification of protein sequences protected by the membrane. Several of the major colicin Ia fragments that remain protected by negatively charged asolectin membranes after 2 hours of pepsin digestion have been identified. These fragments, labeled A-E (Figure 2a), correspond to peptide fragments of Mr 20 kD, 14 kD, 10 kD, ~9 kD and ~8 kD, as determined by SDS-Tricine gel electrophoresis. (The 20 kD and 14 kD bands contain the same peptide as the 23 kD and 15 kD bands of Figure 1, Lane 5, based on N-terminal sequence analysis; data not shown). The same pattern of protection is observed if pepsin proteolysis is carried out in liposomes made from *E. coli* rather than asolectin lipids, as fragments corresponding to A-E (Figure 2b, see arrows) are visible after 30 minutes of digestion (Figure 2b, lane 2). This indicates that colicin Ia associates with both types of membranes in a similar conformation. The rate of proteolysis is more rapid in the presence of *E. coli* liposomes, however, as bands A and B disappear after 2 hours in *E. coli* but remain protected by asolectin for up to 3 hours, even with re-addition of pepsin (Figure 2c). While there were small variations in the abundance of digestion products when comparing different digestions in the presence of asolectin liposomes, the major

protected fragments always included those bands labeled A-E (Figure 2a).

Bands A and B. Bands A and B (Figure 2a) are single peptides, as determined by Edman N-terminal sequence analysis and electrospray mass spectrometry (Table 1). Since the peptide in band A begins at Asp 358 and has a measured mass of 18,122 +/- 10 Da (Figure 3), its C-terminus is therefore Ala 517 (Table 1). Similarly, the single peptide in band B begins at Ser 402, has a mass of 13,134 +/- 10 Da, and therefore also extends to Ala 517 (Table 1). Both peptides contain a portion of an 18 kD channel-forming region of colicin Ia, whose amino terminus is Asp 451 and whose C-terminus is probably the C-terminus of colicin Ia (Ghosh, 1992). In addition, 93 and 49 residues upstream of this 18 kD channel-forming region are protected in fragments A and B, respectively.

Band C. Band C (Figure 2a) is a mixture of several peptides (Table 1), at least three of which begin at successive positions Leu 418, Leu 419, and Lys 420, respectively. Heterogeneity within this sample results from the relatively non-specific proteolytic activity of pepsin, which generally cuts at either side of large aromatic or hydrophobic residues such as Phe, Met, Leu, or Trp, although there are exceptions to this specificity (Sachdev and Fruton, 1970). The presence of several different residues in each sequencing cycle (Table 1) made it difficult to unequivocally identify the N-termini of

components of this mixture using N-terminal sequencing alone. However, in combination with electrospray mass spectrometric analysis, the identity of the peptides in this fraction was confirmed. The mass spectrometry profile reveals several peaks, in three clusters of 2 peptides each (Figure 4a). The masses of the smaller peaks of these clusters, 11,187, 11,298, and 11,412 Da, (Figure 4a) correspond exactly to peptides beginning at Leu 418, Leu 419, and Lys 420, each extending to Ala 517 (Table 1 and Figure 4b).

The masses of the larger peaks (masses 11,204, 11,313, and 11,428 daltons) are all approximately 16 Da +/- 1 greater than the series identified above (Figure 4a). As the sequence between Asn 416 and Ala 517 contains a single methionine, a residue which is readily oxidized, it is probable that these larger peaks also correspond to fraction C peptides, each containing an oxidized methionine.

Band D. Band D (Figure 2a) is also a mixture of peptides, two of which begin at Ala 79 and Tyr 80, respectively (Table 1). Residues consistent with a third peptide beginning at Ser 85 were also present during sequencing, though in lesser amounts (Table 1). Peptides in this band have an approximate mass of 11,450 +/- 250 Da, but precise masses could not be assigned due to sample heterogeneity. Peptides of 11,450 +/- 250 Da that begin at Ala 79 and Tyr 80 would terminate at approximately Ala 179 - Arg 189.

Band E. Band E is very heterogeneous as determined by N-terminal sequencing, with too many peptides present to identify any with absolute certainty. However, peptides beginning at Ser 85, Ala 86, Gln 87 and Glu 521 best account for the majority of residues observed by N-terminal sequencing (Table 1). As bands D and E are not well resolved by electrophoresis, several peptides were common to both bands (peptides beginning at Ala 79 and Tyr 80).

Mass spectrometric data on fraction E also reflected a large degree of heterogeneity, and specific masses could not be assigned to individual peptides. However, the peptides do fall within a mass range of 11,700 +/- 400 Da. Therefore, the C-termini of the protected fragments beginning at Ser 85, Ala 86, and Gln 87 would extend to the region between Glu 184 and Gln 191. A peptide beginning at Glu 521 and extending to the C-terminus of colicin Ia would have a mass of 11,356 Da, consistent with the observed mass range in this fraction.

Summary of protected regions. A summary of the protected peptides is presented in Figure 5a. Peptides identified with certainty span a region which begins at Asp 358 and continues through Ala 517, as well as a region which spans ~100 residues of the N-terminal domain beginning at Ala 79. Sequencing data are also consistent with a protected peptide beginning at Glu 521 and continuing to the C-terminus of the molecule. In contrast, 2 regions (residues 1-77 and residues 191-358) contain no protected peptides, indicating that

these regions are not membrane-associated. The approximate location of the translocation, receptor binding, and channel-forming domains are indicated in Figure 5b.

DISCUSSION

We have identified 2 major regions of colicin Ia that are protected after association with negatively charged asolectin membranes, the largest of which begins at Glu 358 and likely continues to to the C-terminus of colicin Ia. Within this region lies a C-terminal fragment (Asp 451 - Ile 626) which forms ion-conducting channels in planar lipid bilayers (Ghosh, 1992). The conductance properties of these channels differ substantially from those of whole colicin Ia channels, however, suggesting that residues other than Asp 451- Ile 626 are involved in channel formation. As the 93 residues lying directly upstream of Asp 451 are tightly associated with negatively charged membranes, it seems likely that these residues may play a role in channel-formation. This is consistent with the recently solved x-ray structure of whole colicin Ia, which reveals α–helical protein density immediately adjacent to a 10-helix bundle (Ghosh, 1992), the motif identified as the channel-forming domains of both colicins A and E1 (Parker, et al., 1989; Wormald, et al., 1990; Parker, et al., 1992.

The involvement of residues other than those in the C-terminal domain in channel formation has been suggested for colicins A and

E1. Part of the receptor binding domain of colicin A interacts with the channel-forming domain in a pH-dependent fashion, based on fusion and surface pressure studies in planar lipid bilayers and liposomes (Collarini, et al., 1987; Frenette, et al., 1989). Furthermore, an interaction between the C-terminal channel forming domain and the central domain of colicin A has been identified using colicin A-E1 hybrid molecules (Benedetti, et al., 1991). Proteolytic digestion of colicin E1 in the presence and absence of a transmembrane voltage reveals that a region upstream of the channel-forming fragment affects channel-gating (Raymond, et al., 1986). While regions adjacent to the C-terminal domain have been implicated in membrane insertion in each of the above studies, these colicin Ia data provide the first evidence that a portion of the N-terminal domain is membrane-associated as well.

The precise length of the protected region within the N-terminal domain of colicin Ia is not completely defined, though residues 79 - ~190 are resistant to proteolysis. The fact that part of the putative N-terminal translocation domain is protected by membranes suggests that this region may be located near the channel-forming domain in the three dimensional structure of colicin Ia. The C- and N-terminal protected domains may be juxtaposed due to structural constraints, thus forcing the N-terminal portion to be against the membrane and protected. Indeed, Ser 171, which lies within this protected region, appears to be

located near the end of an α–helix that is adjacent to the channel-forming domain in the x-ray crystal structure (Ghosh, 1992).

Colicin Ia associates with membranes in the absence of a transmembrane voltage in these experiments. The voltage-independent membrane-associated conformation of colicins A and E1 has been defined as a "closed" channel state. This state is characterized by large portions of the protein being exposed to the outside or cis side of liposomes or lipid bilayers, as shown by proteolysis (Slatin, et al., 1986) and fluorescence spectroscopy (Lakey, et al., 1991a; Lakey, et al., 1991b) respectively. One model suggests that colicins lie on the membrane surface in an "umbrella" conformation (Parker, et al., 1990). This is in contrast to an "open" channel state, in which portions of the protein are driven into the lipid bilayer in response to a transmembrane voltage as measured by proteolysis (Raymond, et al., 1986) or labeling with a lipophilic probe (Merrill and Cramer, 1990). Thus, colicin Ia is likely in the intermediate voltage-independent closed-channel state in these experiments, with the majority of protein associated with the surface of the lipid bilayer, rather than inserted across the lipid bilayer. Of note is that the amphipathic α–helices that will eventually form the channel are already protected by the membrane in the intermediate, voltage-independent conformation, poised to insert into the membrane upon application of a transmembrane voltage.

We have generated a model for the voltage-independent membrane-associated conformation of colicin Ia (Figure 6). The overall structure is α–helical, based on circular dichroism of colicin Ia in the presence of a hydrophobic detergent environment (Mel, 1992). The lengths of the helices are based on the x-ray crystal structure of the channel fragment of the related molecule colicin A (Parker, et al., 1989; Parker, et al., 1992) and on secondary structure prediction (Stroud PREDICT program), with one exception. This exception is residues 517 - 521 of colicin Ia, which lie within helix #4 of colicin A according to the sequence alignment of Parker et al (Parker, et al., 1989). As pepsin definitely cuts at Ala 517 and likely cuts at Ala 521, Helix #4 of colicin A is instead modeled as a loop region in colicin Ia. By analogy with colicins A and E1, the stretch of 39 hydrophobic residues, Thr 573 - Ile 612, likely spans the membrane as an α–helical hairpin, even in the absence of a transmembrane voltage (Massotte, et al., 1989; Song, et al., 1991). This helical hairpin is not sufficiently long to span the lipid bilayer completely, however, as colicin Ia carries a deletion of 10 residues within this region, when compared with colicin A. Finally, all proteolytic cut sites are indicated with arrows and residue numbers, and are modeled as loop regions between helices.

Three of the protected peptides, A, B, and C, share a common C-terminus at Ala 517, but have different N-termini (Asp 358, Ser 402, Leu 418, Leu 419, Lys 420). That protected sub-fragments of a larger fragment are detected probably reflects different pepsin

cleavage rates at different sites. Differences in cleavage rates were also observed when comparing proteolysis in the presence of liposomes made from *E. coli* with that done in the presence of asolectin lipids. This result could be explained by differential access of pepsin to the surface of these vesicles. The *E. coli* lipid mixture is composed of three major lipid components, PE, PG, and cardiolipin, which together constitute 96% of the membrane lipids (Gennis, 1989). In contrast, asolectin contains a larger variety of components, including ~77 % phospholipids (a mixture of PA, PC, PE, and PI), 13% glycolipids, and 9% plant sugars. As sugar groups tend to be large and bulky, the surface of liposomes made from asolectin will likely be less accessible to pepsin than that of liposomes made from *E. coli* lipids alone. This could result in a decreased rate of proteolysis in the presence of asolectin lipids.

For the first time, both the x-ray structure and a membrane-associated structure of a whole colicin molecule are known. Coupling these data have led to the suggestion that the channel-forming domain of colicin Ia is larger than the equivalent domains of colicins A and E1. Furthermore, for the first time, we find that a portion of the molecule within the N-terminal domain is closely associated with membranes as well. Thus, in the case of colicin Ia, we have increased our understanding of the membrane insertion process by studying the whole molecule rather than a channel-forming fragment.

Figure Legends:

1. Time course (in minutes) of pepsin digestion of colicin Ia in the presence (lanes 1-5) and absence (lanes 6-9) of asolectin liposomes. 0.1 minute reflects the time point taken immediately after pepsin addition. In the presence of lipids, the digestion was carried out at a lipid:protein molar ratio of 1500:1 and a 3:1 molar pepsin:colicin ratio. In the absence of lipids, the pepsin:colicin molar ratio is 2:1. Samples containing approximately 4 µg of protein were added immediately to sample buffer at the appropriate digestion time, then loaded directly onto a 16% SDS-PAGE gel (Laemmli, 1970) without lipid extraction. Molecular weight standards are indicated on the left and pepsin is indicated with an *.

2. A) Colicin Ia peptides that remain protected after 2 hours of pepsin digestion in the presence of asolectin liposomes. The bands labeled A-E are those that have been identified. B) Time course of digestion in liposomes made from *E. coli* lipids. Arrows indicate the protected fragments that migrate at the same M_r as bands A-E. C) Time course of digestion in liposomes made from asolectin lipids. Arrows again indicate fragments A-E. Separation was carried out on 16% SDS-Tricine gels with approximately 40 µg of protein loaded in panel A and approximately 27 µg of protein loaded onto each lane in panels B and C. The numbers at the top refer to minutes of digestion

for panels B and C and pepsin is indicated by an *. Molecular weight standards are indicated at the left of panel A and between panels B and C. Pepsin was added at 120 minutes in B and C as described in Materials and Methods.

3. Electrospray mass spectrum of single peptide in Band A. The family of peaks observed arises from the same peptide having different numbers of protons attached. The measured mass of this peptide is 18,122 +/- 10 Da.

4. A) Deconvoluted electrospray (Mann, et al., 1989) mass spectrum of peptides in band C. The four clusters represent pairs of peptides which differ by 16 +/- 1 Da. The second peak in each cluster is likely an oxidized version of the first peak in each cluster. Underlined masses correspond to peptides illustrated in part B. B) Schematic of the N- and C-termini of the protected peptides from band C.

5. A) Schematic of protected peptides after 2 hours of pepsin digestion in the presence of asolectin liposomes. The solid lines represent termini identified with certainty and the dotted lines represent those that were not. B) Approximate locations of putative domains.

6. Model of colicin Ia in its membrane-associated conformation. Hydrophobic helices are black, amphipathic helices are cross-hatched, and polar helices are white. The numbers and solid arrows indicate the location of definite proteolytic sites while the dashed arrows are likely proteolytic sites.

Acknowledgements:

We would like to thank Dianne DeCamp for assistance with the protease activity assay and Ralph Reid for assistance with N-terminal sequencing. We would further like to thank Debbie Zimmerman and Partho Ghosh for helpful comments on the manuscript and Larry Miercke for many helpful discussions.

Table 1

GEL BAND	SEQUENCING CYCLE[a]										estimated pmoles[b]	OBSERVED MASS (Da)	CALCULATED C-TERMNUS	CALCULATED MASS (Da)
	1	2	3	4	5	6	7	8	9	10				
A	D	V	E	G	D	-	-	-	-	-	4	18,122 +/-10	ALA 517	18,118
	358													
B	S	A	R	T	N	-	-	-	-	-	4	13,134 +/-10	ALA 517	13,134
	402													
C	L	L	K	E	K	E	N	I	R	N	1 (0.2)	11,412 +/-10	ALA 517	11,412
	418													
	L	K	E	K	E	N	I	R	N	Q	0.9 (0.3)	11,298 +/-10	ALA 517	11,299
	419													
	K	E	K	E	N	I	R	N	Q	L	0.4 (0.1)	11,187 +/-10	ALA 517	11,186
	420													
D	A	Y	K	N	T	L	S	-	-	-	2.4 (0.3)	11,450 +/-250	ALA 179 - ARG 189	
	79													
	Y	K	N	T	L	S	A	-	-	-	2.4 (0.3)	SAME	SAME	
	80													
	s	a	q	q	(k)	e	n	-	-	-	1.1 (0.2)	SAME	SAME	
	85													
E	s	a	q	q	( )	e	n	r	n	( )	1 (0.3)	11,700 +/-400	GLU 184 - GLN 191	
	85													
	a	q	q	k	e	n	e	n	( )	r	1.1 (0.3)	SAME	SAME	
	86													
	q	q	k	e	n	e	n	( )	r	t	1.1 (0.3)	SAME	SAME	
	87													
	e	s	v	k	l	s	d	l	s	s	0.8 (0.3)	SAME	ILE 626	11,356
	521													
	y	( )	n	t	l	s	a	q	q	( )	0.5 (0.1)	SAME	GLU 184 - GLN 191	
	80													
	a	y	k	n	t	l	s	a	q	q	0.5 (0.2)	SAME	SAME	
	79													

Table 1 Legend:

[a]Uppercase bold letters represent those sequences identified with certainty. Lowercase non-bold letters identify sequences that are likely present in bands D and E, with the residues in parentheses too weak to identify. The number under the first residue of each peptide identifies that position of that residue in the colicin Ia sequence.
[b]The number of pmoles of each peptide was determined by averaging the pmole quantities of single residues in the peptides. The single peptides in bands A and B comprised > 95% of all material sequenced and those in bands C, D, and E together comprised >80% of all material sequenced in each case.

Figure 1

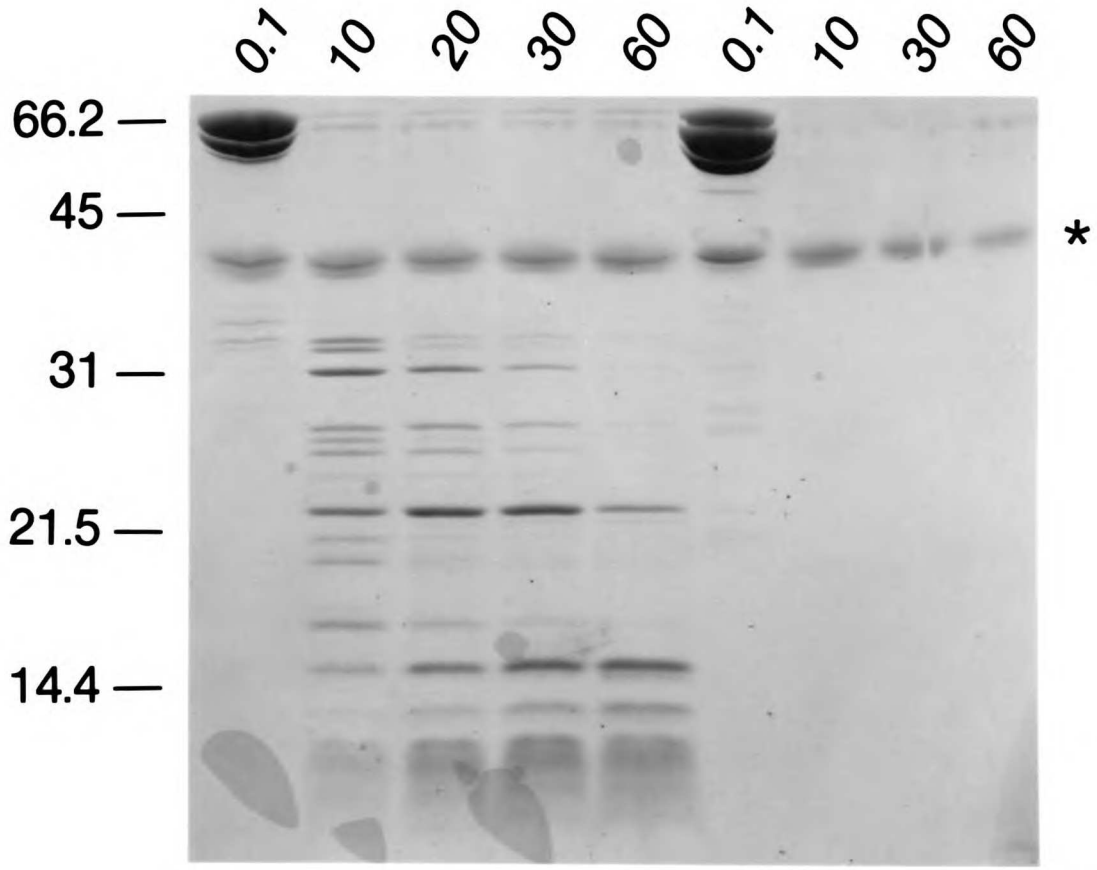


Figure 2

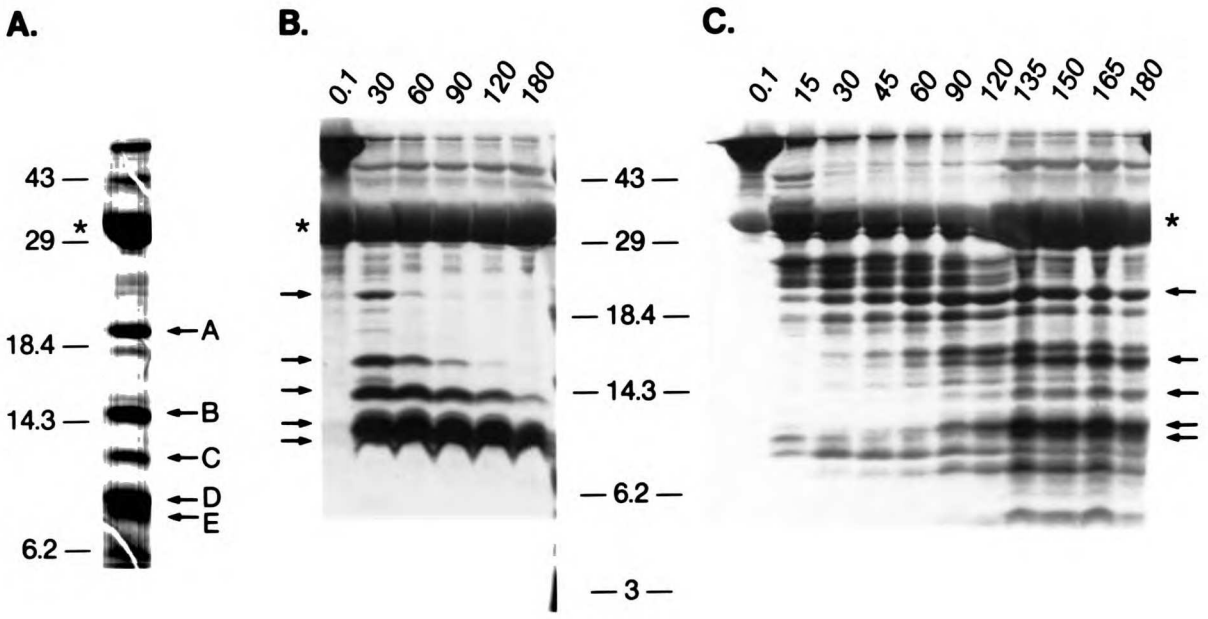


Figure 3

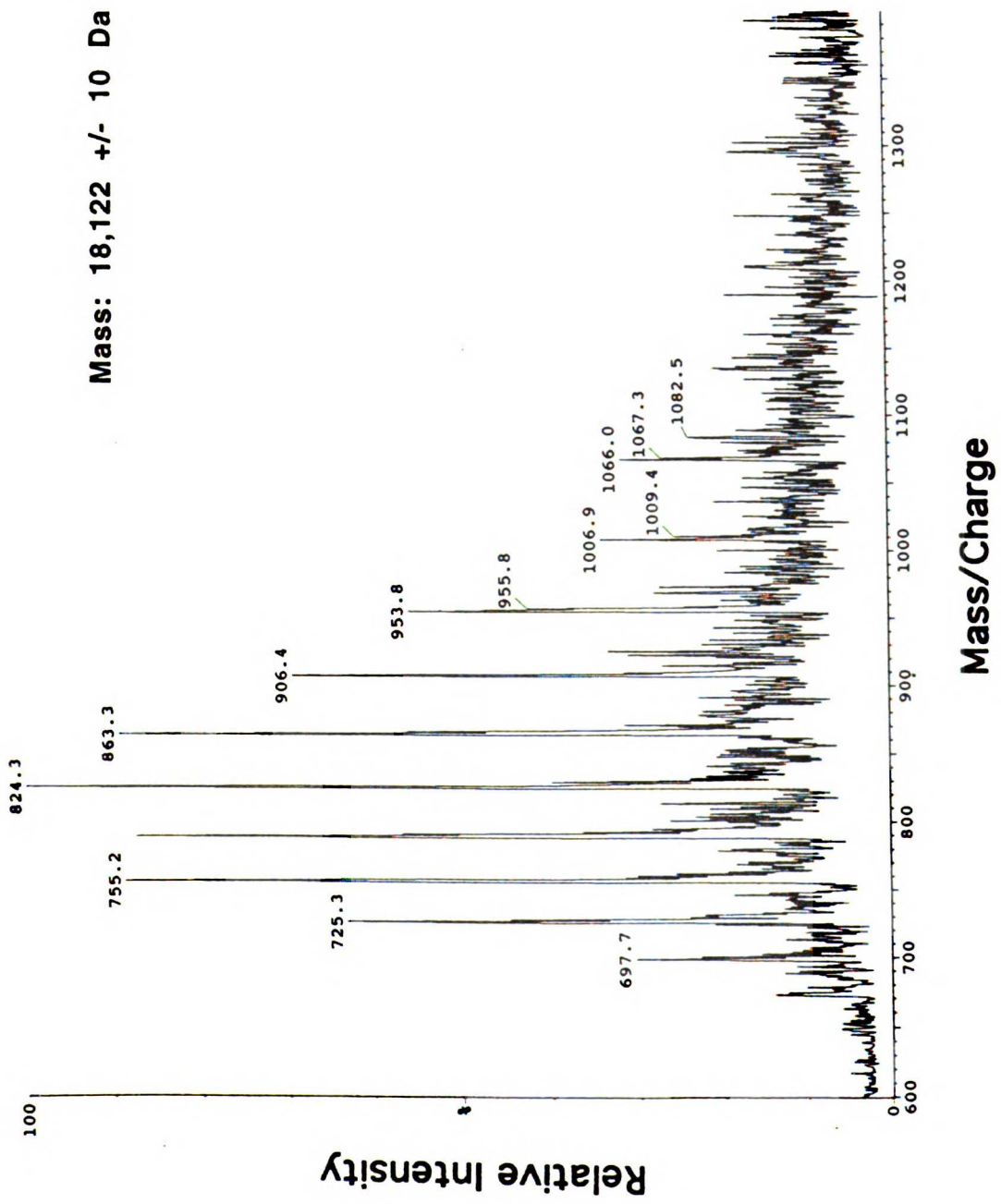
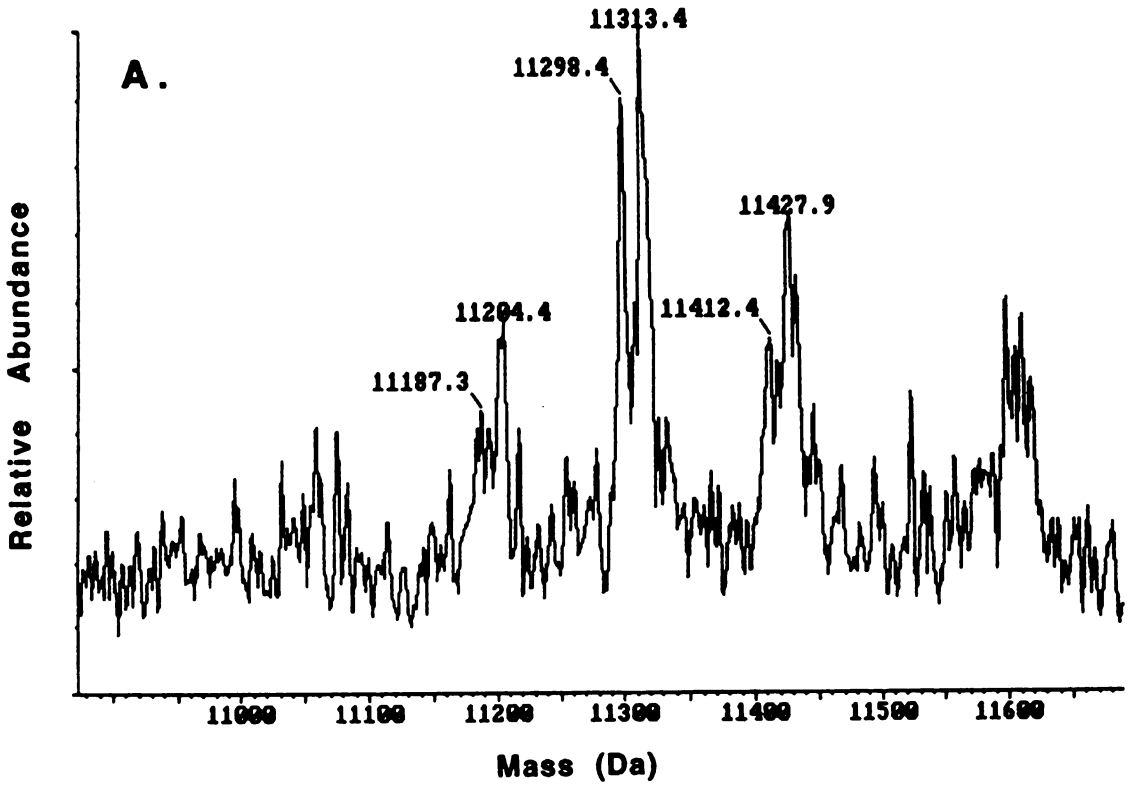

Mass: 18,122 +/- 10 Da
Relative Intensity
Mass/Charge
100
0
600
700
800
900
1000
1100
1200
1300
824.3
863.3
755.2
906.4
725.3
953.8
697.7
955.8
1006.9
1066.0
1009.4
1067.3
1082.5

Figure 4



B.

MASS
11,187
11,298
11,412

Figure 5

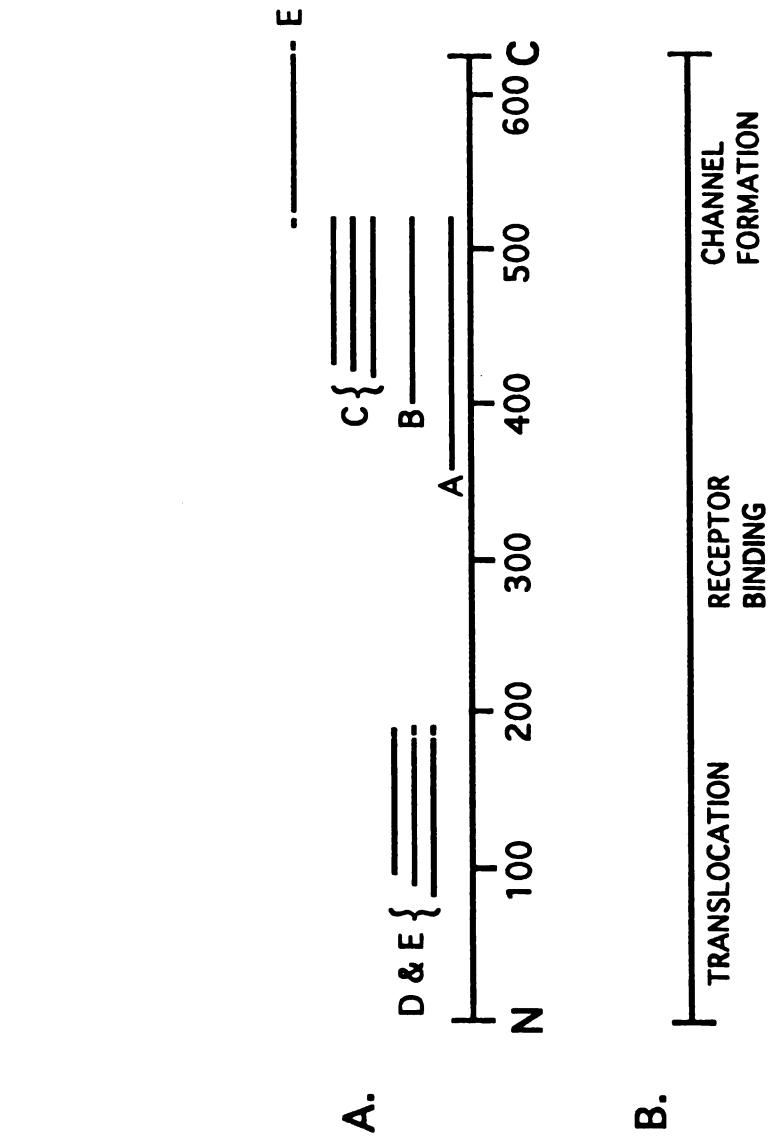
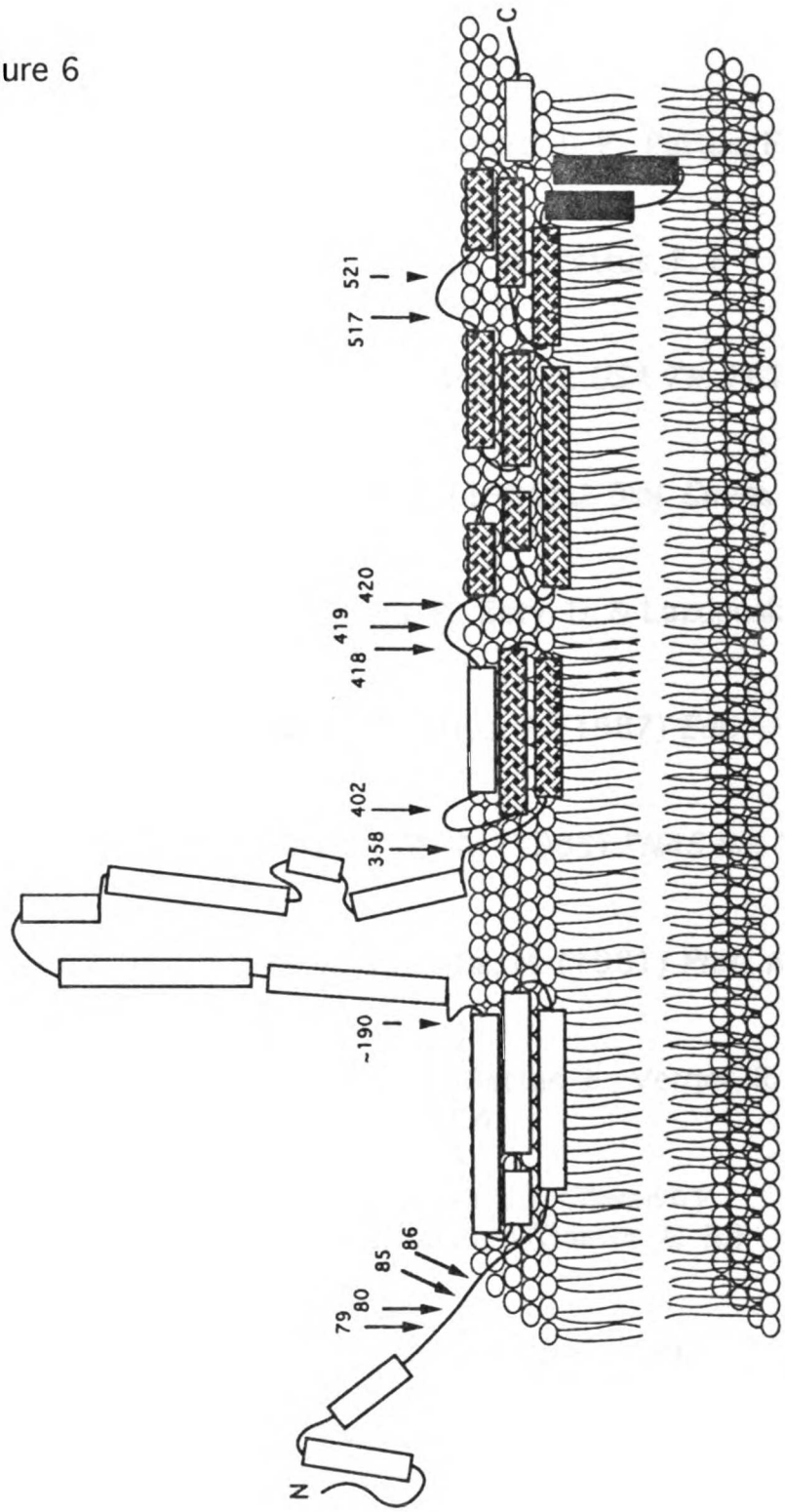

A.
N
100
200
300
400
500
600
C
D & E
B
C
E
B.
TRANSLOCATION
RECEPTOR BINDING
CHANNEL FORMATION

Figure 6



REFERENCES

Baty, D., Frenette, M., Lloubes, R., Geli, V., Howard, S. P., Pattus, F. & Lazdunski, C. (1988) *Mol. Micro. 2*, 807-811.

Benedetti, H., Frenette, M., Baty, D., Knibiehler, M., Pattus, F. & Lazdunski, C. (1991) *J. Mol. Biol. 217*, 429-439.

Bouquet, P. & Duflot, E. (1982) *Proc. Natl. Acad. Sci. USA 79*, 7614-7618.

Brunden, K. R., Cramer, W. A. & Cohen, F. S. (1984) *J. Biol. Chem. 259*, 190-196.

Cavard, D., Crozel, V., Gorvel, J.-P., Pattus, F., Baty, D. & Lazdunski, C. (1986) *J. Mol. Biol. 187*, 449-459.

Collarini, M., Amblard, G., Lazdunski, C. & Pattus, F. (1987) *Eur. Biophys. J. 14*, 147-153.

Davidson, V. L., Brunden, K. R. & Cramer, W. A. (1985) *PNAS 82*, 1386-1390.

Donovan, J. J., Simon, M. I., Draper, R. & Montal, M. (1981) *Proc. Natl. Acad. Sci. USA 78*, 172-176.

Frenette, M., Knibiehler, M., Baty, D., Geli, V., Pattus, F., Verger, R. & Lazdunski, C. (1989) *Biochem. 28*, 2509-2514.

Gennis, R. B. (1989) in *Springer Advanced Texts in Chemistry, Biomembranes: Molecular Structure and Function* (Cantor, C. R.) Springer-Verlag, New York.

Ghosh, P., Ph.D., University of California, San Francisco, 1992.

Holmgren, J. (1981) *Nature 292*, 413-417.

Jennings, M., L. (1989) *Ann. Rev. Biochem. 58*, 999-1027.

Kagawa, Y. & Racker, E. (1971) *J. Biol. Chem. 246*, 5477-5487.

Kayalar, C. & Duzgunes, N. (1986) *Biochim. Biophys. Acta 860*, 51-56.

Konisky, J. (1982) *Ann. Rev. Microbiol. 36*, 125-144.

Laemmli, U. K. (1970) *Nature 227*, 680-685.

Lakey, J. H., Baty, D. & Pattus, F. (1991) *J. Mol. Biol. 218*, 639-653.

Lakey, J. H., Massotte, D., Heitz, F., Dasseux, J.-L., Faucon, J.-F., Parker, M. W. & Pattus, F. (1991) *Eur. J. Biochem. 196*, 599-607.

Liu, Q. R., Crozel, V., Levinthal, F., Slatin, S., Finkelstein, A. & Levinthal, C. (1986) *Proteins 1*, 218-229.

Mann, M., Meng, C. K. & Fenn, J. B. (1989) *Anal. Chem. 61*, 1702-1708.

Martinez, M. C., Lazdunski, C. & Pattus, F. (1983) *EMBO J. 2*, 1501-1507.

Massotte, D., Dasseux, J.-L., Sauve, P., Cyrklaff, M., Leonard, K. & Pattus, F. (1989) *Biochem. 28*, 7713-7719.

Mel, S. F., Ph.D., University of California, San Francisco, 1992.

Merrill, A. R. & Cramer, W. A. (1990) *Biochem. 29*, 8529-8534.

Ohno-Iwashita, Y. & Imahori, K. (1982) *J. Biol. Chem. 257*, 6446-6451.

Parker, M. W., Pattus, F., Tucker, A. D. & Tsernoglou, D. (1989) *Nature 337*, 93-96.

Parker, M. W., Postma, J. P. M., Pattus, F., Tucker, A. D. & Tsernoglou, D. (1992) *J. Mol. Biol. 224*, 639-657.

Parker, M. W., Tucker, A. D., Tsernoglou, D. & Pattus, F. (1990) *TIBS 15*, 126-129.

Pattus, F., Martinez, M. C., Dargent, B., Cavard, D., Verger, R. & Lazdunski, C. (1983) *Biochem. 22*, 5698-5703.

Raymond, L., Slatin, S. L., Finkelstein, A., Liu, Q. & Levinthal, C. (1986) *J. Membrane Biology 92*, 255-268.

Sachdev, G. P. & Fruton, J. S. (1970) *Biochem. 9*, 4465.

Schagger, H. & von Jagow, G. (1987) *Anal. Biochem. 166*, 368-379.

Schein, S. J., Kagan, B. L. & Finkelstein, A. (1978) *Nature 276*, 159-163.

Slatin, S. L., Raymond, L. & Finkelstein, A. (1986) *J. Memb. Biol. 92*, 247-253.

Song, H. Y., Cohen, F. S. & Cramer, W. A. (1991) *J. Bact. 173*, 2927-2934.

Szoka, F. & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. USA 75*, 4194-4198.

Wessel, D. & Flugge, U. I. (1984) *Anal. Biochem. 138*, 141-143.

Wormald, M. R., Merrill, A. R., Cramer, W. A. & Williams, R. J. P. (1990) *Eur. J. Biochem. 191*, 155-161.

CHAPTER 5

SEPARATION AND SOLUBILIZATION OF PROTECTED PEPTIDE FRAGMENTS.

INTRODUCTION:

Before I begin describing experiments, I would like to extend my heartfelt thanks to Larry Miercke and Arnie Falick, without whom this chapter would not exist.

In Chapter 4, I described a series of experiments which led to a preliminary model of the transmembrane topography of colicin Ia. This model was generated by identifying peptide fragments that were protected from the action of pepsin by asolectin membranes. All of the peptides identified were first purified from glycine SDS-PAGE or Tricine-SDS gels; typically 2-3 lanes were blotted onto Immobilon for N-terminal sequencing and 7-10 lanes were electroeluted for subsequent mass determination using electrospray mass spectrometry. While I was able to get N-terminal sequence information and masses on protected fragments from 5 of the gel bands using these techniques, I encountered two major difficulties with this type of experiment. First, many of the single "pure" bands visible on my gels were in fact mixtures of peptides, as was observed for bands C, D, and E. These mixtures were in part due to the relative non-specificity of pepsin, which often cleaved after several adjoining residues in a sequence, leaving peptide fragments with "ragged ends". These ragged-ended fragments did not have molecular weights or structures that were sufficiently different to allow separation by gel electrophoresis. Both N-terminal sequencing and electrospray mass spectrometry data on non-pure samples can

be very difficult, if not sometimes impossible to interpret due to overlap of peaks. Thus, it was of great interest to investigate alternative means of separation, to gain truly pure peptide populations for subsequent N-terminal sequencing and mass spectrometry. Second, after electroelution (using ammonium bicarbonate and SDS) and lyophilization, it was often extremely difficult to resolubilize the peptide fragments in a solvent that was compatible with mass spectrometry. I therefore experimented with several different HPLC column systems, both size exclusion and reverse phase, in an attempt to purify the protected fragments in a solvent which was amenable to mass spectrometry. Finally, for the majority of the following experiments, I turned to proteolytic digestions in the presence of *E. coli* instead of asolectin lipids. Pepsin digestion of colicin Ia in *E. coli* vesicles appears to be more complete than in asolectin, generating fragments that have M_r less than 10 kD (see Chapter 4, figure 2). I reasoned that these fragments would represent the products of a more complete proteolytic digestion, and would thus afford a higher resolution picture of the transmembrane topography of colicin Ia.

I. Chloroform:Methanol:Water (2:5:2) in 0.1% TFA on an HPLC Sizing Column

RATIONALE

Based on evidence that the solvent chloroform:methanol:water in a 2:5:2 ratio, with 0.1% Trifluoroacetic acid (TFA), could readily solubilize and separate the chymotryptic cleavage products C1 and C2 of Bacteriorhodopsin (BR) (Boehm, et al., 1990), we sought to determine if the same would be true for pepsin digestion products of colicin Ia. Our goal was to find a solvent system that could both solubilize and subsequently separate peptide fragments of colicin Ia, and that was compatible with mass spectrometry.

MATERIALS

Samples were run on a 600 x 7.5 mm Bio-Sil 250 HPLC sizing column, with a flow rate of 0.6 ml/min. Detection was at 280 nm, and data were detected at 0.005 or 0.01 absorbance units full scale (AUFS) with a 2.5 mm cell and a time constant of 1 second.

PRELIMINARY TESTS

Initially, several tests were carried out to determine if this solvent system would be suitable for our needs. As the colicin Ia peptide fragments were electroeluted from gels in 50 mM NH_4HCO_3 and 0.1 % SDS in the presence of Serva Blue stain, we first needed to determine how each of these components chromatographed on this column, as we hoped to use this column to separate the peptides away from these reagents. We found that SDS and NH_4HCO_3, either alone or in combination, eluted after approximately 33 minutes. Serva Blue stain also eluted after this time. Using

Bacteriorhodopsin (27 kD), and its proteolytic products C1 (20 kD) and C2 (7 kD) as protein standards, we found that they eluted at approximately 18, 20, and 26 minutes respectively. Therefore, peptide fragments larger than 7 kD, and indeed perhaps even some smaller than 7 kD, can easily be separated away from the reagents SDS, NH_4HCO_3, and Serva Blue.

As an internal control, I treated samples of both Hemoglobin (Hb) and BR in a manner identical to that of the colicin Ia peptide fragments. Specifically, 72 µg of Hb and 72 µg of the BR cleavage products C1 and C2 were chromatographed on a 16% Tricine-SDS gel, electroeluted, and lyophilized as previously described in Chapter 4. To each of the lyophilized samples were added 150 µl of 96% formic acid (to first solubilize the sample) and 200 µl of the 2:5:2 mixture. Samples were centrifuged for 5 min at 8000g in an Eppendorf table top centrifuge and the supernatant was loaded onto the column. Large peaks eluted after ~10 and ~22 minutes, corresponding to fragments C1 and C2 of BO with molecular weights of 20 and 7 kD, respectively. As the molecular weight of a Hb monomer is 16 kD, a peak eluting between the 2 BO peaks would be expected for this sample. No peak eluted within this range. A poorly defined peak eluted between 28 and 32 minutes after injection of the Hb sample, but is likely not related to Hb.

Thus, we can draw several conclusions from these preliminary experiments: 1) it is possible to electroelute peptide fragments from a gel, and subsequently separate away the SDS, NH_4HCO_3, and

Serva Blue stain using size exclusion HPLC; 2) in the case of BR, it is possible to recover pure peptides after gel electrophoresis, electroelution, lyophilization, resolubilization, and subsequent HPLC in 2:5:2. It appears that the 2:5:2 mixture is only amenable to solubilizing hydrophobic peptides, however, as Hb samples did not chromatograph successfully. As the channel forming region of colicin Ia contains a very hydrophobic sequence as well as a number of amphipathic sequences, we thought it likely that at least some colicin peptide fragments would become solubilized under these same experimental conditions, and therefore proceeded with a colicin digestion.

RESULTS OF COLICIN Ia CHROMATOGRAPHY

Digestion in asolectin vesicles: A proteolytic digestion of 7 nmoles of colicin Ia was carried out in the presence of asolectin vesicles, as previously described in Methods in Chapter 4. Six protected fragments were cut out of the gel and electroeluted, lyophilized, and the pellets were re-solubilized in the same mixture of 96% formic acid (150 µl) and 2:5:2 (200 µl) as described above. Of these 6 fragments, only one eluted as a peak from the TSK column, between ~25-32 minutes, indicating a molecular weight range ≤ 7 kD. This peak was broad, with two maxima, indicating that it was not completely pure.

We concluded from this experiment that 1 fragment was sufficiently hydrophobic to be successfully solubilized by the 2:5:2

solvent mixture. The remaining 5 fragments were apparently not solublilized. Indeed, we found that material always precipitated out of solution upon addition of 200 µl of 2:5:2 mixture to the samples which had been solubilized in 150 µl of 96% formic acid, signalling a clear incompatibility with this solvent system. That only 1 of 6 protected fragments chromatographed satisfactorily indicated that this solvent system did not adequately solubilize colicin Ia peptides.

Digestion in E. coli vesicles. One additional attempt was made to purify and identify peptides using this solvent system, this time using peptides that were protected in the presence of *E. coli* instead of asolectin vesicles. As at least some of these fragments were likely to be largely hydrophobic in character, we carried out the following experiment: approximately 7 nmoles of colicin Ia was subjected to pepsin digestion in the presence of *E. coli* vesicles, and 6 protein bands, 1-6, were extracted from the gel and treated as before. When material from bands 4, 5, and 6, all with M_r between 14.3 kD and 6.2 kD, were injected into the column, no peak eluted for peak 4, a single broad peak eluted after ~29 minutes for peak 5 and a peak with a shoulder eluted after ~30 minutes for peak 6. Both peaks 5 and 6 eluted just before the large peak containing SDS, NH_4HCO_3, and Serva Blue stain. It thus appears that fragments 5 and 6 were sufficiently hydrophobic to chromatograph in this system, but that fragment 4 was not. Mass spectra were not obtained on

these samples as they unfortunately were lost in the move when the Stroud Lab was remodeled in 1991.

That peaks 5 and 6 eluted on the shoulder of the reagent peak indicated that the lower molecular weight fragments 1-3 (all less than 6.2 kD) would not be resolved from the reagent peak under these conditions. To confirm this, we injected fragment #1, and no peak eluted from the column. In an attempt to isolate and identify the other two lower molecular weight fragments 2 and 3 (and to eliminate the SDS, NH_4HCO_3, and Serva from these fractions) without using this column, a chloroform/methanol lipid extraction (Wessel and Flugge, 1984) was performed on lyophilized material from bands 2 and 3 instead. These two samples were then subjected to electrospray mass spectrometry, but no masses could be determined because no ion current was observed. This was either due to some (undetected) interference or because little or no protein was injected, indicating that the extraction had failed.

CONCLUSIONS

As only 2 out of 6 colicin Ia fragments from an *E. coli* digest, and only 1 out of 6 colicin Ia fragments from an asolectin digest were amenable to chromatography in this system, due to their size as well as their hydrophobic characteristics, we decided to turn elsewhere, to identify a solvent system that would solubilize both hydrophobic as well as hydrophilic peptides. At this time, a new solvent was brought to my attention, which appeared to be

appropriate for both hydrophobic and hydrophilic peptides. This is described in the following section.

II. Formic acid:Acetonitrile:Isopropanol:Water (FAPH) (50:25:15:10) on an HPLC Sizing Column

RATIONALE

Recently, a method was described in which both hydrophobic and hydrophilic proteins were chemically eluted from gels and subsequently chromatographed on a size exlusion column, with up to 80% recovery of protein (Feick and Shiozawa, 1990). As the 2:5:2 solvent system described in the previous section appeared to solubilize only very hydrophobic peptides, we thought this FAPH solvent a better candidate for colicin Ia solubilization, as colicin contains both hydrophobic and hydrophilic regions. In addition, this solvent appeared to be a good candidate for mass spectrometry.

MATERIALS

Samples were run on a Bio-Sil TSK 250 HPLC sizing column (600 x 7.5 mm, 10 μm particle size) a flow rate of 0.6 ml/minute. Detection was at 280 nm and eluant absorbance was measured at 0.005 AUFS (2.5 mm cell) with a time constant of 2 sec.

RESULTS

Colicin Ia chromatography. After a pepsin digest of 7 nmoles of colicin Ia in the presence of *E. coli* lipids, 10 protected peptides (A-D, 1-6) which remained visible on a 16% Tricine-SDS gel were cut out and extracted with FAPH, according to the method referenced above. To solubilize the peptides for chromatography, 450 µl of FAPH was added to each of the dried peptide pellets. Samples were then centrifuged for 5 minutes (Eppendorf tabletop) and all of the supernatant was loaded onto the column. Of the 10 protected fractions, 7 eluted as peaks from the column! In preparation for mass spectrometry, the material eluted from each peak was dried in a speed-vac, then resolubilized in 20 µl of a 50:50 mixture of Acetonitrile + 0.8% TFA:0.1% TFA. Approximate masses were obtained on 3 out of 7 of the peaks. Electrospray mass spectrometric analysis revealed that fragment #1 contained a component with mass ~ 5 kD, fragment B a component of ~9.1 kD, and fragment C and component of ~ 9 kD. Masses were not obtained for the remaining 4 fragments, as explained below.

Despite some success in separation, solubilization, and mass determination of protected fragments of colicin Ia, several problems remained. First, exact masses could not be assigned to any of the fragments due to a large degree of hetrogeneity within the samples. Assigning masses for a protein mixture containing multiple similar masses using electrospray mass spectrometry is very difficult. While a sizing column is appropriate for separating mixtures which contain components of widely varying molecular weights, peptides

which differ by only a few amino acids will not separate on this column. While the peaks eluting from the column appeared to be pure, they in fact contained mixtures of peptides. Thus, this sizing column did not offer sufficient resolution of peptides of similar molecular weights.

Secondly, the mass spectrometer signals were very weak for these samples. This is very likely due to the fact that the samples were dried down after elution from the FAPH column and not adequately resolubilized for mass spectrometry. Ideally, a drying step would not be included in this procedure, as proteins are frequently more difficult to resolubilize once they have been dehydrated. However, in the case of colicin Ia, the samples eluting from the column were very dilute (in several ml); the volume of each of the column peaks therefore needed to be greatly reduced, to ~ 5 µl, for injection into the mass spectrometer. This requires a speed-vac or lyophilization step. Future experiments should address diffferent ways of reducing the sample volume while avoiding complete dehydration of the sample, possibly by lyophilizing the sample rather than using a Speed-vac.

Even if problems due to dehydration could be eliminated, exposure to organic solvents may cause problems. As Feick and Shiozawa describe: "one problem we encountered was that proteins or polypeptide fragments previously exposed to FAPH or other organic solvents were very difficult to completely resolubilize directly in the SDS solubilization buffer." They went on to describe

several postchromatographic sample treatments for resolubilization of peptides, which included treatment with reagents such as SDS, urea, NaOH, NH_4OH, and/or heat. These treatments are generally not compatible with mass spectrometry.

Thus, while three (approximate) masses of colicin Ia fragments were successfully determined using the FAPH extraction method coupled with size exclusion chromatography and mass spectrometry, the problems of heterogeneity within the eluted column fractions and low mass spectrometry signals remained. We carried out one final experiment in which a sample of myoglobin (Mb) was run in parallel on the gel along with the protected fragments of colicin Ia resulting from a digestion in *E. coli* lipids. Mb was used to determine how a known protein would behave in this solvent system. Mb and four colicin Ia digestion products were cut out of the gel, extracted with FAPH, and loaded onto the column. Peaks eluted for all of these samples. These peaks were dried; 5 µl of FAPH was then immediately added to each sample for mass spectrometric analysis, which yielded no masses for any of the fragments.

CONCLUSIONS

We conclude from these experiments that FAPH is indeed a good solvent for both hydrophobic and hydrophilic peptides, likely due to the high percentage of formic acid which has excellent solubilizing properties. However, problems with solubility but more importantly sample heterogeneity remain, making this system

inappropriate for our needs. In an effort to reduce the problems due to sample heterogeneity, we turned to reverse phase chromatography.

III. Reverse Phase Chromatography

RATIONALE

As we found that an HPLC sizing column does not adequately separate mixtures of peptide fragments which chromatograph as a single band on a gel, we turned to reverse phase chromatography, which separates peptides on the basis of both shape and hydrophobicity. By including formic acid in the gradient, we also hoped to improve peptide solubilization.

A. FORMIC ACID/ACETONITRILE GRADIENT

MATERIALS AND METHODS

Chromatography. Separation of peptides was carried out either on a 4.6 x 250 mm Vydac C-18 column with 300 Å pores and 10 µm silica particles, or a Rainin C-4 column with 5 µm silica particles and 300 Å pores. The gradient was 1 % B/minute, with A = 5% formic acid, B = 5% formic acid in ACN, at a flow rate of 1 ml/min. Detection was at 280 nm due to high absorbance of formic acid at 215 nm, and data were recorded at 0.1 AUFS using a 10 mm path flow cell.

EXPERIMENT #1

Sample Preparation. Seven nmoles of colicin Ia were proteolyzed by pepsin in the presence of *E. coli* lipids, as described in Chapter 4. After two hours of digestion, the digestion mixture was divided into several tubes (200 µl each) and spun at 30 psi for one hour in an airfuge (micro-ultracentrifuge). This spin was to separate the lipid pellet containing the protected peptides from the supernatant fraction which contained all of the pepsin and the peptides of colicin Ia not protected by the membrane. The only band visible in the supernatant fraction is that of pepsin. Lipids were then extracted from the pellet. After extraction, peptides were dried in a hood, then resolubilized in a total of 6 µl of 96% formic acid (Aldrich) in preparation for reverse phase chromatography. The samples sat at room temperature for 2 hours at which time they were diluted to 5.4 % formic acid with a total of 104 µl of water; samples then sat an additional 2 hours at room temperature before they were placed at 4°C for one week. After one week, all tubes were combined. Before loading onto the column, the sample was centrifuged for 5 minutes (eppendorf table top) to remove any insoluble material, and the supernatant removed for subsequent chromatography. After centrifugation, a pellet of insoluble material remained. To this pellet was added an additional 10 µl of 96% formic acid, which solublilzed the remaining pellet. This material was then combined with the original sample that was at 5.4% formic

acid, for a total of 120 µl of sample in 13% formic acid. This material, after centrifugation, was loaded onto a C-18 column.

RESULTS

This first attempt at reverse phase chromatography appeared to be successful. Not only did a number of peaks elute from the column (Figure 1a), but we were able to distinguish individual peptide masses in 11 of the peaks (see table I). Of note is that a number of peaks corresponded to contaminants in formic acid and not to colicin Ia peptides (see blank, Figure 1b).

To assign amino acid sequence to each of the peptides in Table I, it was first necessary to generate a list of all potential peptides within the colicin Ia sequence which corresponded to each of the masses. Such a list of peptides must take into account the known cut sites of the enzyme used; however as pepsin cleavage is highly non-specific, we did not specify particular cut sites but instead generated a list of all of the peptides within colicin Ia at any given mass. A survey of the colicin Ia sequence reveals that there exist between 6-18 peptides at any given mass. An example of one of these lists appears in figure 2. While some of the cut sites are perhaps more likely to occur as a result of pepsin digestion than others, it was not possible to unequivocally specify which of the sequences corresponded to the peptide fragments which eluted from the column, without N-terminal sequence analysis. Furthermore, these peaks still contained multiple peptides. Thus, the experiment

was repeated with the intention of generating enough material for both mass spectrometric as well as N-terminal sequence analysis, as well as to improve separation of peptides.

EXPERIMENT #2

RATIONALE/METHODS

The above experiment was repeated though with several small changes in the procedure in an attempt to improve separation and yield of protected peptides. A C-4 rather than a C-18 column was used, as C-4 is more likely to yield improved separation of the less hydrophobic peptides. Furthermore, the gradient time was doubled to 0.5 % B/minute in an attempt to decrease the heterogeneity within each of the peaks. Finally, as Aldrich formic acid contains a number of impurities which chromatograph along with the peptides (see figure 1b), Kodak formic acid was tried, based on the observation that it contains fewer impurities (Dr. Brad Gibson, personal communication).

The pepsin digestion and subsequent sample treatment of colicin Ia were as previously described, though the samples did not sit in the refrigerator for one week, but instead at room temperature for 8 hours. We also reduced the final formic acid concentration in the sample to be loaded onto the column to 5% rather than 13% that was previously used. This was done in an effort to minimize the chances of colicin Ia peptides preciptating

out of solution, as the concentration of formic acid in the column gradient was 5%.

RESULTS

The results of this experiment were not satisfactory. While some very small peaks did elute from the column, they were few in number, and small in height. It was subsequently noted after repeating this experiment that the back pressure of the column was extremely high after sample loading, suggesting that some peptide material may have precipitated out in the guard column and clogged the system.

EXPERIMENT #3

RATIONALE/METHOD/RESULTS

As the last experiment did not work as well as the first time when a C-18 column was used, we repeated the original experiment exactly, using Aldrich formic acid and a 1% B/minute gradient. Furthermore, the sample sat at 4°C for 1 week, and was loaded onto the column in a final concentration of 13% formic acid, as had been previously done. The results of this digestion are shown in figure 3. While the elution profile is not as extensive as was previously seen, several peaks did elute, and masses were measured for 4 of them (table 2). N-terminal sequence analysis yielded no results, likely due to not enough sample.

ADDITIONAL EXPERIMENTS

Several additional digestions were carried out in an attempt to improve the yield of pure peptides:

- Colicin Ia protected peptides were generated as before but loaded onto the column in 70% formic acid rather than the previously tried 5% or 13% formic acid. This was attempted because of the excellent solubilizing properties of formic acid. We had noted that, during sample preparation, the pellet of peptides appeared to dissolve completely upon addition of 96% formic acid. However, a precipitate was always noticed after water was added, when reducing the concentration of formic acid from 96% to 5% or 13% for column loading. Therefore, it appears that 96% formic acid completely solubilizes the peptides while lower concentrations of formic acid do not. The addition of 70% formic acid appeared to solubilize the pellet. However, after chromatography, no peptides eluted, and the back pressure of the column increased dramatically, suggesting that peptides had precipitated out of solution upon injection into the column. As the concentration of formic acid is 5% in the column, this is not unexpected.

- The experiment which generated the profile in figure 1 was repeated exactly except that the sample was loaded immediately onto the column after solubilizing the peptide pellet, rather than

sitting for one week at 4°. Very little material eluted from the column.

- I discovered that the injection loop on the HPLC column was filled with 10% formic acid in ACN for the first experiment that generated the profile in figure 1 but thereafter, it was filled with 5% formic acid in water. Expecting that this mistake might have been the cause of sample precipitation upon column loading, I repeated the original procedure that had generated the profile seen in figure 1. The elution profile did not exhibit satisfactory peaks.

- We observed no apparent difference in the solubilizing capability of Aldrich vs. Kodak formic acid. Both formic acids had contaminants which were visible at 0.005 AUFS, but these did not interfere with peptide peak identification. Furthermore, we observed no dramatic differences between C4 and C18 chromatography.

CONCLUSIONS

It seems that the problem we are facing is occuring at one of two places in the experiment. Either the peptides are not becoming solubilized in the first place and are therefore never getting loaded onto the column. Alternatively, the peptides may be completely solubilized and are therefore all loaded onto the colum, but are not being successfully chromatographed. The best elution profiles were generated after samples had been at 4°C for 1 week before being chromatographed. Therefore, it is possible that the long time in

contact with solvent is important for peptide solubilization. The solubility question is addressed in an experiment in a following section.

For reasons that I do not entirely understand, we were never able to recover as much peptide as we did in the first experiment, despite repeating the procedure exactly.

B. TRIFLUOROACETIC ACID/TETRAHYDROFURAN (TFA/THF) GRADIENT

METHOD

As colicin Ia peptides will become solubilized in 96% formic acid, we turned to a recently developed system which successfully solubilizes and separates bacteriorhodopsin-derived hydrophobic peptides after loading samples onto a column in 96% formic acid (Hunt, 1992). Hunt employed a C-18 column and a 20-100% B TFA/THF gradient, where A = 0.1% TFA and B = 0.1% TFA, 95% THF, 5% water. Samples are loaded in a 1-2 ml loop in 96% formic acid. After sample injection, the column is held at 20% B until the large formic acid peak elutes, at which time the gradient is started. Hunt found that formic acid did not interfere with the hydrophobic interaction between bacteriorhodopsin peptides (~19-mers) and the column matrix, as peptides were found to stick to the column, despite being solubilized in 96% formic acid during loading.

Based on these data, I tried the same solvent system and gradient on a C-18 column. The result was that no peptide peaks eluted from the column. This could be explained by the fact that colicin Ia is not a highly hydrophobic protein, with the exception of one stretch of ~ 40 amino acids at the C-terminus. As the remainder of the sequence is more amphipathic in nature, it would therefore be less likely to interact with the column matrix, especially in the presence of 96% formic acid. Thus, it seems likely that no colicin Ia peptides eluted with the gradient because they all remained soluble in the 1 ml slug of 96% formic acid in which the sample was loaded. This formic acid slug eluted from the column even before the gradient was started. We concluded that, while this gradient is approriate for small hydrophobic peptides, it is not appropriate for larger more amphipathic peptides.

C. N-PROPANOL/ETHANOL

A third attempt at solubilizing and chromatographing peptides from an *E. coli* digest was carried out using a 1% B/minute gradient where A = 5% formic acid and B = 5% formic acid, 67% N-propanol, 27% Ethanol. A combination of N-propanol and ethanol has been successfully used to solubilize and separate peptides with the same efficiency as does an acetonitrile gradient (Feldhoff, 1991). This solvent system did not improve the yield of separated peptides.

IV. SOLUBILITY TESTS

It was important to isolate the problem of low peptide recovery after chromatography. Solubility tests were carried out on the peptides fragments using 5 different solvents. The motive was to determine if the low yields after chromatography were due to inadequate solubilization of the peptides pre-HPLC, or to inadequate chromatography.

METHODS

A pepsin digest was carried out as before, and the protected peptide pellets were separated into 6 tubes, each with approximately 1 nmole of protein. The ability of each solvent mixture to solubilize peptides was measured by gel electrophoresis. After treatment with a particular solvent, samples were centrifuged in an eppendorf table top centrifuge. Those peptides that become solubilized will be in the supernatant while the insoluble peptides will remain in the pellet. Thus, gel electrophoresis of the supernatant and pellet fractions will indicate which of the solvent systems has successfully solubilized the peptide. Samples were treated as follows: To tube

1) 10 µl of 0.25% SDS in $NaPO_4$ buffer was added as a control. SDS will solubilize all peptides. After two hours, sample was centrifuged and supernatant removed;

2) 10 µl of 96% formic acid was added for 10 minutes. Sample was then centrifuged and the supernatant removed;

3) 10 µl of 96% formic acid was added and the sample was centrifuged after 2 hours, the supernatant was then removed;

4) 10 µl of 96% formic acid was added. Sample incubated 10 minutes at room temperature at which time the formic acid was diluted to 15% with water. Sample then sat an additional 2 hours, at which time it was centrifuged and the supernatant removed.

5) 10 µl of 5% formic acid was added, sample sat for 2 hours, then centrifuged;

6) 10 µl of a 2:1 mixture of isopropanol:ethanol in 0.1% TFA was added, sample sat for 2 hours, then centrifuged.

After centrifugation, all supernatants were removed and dried in a Speed-Vac. The remaining pellets were also dried. The dried samples (supernatants and pellets) were then resolubilized in 10 µl of 0.25% SDS and 5 µl of sample buffer, in preparation for gel electrophoresis.

RESULTS/CONCLUSIONS:

We found that the only solvent which completely solubilized the peptide fragments (as much as did SDS) was 96% formic acid. While the majority of the fragments were solubilized after 10 minutes in 96% formic acid (sample 2), some material remained in

the pellet fraction. After 2 hours in formic acid, all peptide fragments were in the supernatant fraction (sample 3).

In each of the other samples 4-6, only one major peptide fragment appeared to become solubilized and was apparent in the supernatant fraction after gel electrophoresis. The majority of the peptide fragments remained with the pellet fraction in fractions 4-6.

CONCLUSIONS

We have found that 96% formic acid is indeed an excellent solvent and will solubilize all of the protected colicin Ia fragments. When decreased to 15%, colicin Ia peptides precipitate out of solution. Thus, we have determined that the problem in generating material for mass spectrometry and N-terminal sequencing in these experiments is at the level of solubility. It appears that the peptides are never becoming solubilized in the first place, and therefore never getting loaded onto the column.

The results of this experiment lead to a re-evaluation of the peaks obtained using the formic acid/acetonitrile gradient (figure 1). While a number of peaks eluted from the column, and indeed, masses were assigned to some of them,it is probable that those peaks did not represent all of the protected fragments of colicin Ia. This is based on the fact that the samples were loaded in 13% formic, which does not effectively solubilize all of the protected fragments, as seen in these experiments.

GENERAL CONCLUSIONS/ FUTURE EXPERIMENTS:

It appears that the major problem that we are faced with in these experiments is one of peptide solubility. The problem of how to solubilize peptides, in particular hydrophobic peptides and peptides that have been dehydrated or have come into contact with organic solvents, is one that has met with little success over the years. During the course of these experiments, we learned several important things and given more time, I would concentrate on the following items:

a) determine the lowest concentration of formic acid that will keep all colicin Ia peptides in solution. If low enough, it may be possible to load colicin Ia onto a reverse phase column in this concentration of formic acid and have the peptides bind to the column; an organic gradient containing the appropriate formic acid concentration would follow.

b) try to eliminate dehydration steps in the experimental protocol. It is well established that peptides are more difficult to resolubilize once they have been dried. Therefore, it might be possible to dry samples down, but never to complete dryness, after the lipid extraction step. This might aid in solubilization in an appropriate solvent for subsequent chromatography.

c) try digesting colicin Ia with a more specific enzyme to eliminate the problem of "ragged-ends" that results from pepsin digestion.

d) finally, many different solvent /gradient /column combinations must be tried.

FIGURE LEGENDS

1. A) Peaks eluting from C-18 column, as described in text. B) Baseline, showing contaminants from formic acid. Abs is absorbance.

2. Predicted sequences of all of the colicin Ia peptides that have masses that range between 9353.3 daltons and 9357.7 daltons.

3. Peaks eluting from C-18 column, as described in text. Abs is absorbance.

TABLE 1

PEAK #	MASSES
13	3640.9
14	3640.5, 3975.8
15	5797.5, 5823.6
16	6540.1, 6726.5?, 6008.3?
17	6539.8, 5160.1
18	5003.1, 5031.3
19	lost, computer glitch
20	5003, 4865 (weak), 4557?
21	9715, 9097, 8936
22	9714, 9748
23	9212, 9830, 9743
24	8974, 8353, 9001.5, 9991?
25	9976, 9357, 10,006?, 9199?
26	9975, 9354, 9203,

TABLE 2

PEAK #	MASSES
6	nothing
13	9731
14	9717, 9716, 9608
15	13,138
7	non-peptide, probably polymer
12	5002
6	nothing
FAPH	polymer

Figure 1

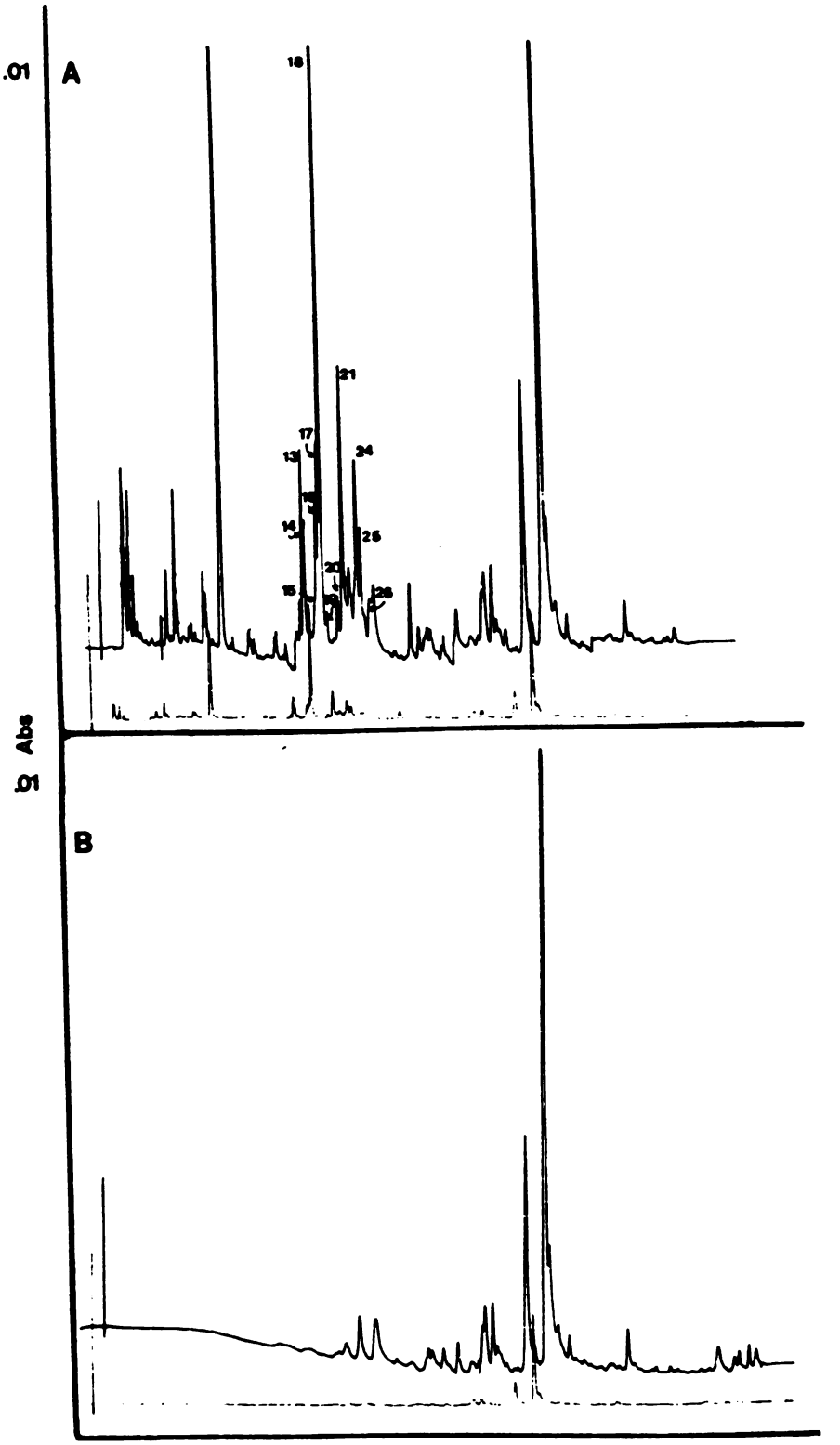
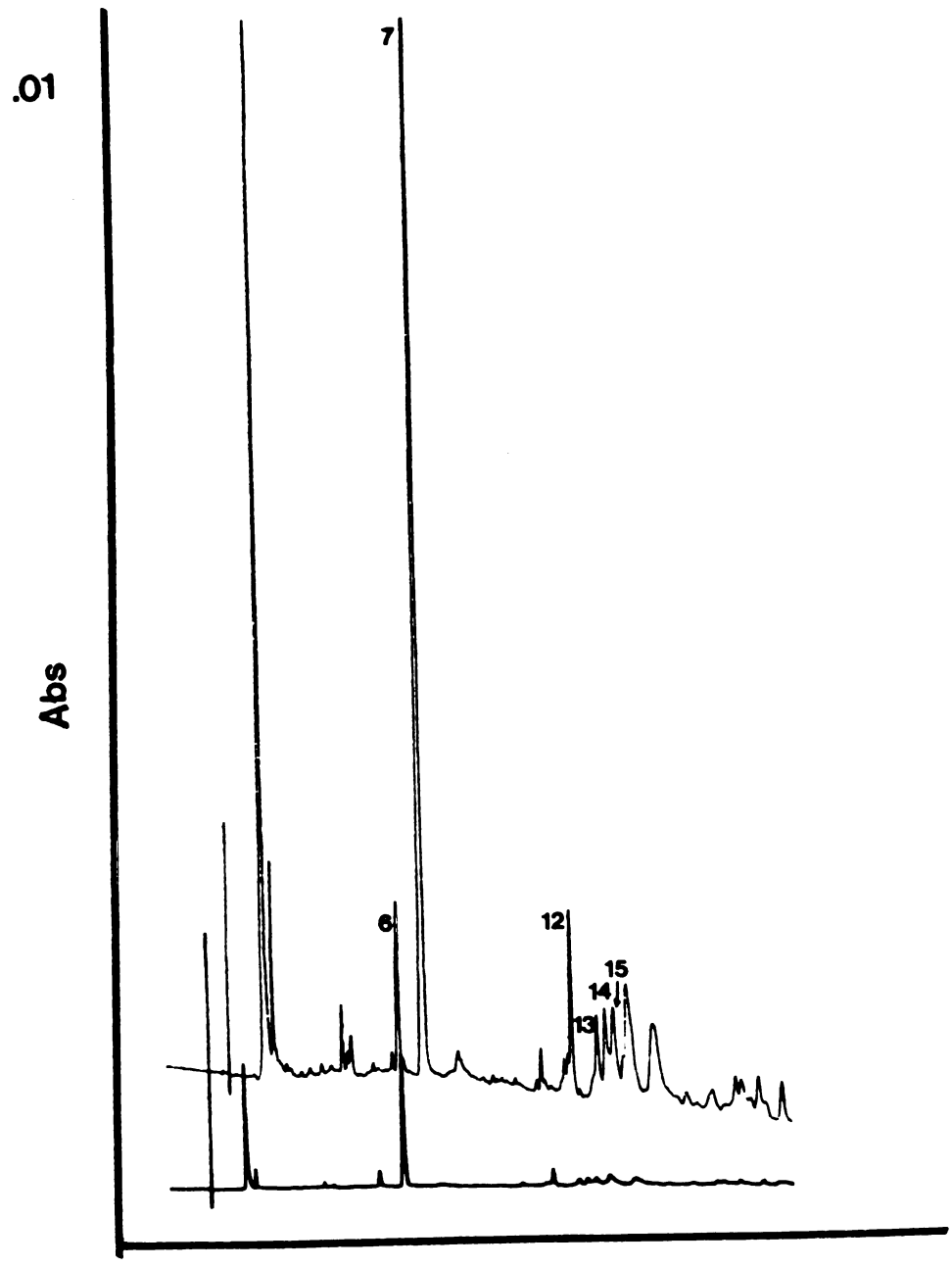


Figure 2

For Protein 'colicin.seq', Fragments within the range of 9353.5 and 9357.5 amu are:

Num.	Fragment	MH+ (mass)	Sequence
1	22 - 103	9354.64	(G) HEIMAVDIYVNPPRVDVFHGTPPAWSSFGNKTIWGGNEWV DDSPTRSDIEKRDKEITAYKNTLSAQQKENENKRTEAGKR LS (A)
2	28 - 110	9356.69	(V) DIYVNPPRVDVFHGTPPAWSSFGNKTIWGGNEWVDDSPTR SDIEKRDKEITAYKNTLSAQQKENENKRTEAGKRLSAAIA ARE (K)
3	34 - 116	9355.69	(P) PRVDVFHGTPPAWSSFGNKTIWGGNEWVDDSPTRSDIEKR DKEITAYKNTLSAQQKENENKRTEAGKRLSAAIAAREKDE NTL (K)
4	74 - 156	9356.84	(R) DKEITAYKNTLSAQQKENENKRTEAGKRLSAAIAAREKDE NTLKTLRAGNADAADITRQEFRLLQAELREYGFRTEIAGY DAL (R)
5	215 - 300	9356.92	(L) DTRLSELEKNGGAALAVLDAQQARLLGQQTRNDRAISEAR NKLSSVTESLNTARNALTRAEQQLTQQKNTPDGKTIVSPE KFPGRS (S)
6	299 - 381	9356.88	(G) RSSTNDSIVVSGDPRFAGTIKITTSAVIDNRANLNYLLSH SGLDYKRNILNDRNPVVTEDVEGDKKIYNAEVAEWDKLRQ RLL (D)
7	308 - 390	9353.92	(V) VSGDPRFAGTIKITTSAVIDNRANLNYLLSHSGLDYKRNI LNDRNPVVTEDVEGDKKIYNAEVAEWDKLRQRLLDARNKI TSA (E)
8	335 - 417	9355.80	(Y) LLSHSGLDYKRNILNDRNPVVTEDVEGDKKIYNAEVAEWD KLRQRLLDARNKITSAESAVNSARNNLSARTNEQKHANDA LNA (L)
9	336 - 418	9355.80	(L) LSHSGLDYKRNILNDRNPVVTEDVEGDKKIYNAEVAEWDK LRQRLLDARNKITSAESAVNSARNNLSARTNEQKHANDAL NAL (L)
10	337 - 419	9355.80	(L) SHSGLDYKRNILNDRNPVVTEDVEGDKKIYNAEVAEWDKL RQRLLDARNKITSAESAVNSARNNLSARTNEQKHANDALN ALL (K)
11	385 - 467	9356.90	(R) NKITSAESAVNSARNNLSARTNEQKHANDALNALLKEKEN IRNQLSGINQKIAEEKRKQDELKATKDAINFTTEFLKSVS EKY (G)
12	389 - 472	9356.86	(T) SAESAVNSARNNLSARTNEQKHANDALNALLKEKENIRNQ LSGINQKIAEEKRKQDELKATKDAINFTTEFLKSVSEKYG AKAE (Q)
13	413 - 495	9356.06	(N) DALNALLKEKENIRNQLSGINQKIAEEKRKQDELKATKDA INFTTEFLKSVSEKYGAKAEQLAREMAGQAKGKKIRNVEE ALK (T)
14	431 - 512	9357.02	(S) GINQKIAEEKRKQDELKATKDAINFTTEFLKSVSEKYGAK AEQLAREMAGQAKGKKIRNVEEALKTYEKYRADINKKINA KD (R)
15	435 - 516	9356.07	(Q) KIAEEKRKQDELKATKDAINFTTEFLKSVSEKYGAKAEQL AREMAGQAKGKKIRNVEEALKTYEKYRADINKKINAKDRA AI (A)
16	500 - 585	9355.03	(K) YRADINKKINAKDRAAIAAALESVKLSDISSNLNRFSRGL GYAGKFTSLADWITEFGKAVRTENWRPLFVKTETIIAGNA ATALVA (L)
17	508 - 595	9355.04	(K) INAKDRAAIAAALESVKLSDISSNLNRFSRGLGYAGKFTS LADWITEFGKAVRTENWRPLFVKTETIIAGNAATALVALV FSILTGSA (L)
18	509 - 596	9355.04	(I) NAKDRAAIAAALESVKLSDISSNLNRFSRGLGYAGKFTSL ADWITEFGKAVRTENWRPLFVKTETIIAGNAATALVALVF SILTGSAL (G)

Figure 3



REFERENCES

Boehm, M. F., Gawinowicz, M. A., Foucault, A., Derguini, F. & Nakanishi, K. (1990) *J. Am. Chem. Soc. 112*, 7779.

Feick, R. & Shiozawa, J. (1990) *Anal. Biochem. 187*, 205-211.

Feldhoff, R. (1991) in *Techniques in Protein Chemistry II*, Academic Press, Inc.

Hunt, J., Ph.D. thesis, Yale, 1992.

Wessel, D. & Flugge, U. I. (1984) *Anal. Biochem. 138*, 141-143.

APPENDIX

A CARBOXY-TERMINAL FRAGMENT OF COLICIN Ia FORMS ION CHANNELS

Partho Ghosh, Stephanie F. Mel[#], and Robert M. Stroud[+]

Departments of Biochemistry and Biophysics and Experimental Pathology[#]
University of California, San Francisco
San Francisco, California 94143-0448

[+]Author to whom correspondence should be addressed

Running Title: Ion channel-forming domain of colicin Ia

SUMMARY

A carboxy-terminal, 18 kD fragment of colicin Ia, a bacterial toxin, forms ion channels in artificial phospholipid bilayers. This fragment, which comprises a quarter of the intact 70 kD molecule, is resistant to extensive protease digestion and probably constitutes a structural domain of the protein. The ion channels formed by the 18 kD fragment are functionally heterogeneous, having conductances that range from 15 to 30 pS at positive voltages and from 70 to 250 pS at negative voltages, and open lifetimes that range from at least 25 milliseconds to 5 seconds. In contrast, ion channels formed by whole colicin Ia open only at negative voltages, at which their conductances range from 6 to 30 pS, and their open lifetimes range from 1 second to 3 minutes. Additionally, the open state of the 18 kD fragment channel is characterized by noisy fluctuations in current, while the open state of the whole molecule ion channel is often marked by numerous, stable sub-conductance states. Since the properties of the fragment channel differ substantially from those of the whole molecule channel, we suggest that portions of the molecule outside of the 18 kD fragment are involved in forming the whole molecule ion channel.

INTRODUCTION

Ion channel proteins are significant transducers of signals across membranes. In order to understand the structural basis for

ion channel formation and function, we have studied the bacterial toxin colicin Ia, a member of the ion channel-forming family of colicins. This family includes colicins A, B, E1, Ib, K and N as well (for review see Cramer, et al., 1990; Pattus, et al., 1990). The colicins are plasmid-encoded, soluble proteins of 42 to 70 kD which are synthesized upon stress during the SOS response (Morlon, et al., 1983; Waleh & Johnson, 1985; Mankovich, Hsu & Konisky, 1986; Salles, Weisemann & Weinstock, 1987). These proteins are secreted into the media, bind to an outer membrane receptor of target bacteria, and translocate across the periplasmic space to the inner plasma membrane, where they form lethal, transmembrane ion channels. Colicin ion channels are voltage-gated and relatively non-selective, as determined by recordings from artificial membranes (Schein, Kagan & Finkelstein, 1978; Weaver, et al., 1981; Seta, et al., 1983; Raymond, Slatin & Finkelstein, 1985).

Proteolytic digestion (Carmen Martinez, Lazdunski & Pattus, 1983) and genetic truncation (Baty, et al., 1988; Baty, et al., 1990) of colicin A have identified the carboxy-terminal third of the protein as the ion channel-forming region. Similarly, proteolytic cleavage of colicin E1 has shown that the carboxy-terminal third of colicin E1 is responsible for forming ion channels (Ohno-Iwashita & Imahori, 1982). The functional properties of the ion channel-forming domains of colicins A and E1 are generally similar to those of each respective whole molecule, although some minor differences do exist in voltage-dependence, gating kinetics, and ion selectivities (Carmen Martinez,

et al., 1983; Cleveland, et al., 1983). These functional ion channel-forming regions appear to be equivalent to structural domains. The x-ray crystal structure of the ion channel-forming portion of colicin A reveals that it is a compact bundle of 10 α–helices (Parker, et al., 1989; Parker, et al., 1990), and nuclear magnetic resonance (NMR) shows that the ion channel-forming portion of colicin E1 is structurally similar to the colicin A fragment (Wormald, et al., 1990).

We asked whether a carboxy-terminal portion of the 70 kD protein colicin Ia is also capable of forming ion channels. We report that a tryptic, carboxy-terminal fragment of colicin Ia does form ion channels, but that these channels differ substantially from those formed by intact colicin Ia. These results are discussed in the context of other channel-forming colicins and their channel-forming fragments.

MATERIALS AND METHODS

Probing the structure of colicin Ia with trypsin and V8. Colicin Ia was expressed and purified (Mel & Stroud, 1992) from plasmid pJK5 in *E. coli* strain 294 (Weaver, Redborg & Konisky, 1981). The purified molecule was digested at 37° C with bovine trypsin (Worthington) at a 1:3 trypsin:colicin Ia molar ratio, with colicin Ia at a concentration of 270 μM in 50 mM NaCl, 20 mM citrate buffer, pH 5.2. The reaction was stopped by the addition of leupeptin to a final concentration of 90 μM.

For digestion with *S. aureus* V8 protease (Endoproteinase Glu-C, Boehringer Mannheim), the reaction was carried out at 37° C at a V8:colicin Ia molar ratio of 1:3, with colicin Ia at a concentration of 44 μM in 50 mM phosphate buffer, pH 7.8, and 2.4 M urea. The products of both reactions were examined by SDS-PAGE (Laemmli, 1970).

Purification of fragments. Purified colicin Ia was digested with trypsin as above, except at a lower trypsin:colicin Ia molar ratio of 1:30. The products of the digest were purified by size-exclusion HPLC on two, tandem Biosil TSK 250 columns (each 21.5 mm x 60 cm), which had been equilibrated with 50 mM NaCl, 20 mM citrate buffer, pH 5.2. Three resolved fractions, which eluted at 117, 141, and 160 minutes at a column flow rate of 2 ml/min, were collected and subjected to amino-terminal sequencing and relative molecular weight determination by SDS-PAGE. Only the leading edge of the peak eluting at 160 minutes was collected, since the trailing edge of this peak overlaps with another peak.

Formation of Artificial Phospholipid Bilayers. Artificial phospholipid bilayers were formed on the tip of tight-seal pipets (Coronado & Latorre, 1983; Suarez-Isla, et al., 1983) as described in Ghosh & Stroud (1991) using soybean asolectin (Associated Concentrates) which had been purified (Kagawa & Racker, 1971). This procedure yielded membrane-sealed tips having a resistance of 5-100 GΩ with

a success rate of 80-90%. Colicin Ia and the tryptic fragments were added to the unstirred, 2 ml bath solution containing the membrane-sealed tip to a final concentration ranging from 0.03-145 μg/ml. Ion channels typically appeared after 10 to 20 minutes after the introduction of the protein into the bath solution.

Channel Recordings. A voltage-clamp amplifier (EPC-5, List Electronics) was used to set the transmembrane potential and to measure ionic currents. Ground potential was defined as that of the bath, so the reported potentials represent those of the trans compartment; the cis compartment is defined as the compartment to which protein was added. The sign convention is such that current into the pipet (from cis to trans) is defined as positive and shown as an upward deflection. Ion channels were recorded with cis and trans compartments containing symmetrical solutions of either 500 mM or 1000 mM NaCl and 1 mM $CaCl_2$, 10 mM dimethylglutarate, pH 4.0. Current and voltage records were recorded on chart paper with a Gould 220 brush recorder resulting in 40 Hz filtering of data.

RESULTS

The carboxy-terminal quarter of colicin Ia is resistant to proteolysis. Tryptic digestion of colicin Ia yields two major products, a protease-resistant fragment of M_r 18.0 ± 0.4 kD (n=7) and a less resistant fragment of M_r 34.1 ± 1.0 kD (n=6) (Figure 1a). The 34 kD fragment disappears after two hours of digestion at 37° C at a trypsin:colicin Ia

molar ratio of 1:3, while the 18 kD fragment persists for at least 4 hours. The amino-terminus of the 34 kD fragment is residue 7, having been cleaved at arginine 6, as determined by amino-terminal sequencing. Its carboxy-terminus probably extends to arginine 313, as determined from its relative molecular weight and the specificity of trypsin for lysines and arginines. However, given the errors in determining the relative molecular weight, it is possible that either arginine 299 or arginine 313 could be the carboxy-terminus of the 34 kD fragment. The 18 kD fragment begins at aspartate 451 and likely extends to the carboxy terminus of the intact molecule, isoleucine 626. However, the fragment may be shorter, having been cleaved at either arginine 619 or lysine 622. Even with the uncertainty in the carboxy-terminii of these fragments, it is clear that the 34 kD fragment constitutes the amino-terminal half of colicin Ia while the 18 kD fragment constitutes the carboxy-terminal quarter.

The structural stability of the carboxy-terminal quarter is confirmed by a more rigorous digest in the presence of 2.4 M urea using V8 (Figure 1b). After 5 minutes of digestion at 37° C at a V8:colicin Ia molar ratio of 1:3, only a 20.4 ± 0.2 kD M_r (n=4) fragment as well as several lower molecular weight fragments remain. The 20 kD fragment, whose amino-terminus is residue 446 and whose carboxy-terminus probably extends to the carboxy-terminus of intact colicin Ia, remains uncleaved for at least 2 hours. The 20 kD V8 fragment is longer by 6 residues at its amino-terminal

end and probably by 7 residues at its carboxy-terminal end than the 18 kD tryptic fragment. Therefore, as determined from tryptic and V8 proteolysis, the carboxy-terminal quarter of colicin Ia appears to constitute a stable, structural domain.

The 18 kD colicin Ia fragment forms ion channels. In order to determine whether the carboxy-terminal quarter of colicin Ia possesses ion channel-forming activity, the 18 kD tryptic fragment was purified by size-exclusion HPLC (Figures 2a and b) and assayed for ion channel-forming activity by single channel recording techniques. The addition of the 18 kD, carboxy-terminal fragment to artificial phospholipid bilayers results in the appearance of ion channels which are characterized by noisy open states (Figures 3a, b, and c). The current flowing through these channels is not stable, but instead fluctuates at a rate greater than the resolution limit of our recording system, 25 milliseconds. These fluctuations can be as great as the open channel current. Furthermore, ion channels formed by the 18 kD fragment are functionally heterogeneous such that a range of conductance rather than a single conductance value is observed. The range of conductances is dependent on the polarity of the voltage. At positive voltages, the conductance ranges from 15 to 30 pS (Figure 3a) in 500 mM NaCl, pH 4.0, as determined from 17 different membranes and 153 channels. However, at negative voltages, the conductance is higher, ranging from 70 to 250 pS (Figures 3b and c), as determined from 21 different membranes and

204 channels. Infrequently, channels of much smaller conductance, approximately 10 pS, are observed at negative voltages (Figure 3c). The open lifetimes of these fragment channels range from at least 25 milliseconds to approximately 700 milliseconds at positive voltages and from 25 milliseconds to approximately 5 seconds at negative voltages (Figures 3a, b, and c).

To determine whether these ion channels result uniquely from the action of the 18 kD fragment and to determine whether other portions of the molecule are capable of forming ion channels, the amino-terminal 34 kD fragment was also assayed for ion channel-forming activity. This fragment was generated by the same tryptic digest yielding the 18 kD fragment and purified identically to the 18 kD fragment (Figures 2a and b). In contrast to the 18 kD fragment, the 34 kD fragment does not form detectable ion channels, those greater than 1 pS, within the 6 hour duration of an experiment (data not shown). Therefore, it appears that only the carboxy-terminal quarter of colicin Ia is required to form ion channels.

Channels formed by the 18 kD fragment are dissimilar to channels formed by whole colicin Ia. Ion channels formed by the 18 kD fragment, however, differ considerably from those formed by whole colicin Ia. As determined from 10 different membranes and 28 different channels, the conductance of whole colicin Ia at negative voltages in 500 mM NaCl, pH 4.0 ranges from 6 to 30 pS (Figures 4a and b), approximately 10-fold less than that for the 18 kD fragment

at negative voltages. A single conductance value is difficult to assign to these channels, since a variety of conductance states are found (Figures 4a and b). Some of these are sub-conductance states of the same channel rather than separate conductance states of a number of channels. This was determined from the observation that closure to the zero current level often occurs from a state other than the lowest conductance state. One channel was observed to vary among several states and then to close from the second largest sub-conductance state (Figure 4b); the lifetime of each of these sub-conductance states is on the order of seconds. The open lifetime of the whole colicin Ia channel ranges from 1 second to 3 minutes, contrasting with the much shorter lifetimes of the 18 kD fragment.

In further contrast, the opening of the colicin Ia channel but not of the 18 kD fragment is dependent on voltage. Membranes in which several whole colicin Ia channels are active show that the channel conducts current only at negative voltages (Figure 5a and 5b). Whole colicin Ia channels close at positive voltages but reversibly re-open at negative voltages. In contrast, the 18 kD fragment conducts ions at either positive or negative voltages (Figures 3a, b, and c), although its conductance is diminished at positive voltages.

DISCUSSION

We have found from proteolytic digestion and single channel recordings that the carboxy-terminus of colicin Ia is capable of

forming ion channels, as are the carboxy-terminal portions of colicins A and E1. However, in contrast to colicins A and E1, the ion channel properties of this colicin Ia carboxy-terminal fragment differ greatly from those of the whole molecule.

The ion channel properties found in this and other studies (Bullock & Cohen, 1986; Nogueira & Varanda, 1988) for whole colicin Ia are generally similar to those found for whole A and E1. In comparison to the range of 6 to 30 pS found for colicin Ia in 500 mM NaCl, pH 4.0, colicin A has a conductance of 13 pS in 1 M KCl, pH 6.2 (Carmen Martinez, et al., 1983), and colicin E1 has a conductance of 21 pS in 1 M NaCl, pH 6 (Bullock, et al., 1983). The recordings in this study were carried out at pH 4 since colicins insert preferentially into membranes at pH values ≤ 4 (Pattus, et al., 1983; Davidson, et al., 1984; Mel & Stroud, 1992). The finding that the whole colicin Ia channel can adopt a number of sub-conductance states with lifetimes in the range of seconds has also been noted for colicin E1 (Raymond, et al., 1986). In general, channels formed by whole colicins A, E1, and Ia have similar conductances, a dependence on negative voltage for opening, and open lifetimes in the range of seconds to minutes.

However, unlike colicins A and E1, colicin Ia's channel-forming fragment has ion channel properties which differ markedly from those of the whole molecule. The single channel conductance of the 18 kD fragment is 10-fold greater at negative voltages than that of the whole molecule, and the open lifetimes are much shorter. Therefore, although the flux of ions through the fragment channel is

greater than through the whole molecule channel, the open fragment channel is much less stable than the open whole molecule channel. In parallel with the reduced stability, the large fluctuations of current through the fragment channel may indicate of greater conformational flexibility in the open state of the fragment channel than of the whole molecule channel (Sigworth, 1985). The gating of the fragment ion channel also differs from that of the whole molecule. While the whole molecule ion channel closes at positive voltages, the 18 kD fragment ion channel continues to conduct current, although the magnitude of its conductance is diminished by at least 2-fold.

It is possible that these differences arise from an alteration in the mechanism of ion channel formation. While whole colicin Ia probably forms channels as a monomer by analogy to colicin E1 (Bruggemann & Kayalar, 1986; Peterson & Cramer, 1987; Slatin, 1988; Levinthal, et al., 1991), it is possible that the 18 kD fragment forms channels as an oligomer. Peptides with sequences capable of forming amphipathic α–helices have been shown to form ion channels most likely as oligomers (Lear, Wasserman & DeGrado, 1988; Oiki, et al., 1988; Oiki, Danho & Montal, 1988; Ghosh & Stroud, 1991), and the 18 kD fragment contains such sequences. Our experiments, however, do not allow us to distinguish the association state of the channel, since addition of too few molecules leads to no channel formation within the 6 hours of an experiment and addition of too many molecules leads to lysis of the membrane.

A more likely possibility is that the differences between the 18 kD fragment and the whole molecule channel indicate that portions of the protein outside this ion channel-forming fragment are involved in forming the whole molecule channel. Studies with colicin A truncation mutants (Frenette, et al., 1989) and colicin A-E1 fusion proteins (Benedetti, et al., 1991) indicate that the colicin A channel-forming domain interacts with the middle third of the protein, the receptor-binding domain. Analogously, portions amino-terminal to the colicin Ia 18 kD fragment may interact with this channel-forming domain and may be necessary to form the whole colicin Ia channel. These portions may confer stability to the open ion channel, perhaps by anchoring it in the membrane, and may also be involved in some aspects of the gating of the whole molecule.

Structurally, the 18 kD fragment of colicin Ia probably constitutes a stable core of the protein as seen from its protease resistance, and is probably similar to the compact, structural domain of the colicin A channel-forming fragment (Parker, et al., 1989; Parker, et al., 1990). The colicin A fragment is composed of 10 α-helices arranged in a three-layer bundle, with helices 8 and 9 forming the hydrophobic core. The channel-forming fragment of colicin E1 as determined by NMR is consistent with this structure, except that helices 9 and 10 are slightly rearranged relative to colicin A (Wormald, et al., 1990). The channel-forming colicin Ia fragment is slightly shorter than the colicin A fragment but about the same size as the colicin E1 fragment. By sequence comparison, the amino-

terminus of the 18 kD fragment corresponds to helix 1 of the colicin A fragment without the first 7 residues; its likely carboxy-terminus at residue 626 corresponds to the colicin A fragment without its last 3 residues. Therefore, the colicin Ia fragment is probably structurally similar to both the colicin A and E1 fragments, although confirmation of this awaits the x-ray crystal structure of colicin Ia (see Chapter 3).

The marked difference in functional properties between the 18 kD fragment and the colicin A and E1 fragments then likely arise from small, specific differences in structure arising from differences in sequence. However, it is also possible that although the soluble structures of these fragments may be similar, the membrane-inserted structures could vary considerably from each other. The channel-forming domains of the colicins share little sequence identity, about 25-30%, but have in common sequences that are capable of forming amphipathic α–helices and a hydrophobic stretch of about 35 amino acids. Since their secondary and tertiary structure are likely to be similar but their ion channel properties differ, the channel-forming domains of these colicins provide a useful way to identify sequence elements responsible for specific ion channel properties.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health Grant GM24485 (to R.M.S.). P.G. is the recipient of an NSF Predoctoral

Fellowship and was partially supported by a grant from the Lucille P. Markey Charitable Trust. S.F.M. is the recipient of a Claypole Fellowship and a U.C.S.F. Mentorship Award. We thank Jordan Konisky for plasmid pJK5, A. J. Hudspeth for advice and the use of his lab for carrying out channel recordings, and Larry Miercke for assistance in HPLC purification. We also thank Juan Korenbrot, Michael Shuster, and Michael Wiener for comments on the manuscript.

REFERENCES

Baty, D., Frenette, M., Lloubes, R., Geli, V., Howard, S. P., Pattus, F., Lazdunski, C. 1988. Functional domains of colicin A. *Mol. Microbiol.* **2**:807-811

Baty, D., Lakey, J., Pattus, F., Lazdunski, C. 1990. A 136-amino-acid-residue COOH-terminal fragment of colicin A is endowed with ionophoric activity. *Eur. J. Biochem.* **189**:409-413

Benedetti, H., Frenette, M., Baty, D., Knibiehler, M., Pattus, F., Lazdunski, C. 1991. Individual domains of colicins confer specificity in colicin uptake, in pore-properties and in immunity requirement. *J. Mol. Biol.* **217**:429-439

Bruggemann, E. P., Kayalar, C. 1986. Determination of the molecularity of the colicin E1 channel by stopped-flow ion flux kinetics. *Proc. Nat. Acad. Sci. USA* **83**:4273-4276

Bullock, J. O., Cohen, F. S. 1986. Octylglucoside promotes incorporation of channels into neutral planar phospholipid bilayers. Studies with colicin Ia. *Biochim. Biophys. Acta* **856**:101-108

Bullock, J. O., Cohen, F. S., Dankert, J. R., Cramer, W. A. 1983. Comparison of the macroscopic and single channel conductance

properties of colicin E1 and its COOH-terminal tryptic peptide. *J. Biol. Chem.* **258:**9908-9912

Carmen Martinez, M., Lazdunski, C., Pattus, F. 1983. Isolation, molecular and functional properties of the C-terminal domain of colicin A. *EMBO J.* **2:**1501-1507

Cleveland, M. v., Slatin, S., Finkelstein, A., Levinthal, C. 1983. Structure-function relationships for a voltage-dependent ion channel: Properties of COOH-terminal fragments of colicin E1. *Proc. Nat. Acad. Sci. USA* **80:**3706-3710

Coronado, R., Latorre, R. 1983. Phospholipid bilayers made from monolayers on patch-clamp pipettes. *Biophys. J.* **43:**231-236

Cramer, W. A., Cohen, F. S., Merrill, A. R., Song, H. Y. 1990. Structure and dynamics of the colicin E1 channel. *Mol. Microbiol.* **4:**519-526

Davidson, V. L., Cramer, W. A., Bishop, L. J., Brunden, K. R. 1984. Dependence of the activity of colicin E1 in artificial membrane vesicles on pH, membrane potential, and vesicle size. *J. Biol. Chem.* **259:**594-600

Frenette, M., Knibiehler, M., Baty, D., Geli, V., Pattus, F., Verger, R., Lazdunski, C. 1989. Interaction of colicin A domains with

phospholipid monolayers and liposomes: Relevance to the mechanism of action. *Biochemistry* **28:**2509-2514

Ghosh, P., Stroud, R. M. 1991. Ion channels formed by a highly charged peptide. *Biochemistry* **30:**3551-3557

Kagawa, Y., Racker, E. 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation. *J. Biol. Chem.* **246:**5477-5487

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680-685

Lear, J. D., Wasserman, Z. R., DeGrado, W. F. 1988. Synthetic amphiphilic peptide models for protein ion channels. *Science* **240:**1177-1181

Levinthal, F., Todd, A. P., Hubbell, W. L., Levinthal, C. 1991. A single tryptic fragment of colicin E1 can form an ion channel: Stoichiometry confirms kinetics. *Proteins* **11:**254-262

Mankovich, J. A., Hsu, C., Konisky, J. 1986. DNA and amino acid sequence analysis of structural and immunity genes of colicins Ia and Ib. *J. Bacteriol.* **168:**228-236

Mel, S., Stroud, R. M. 1992. Colicin Ia inserts into negatively charged membranes at low pH with a tertiary but little secondary structural change. *Biochemistry*, submitted

Morlon, J., Lloubes, R., Varenne, S., Chartier, M., Lazdunski, C. 1983. Complete nucleotide sequence of the structural gene for colicin A, a gene translated at non-uniform rate. *J. Mol. Biol.* **170:**271-285

Nogueira, R. A., Varanda, W. A. 1988. Gating properties of channels formed by colicin Ia in planar lipid bilayer membranes. *J. Membr. Biol.* **105:**143-153

Ohno-Iwashita, Y., Imahori, K. 1982. Assignment of the functional loci in the colicin E1 molecule by characterization of its proteolytic fragments. *J. Biol. Chem.* **257:**6446-6451

Oiki, S., Danho, W., Madison, V., Montal, M. 1988. M2, a candidate for the structure lining the ionic channel of the nicotinic cholinergic receptor. *Proc. Nat. Acad. Sci. USA* **85:**8703-8707

Oiki, S., Danho, W., Montal, M. 1988. Channel protein engineering: Synthetic 22-mer peptide from the primary structure of the voltage-sensitive sodium channel forms ionic channels in lipid bilayers. *Proc. Nat. Acad. Sci. USA* **85:**2393-2397

Parker, M. W., Pattus, F., Tucker, A. D., Tsernoglou, D. 1989. Structure of the membrane-pore-forming fragment of colicin A. *Nature* **337**:93-96

Parker, M. W., Tucker, A. D., Tsernoglou, D., Pattus, F. 1990. Insights into membrane insertion based on studies of colicins. *Trends Biochem. Sci.* **15**:126-129

Pattus, F., Martinez, M. C., Dargent, B., Cavard, D., Verger, R., Lazdunski, C. 1983. Interaction of colicin A with phospholipid monolayers and liposomes. *Biochemistry* **22**:5698-5703

Pattus, F., Massotte, D., Wilmsen, H. U., Lakey, J., Tsernoglou, D., Tucker, A., Parker, M. W. 1990. Colicins: Prokaryotic killer-pores. *Experientia* **46**:180-192

Peterson, A. A., Cramer, W. 1987. Voltage-dependent, monomeric channel activity of colicin E1 in artificial membrane vesicles. *J. Membr. Biol.* **99**:197-204

Raymond, L., Slatin, S. L., Finkelstein, A. 1985. Channels formed by colicin E1 in planar lipid bilayers are large and exhibit pH-dependent ion selectivity. *J. Membr. Biol.* **84**:173-181

Raymond, L., Slatin, S. L., Finkelstein, A., Liu, Q., Levinthal, C. 1986. Gating of a voltage-dependent channel (colicin E1) in planar lipid bilayers: translocation of regions outside the channel-forming domain. *J. Membr. Biol.* **92:**255-268

Salles, B., Weisemann, J. M., Weinstock, G. M. 1987. Temporal control of colicin E1 induction. *J. Bacteriol.* **169:**5028-5034

Schein, S. J., Kagan, B. L., Finkelstein, A. 1978. Colicin K acts by forming voltage-dependent channels in phospholipid bilayer membranes. *Nature* **276:**159-163

Seta, P., d'Epenoux, B., Sandeaux, R., Pattus, F., Lazdunski, C., Gavach, C. 1983. Voltage and time dependence of the conductance of planar lipid bilayers doped with colicin A. *Biochem. Biophys. Res. Commun.* **113:**765-771

Sigworth, F. J. 1985. Open channel noise. *Biophys. J.* **47:**709-720

Slatin, S. L. 1988. Colicin E1 in planar lipid bilayers. *Int. J. Biochem.* **20:**737-744

Suarez-Isla, B. A., Wan, K., Lindstrom, J., Montal, M. 1983. Single-channel recordings from purified acetylcholine receptors

reconstituted in bilayers formed at the tip of patch pipets. *Biochemistry* **22:**2319-2323

Waleh, N. S., Johnson, P. H. 1985. Structural and functional organization of the colicin E1 operon. *Proc. Nat. Acad. Sci. USA* **82:**8389-8393

Weaver, C. A., Kagan, B. L., Finkelstein, A., Konisky, J. 1981. Mode of action of colicin Ib. *Biochim. Biophys. Acta* **645:**137-142

Weaver, C. A., Redborg, H., Konisky, J. 1981. Plasmid-determined immunity of *Escherichia coli* K-12 to colicin Ia Is mediated by a plasmid-encoded membrane protein. *J. Bacteriol.* **148:**817-828

Wormald, M. R., Merrill, A. R., Cramer, W. A., Williams, R. J. P. 1990. Solution NMR studies of colicin E1 C-terminal thermolytic peptide: Structural comparison with colicin A and the effects of pH changes. *Eur. J. Biochem.* **191:**155-161

FIGURE LEGENDS

Figure 1. Proteolytic digestion of colicin Ia. (a) 14% SDS-PAGE of tryptic digest of colicin Ia. Colicin Ia was digested with trypsin at a trypsin:colicin Ia molar ratio of 1:3 with colicin at 270 μM at 37° C, and time points were collected as indicated on the figure. The 18 kD colicin Ia fragment, whose amino-terminus is residue 451, persists for 4 hours, while the 34 kD fragment, whose amino-terminus is residue 7, persists for 1 hour. The band at 28 kD corresponds to trypsin. (b) 16% SDS-PAGE of V8 digest of colicin Ia in 2.4 M urea. Colicin Ia was digested with V8 at a V8:colicin Ia molar ratio of 1:3 with colicin Ia at 44 μM at 37° C, and time points were collected as indicated on the figure. A 20 kD fragment, whose amino-terminus is residue 446, and several lower molecular weight fragments persist through 2 hours of digestion.

Figure 2. Tryptic digestion and purification of 34 and 18 kD fragments. (a) Size exclusion HPLC of tryptic digest of colicin Ia. The extent of the fractions collected are indicated by shading. Digestion conditions were as in the legend to Figure 1a except that the trypsin:colicin Ia molar ratio was 1:30. The digest was stopped at 4 hours, and the products were applied to two, tandem Biosil TSK 250 columns at a flow rate of 2 ml/min. The peaks eluting are: Peak 1, colicin Ia at 117 minutes; Peak 2, 34 kD fragment at 141 minutes;

and Peak 3, 18 kD fragment at 160 minutes. (b) 16% SDS-PAGE of peak fractions from size exclusion HPLC. Lane 1 contains Peak 1, 117 minutes; lane 2 contains Peak 2, 141 minutes; and lane 3 contains Peak 3, 160 minutes.

Figure 3. 18 kD fragment ion channels. The three traces are from three different membranes. (a) Single channel currents resulting from the 18 kD fragment in symmetrical 500 mM NaCl, 1 mM $CaCl_2$, 10 mM dimethylglutarate, pH 4.0, at a holding potential of +78 mV. The horizontal bar represents the closed state of the channel, and the downward deflections indicate opening of the channel. The conductances of these channels range from 15 to 30 pS and their open times range from 25 to 400 milliseconds. (b) Single channel currents resulting from the 18 kD fragment in the same conditions as in 4a, except that the holding potential is -55 mV. The conductance of these channels is approximately 100 pS and their open times range from approximately 200 milliseconds to 4 seconds. Large fluctuations in current flowing through the open channel are evident. (c) Single channel currents resulting from the 18 kD fragment in the same conditions as in 4a, except that the holding potential is -45 mV. The channels displayed range between approximately 60 pS and 180 pS, although smaller channels of 10 pS are evident.

Figure 4. Whole colicin Ia channels. The two traces are from two different membranes. (a) Single channel currents resulting from

whole colicin Ia in symmetrical 500 mM NaCl, 1 mM $CaCl_2$, 10 mM dimethylglutarate, pH 4.0, at a holding potential of -93 mV. The current was filtered at 10 Hz with an 8-pole Butterworth filter resulting in the low current noise level in this trace. The horizontal bar represents the closed state of the channel, and upward deflections represent channel openings. At the beginning of the record, the closure of a previously open channel is followed by the opening of another channel or the same channel to a sub-conductance state. Various conductance states are evident with the lowest conductance state being 11 pS. (b) Single channel currents resulting from whole colicin Ia in the same conditions as the legend to Figure 4a, except that the holding potential is -80 mV. A previously open channel transits through various sub-conductance states and finally closes from the second largest sub-conductance state, which is 25 pS.

Figure 5. Whole colicin Ia opens only at negative voltages. The two traces are from the same membrane. (a) Single channel currents resulting from whole colicin Ia in symmetrical 1 M NaCl, 1 mM $CaCl_2$, 10 mM dimethylglutarate, pH 4.0. The voltage was ramped between +75 mV and -80 mV. About 5 to 20 channels are open, resulting in a peak total conductance of 140 pS at -80 mV. Upward deflections at negative voltages, which represent channel openings, are evident. The current at positive voltages is saturated, and therefore an off-set to the amplifier was applied in (b), shown at the arrow. Here,

current at positive voltages is no longer saturated, although current at negative voltages is partially saturated. This record shows that no channel activity is found at positive voltages while upward deflections, indicative of channel openings, are still found at negative voltages.

FIGURE 1a

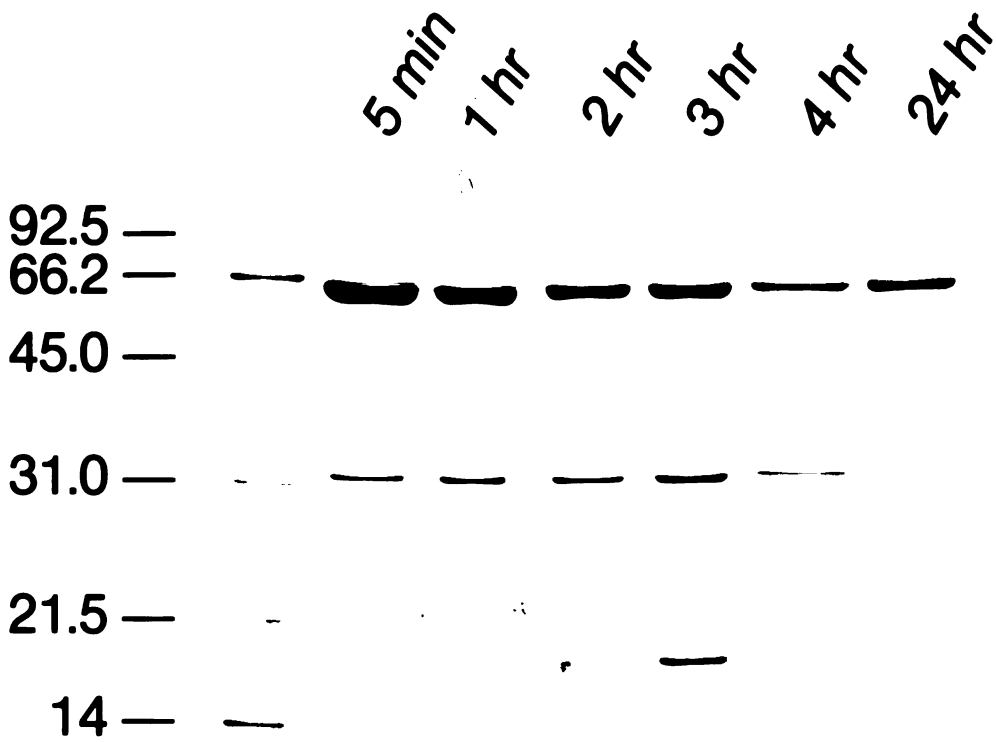


FIGURE 1b

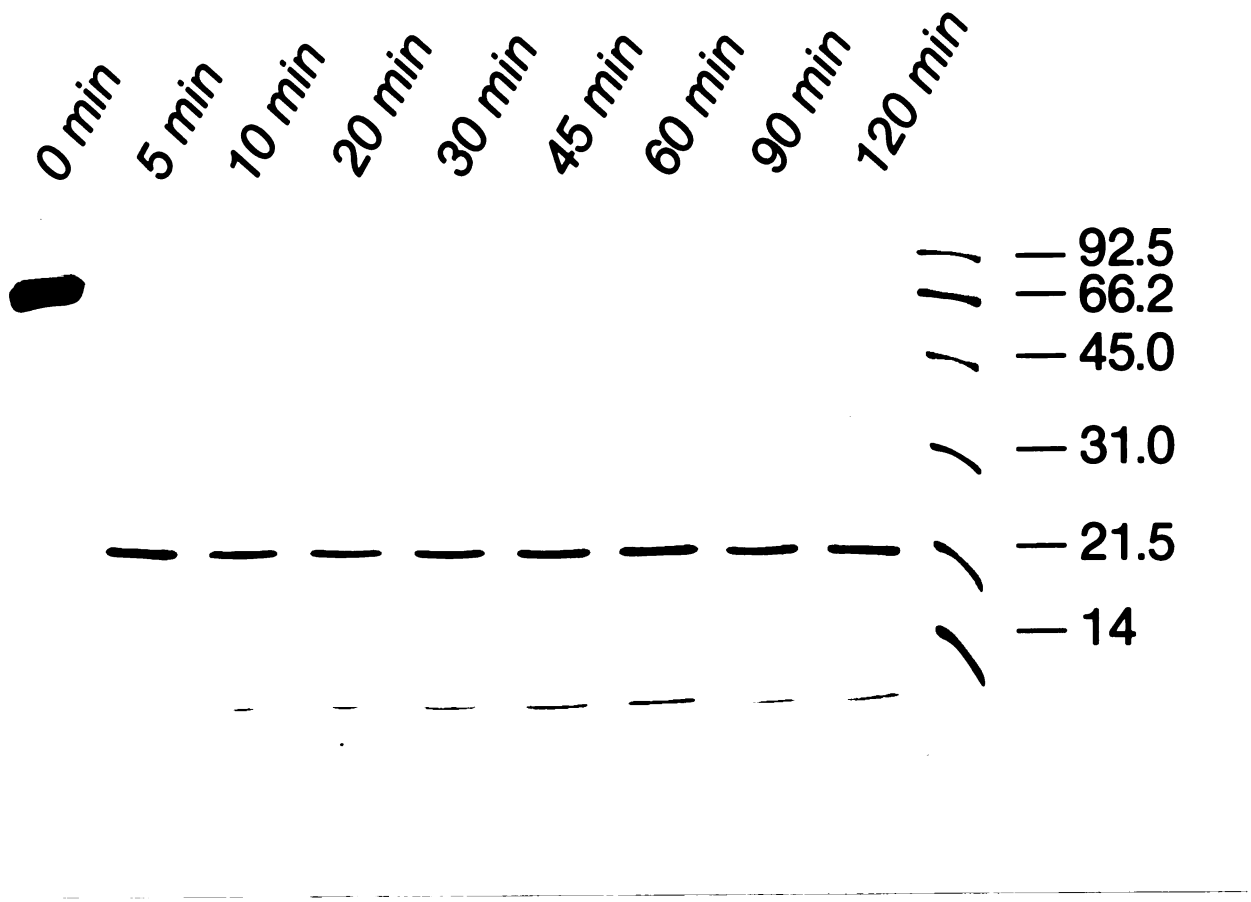

0 min
5 min
10 min
20 min
30 min
45 min
60 min
90 min
120 min
92.5
66.2
45.0
31.0
21.5
14

FIGURE 2a

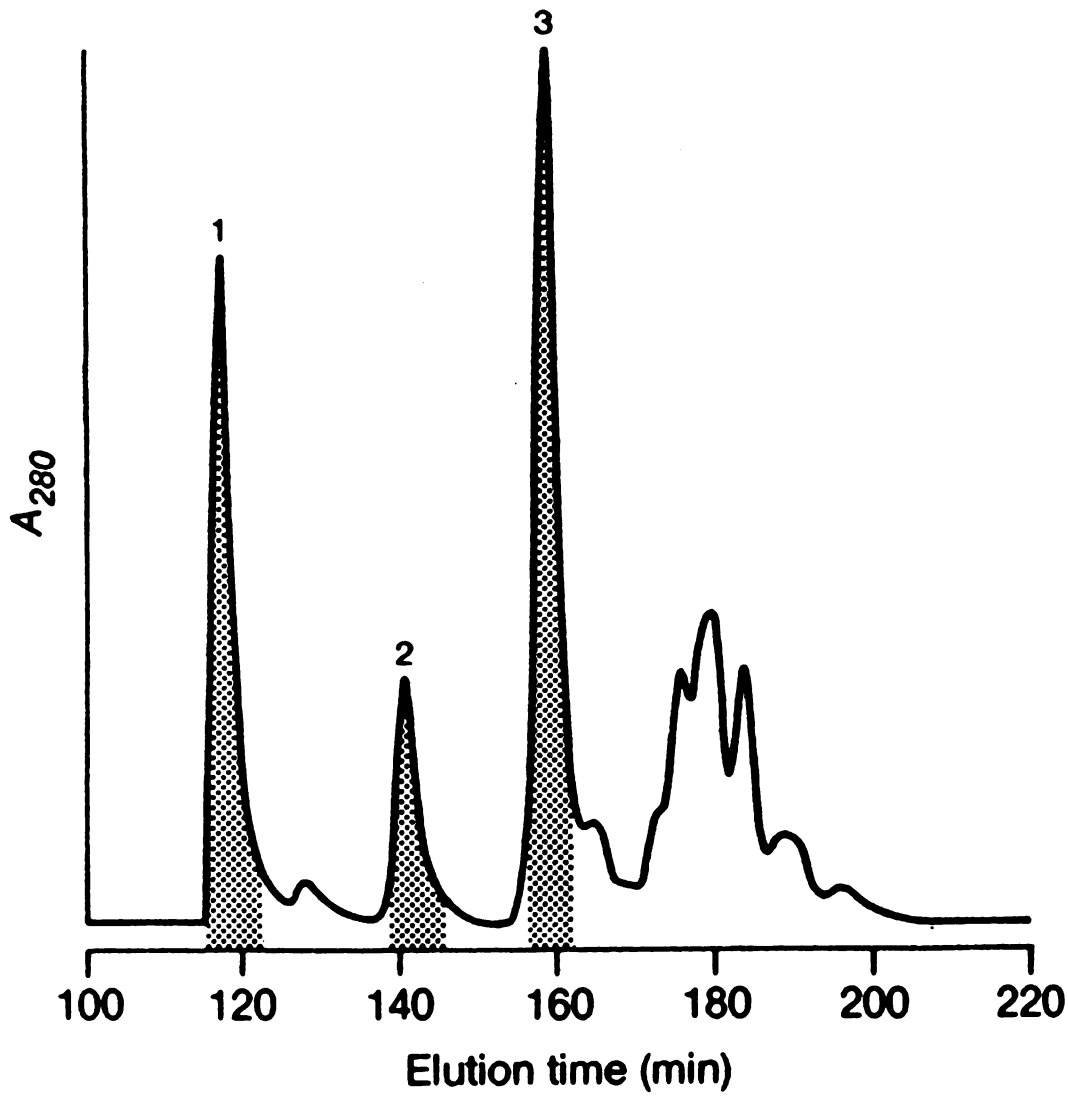
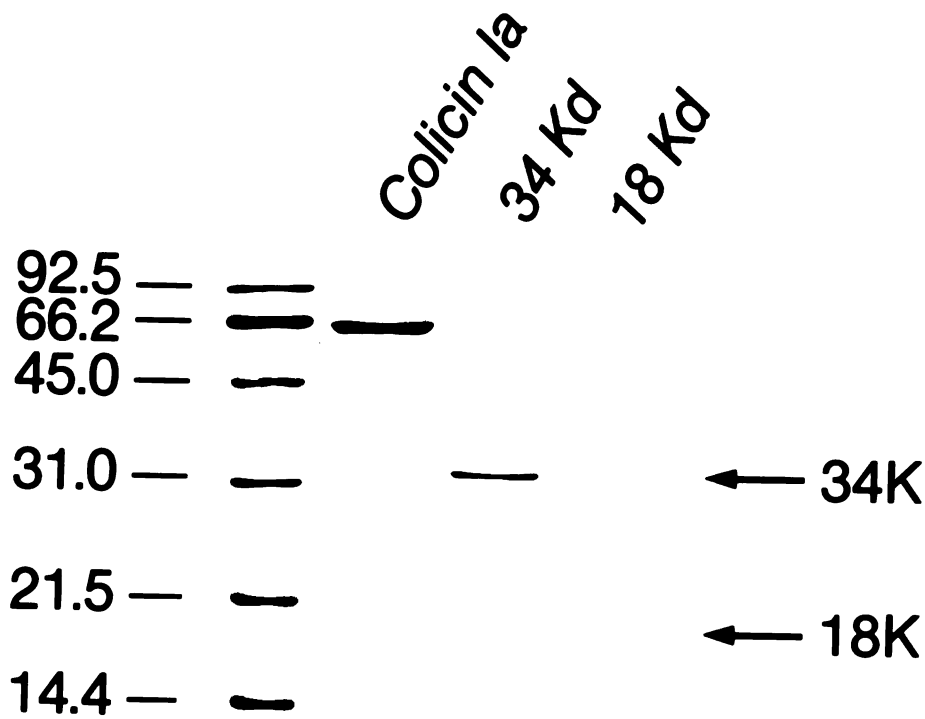
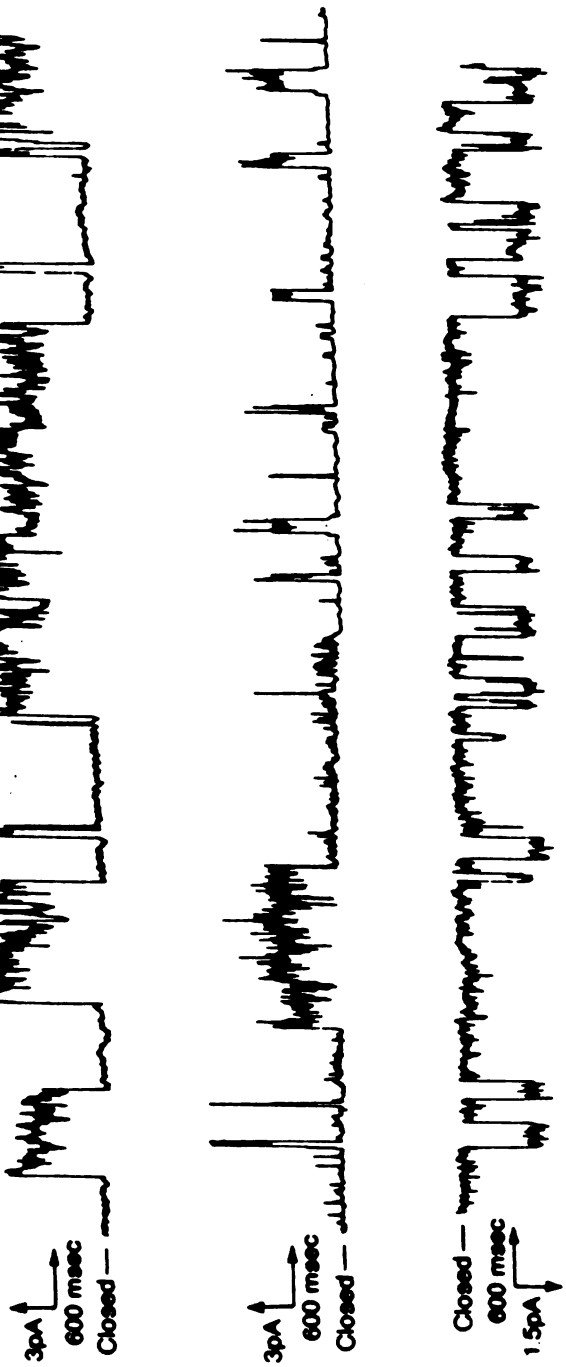


FIGURE 2b

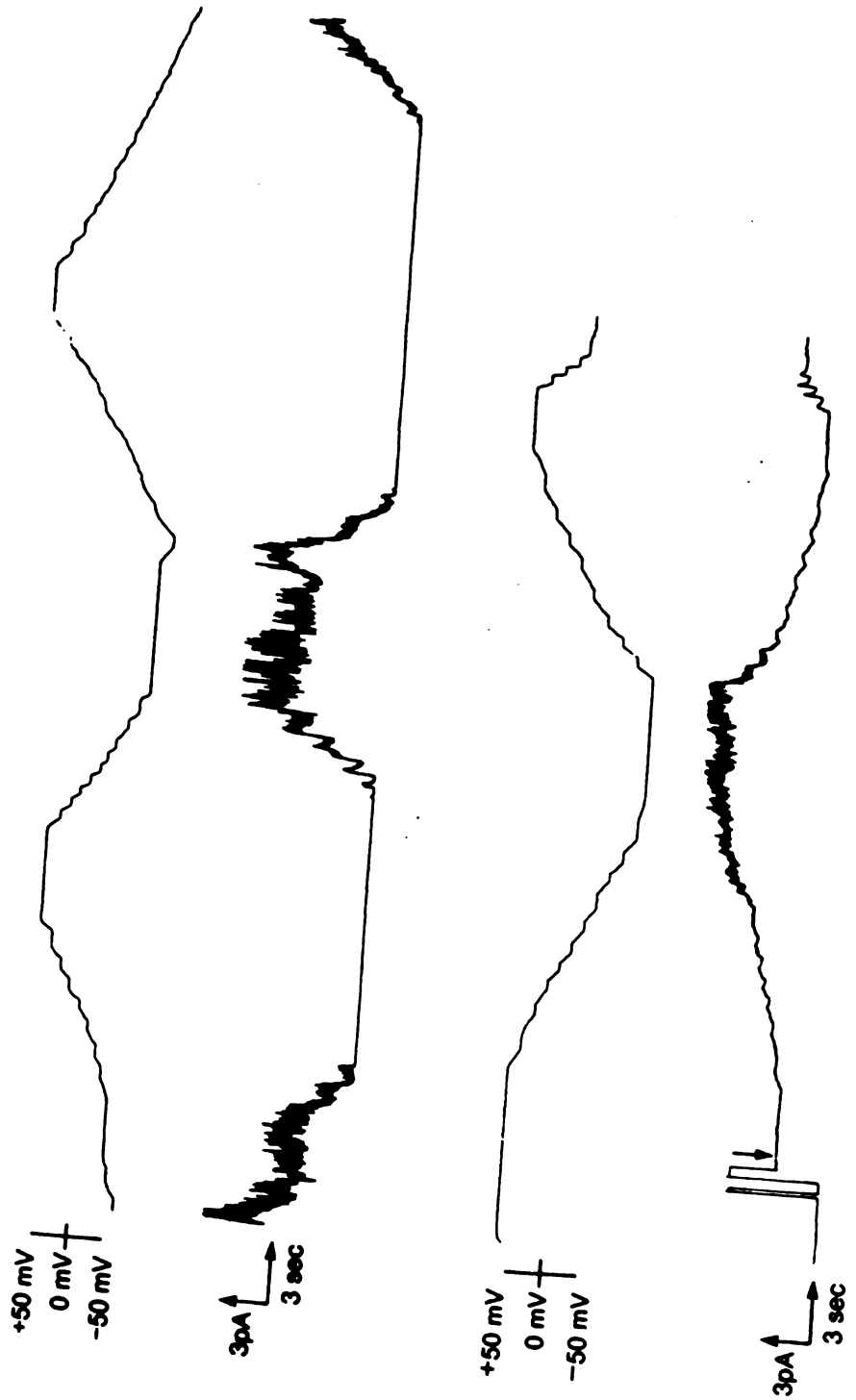


FIGURES 3a, b , and c



FIGURES 4a and b

FIGURES 5a and b



UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

BERKELEY • DAVIS • IRVINE • LOS ANGELES • RIVERSIDE • SAN DIEGO • SAN FRANCISCO SANTA BARBARA • SANTA CRUZ

SCHOOL OF MEDICINE
DEPARTMENT OF BIOCHEMISTRY AND BIOPHYSICS
SAN FRANCISCO, CALIFORNIA 94143-0448
(415) 476-4324 FAX # (415) 476-0961

27 Aug. 1992

Harcourt Brace Jovanovich, Publishers
6277 Sea Harbor Drive
Orlando, FL. 32887

To whom it may concern:

I would like written permission to include in my dissertation a copy of the paper cited below, which was published in

Techniques in Protein Chemistry, Edited by Tony E. Hugli
Academic Press, Inc. 1989

Arnold M. Falick, Stephanie F. Mel, Robert M. Stroud, and A. L. Burlingame, (1989) A New Strategy for Mapping the Topography of a Transmembrane Protein using Mass Spectrometry. pp. 152-159.

The dissertation will be microfilmed by University Microfilms Incorporated, and they request permission to supply single copies on demand.

Thank you for your consideration.

Stephanie Mel

September 10, 1992

PERMISSION GRANTED, provided that 1) complete credit is given to the source, including the Academic Press copyright notice; 2) the material to be used has appeared in our publication without credit or acknowledgement to another source and 3) if commercial publication should result, you must contact Academic Press again.

We realize that University Microfilms must have permission to sell copies of your thesis, and we agree to this. However, we must point out that we are not giving permission for separate sale of your article.

Martha Strassberger
Contracts, Rights and Permissions
Academic Press, Inc.
Orlando, FL 32887