Structure-function studies on the mechanism of action of staphylococcal alpha toxin.

Terence E. Hebert

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STRUCTURE-FUNCTION STUDIES ON THE MECHANISM OF ACTION OF STAPHYLOCOCCAL ALPHA TOXIN

by
Terence, E. Hebert

A Thesis submitted to the Faculty of Graduate Studies through the Department of Biological Sciences in partial fulfillment for the degree of Master of Science at the University of Windsor Windsor, Ontario Canada 1987
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Abstract

The effects of 1,2-cyclohexanediolene and phenylglyoxal on staphylococcal α-toxin were studied. Modification of one arginine residue in α-toxin was sufficient to render the toxin nonhemolytic with no conformational change. Modification of the same arginine also abrogated the cytotoxic effect of α-toxin in VERO cells and perfused rat atria. Modified α-toxin did not protect cells from hemolysis or cytotoxicity by native α-toxin. An arginine residue is therefore at or near the binding site of α-toxin. Trypsin digestion of modified α-toxin generated a Mr 20 kD fragment which was isolated using a boric acid gel column. Upon regeneration of the modified arginine residue this 20 kD fragment was not recognized by a population of antibodies which prevented α-toxin binding. The fragment was recognized by antibodies directed against post-binding events. However, the antibinding antibodies recognized the intact modified toxin. This leads us to conclude that antibinding determinants are not found directly in the binding site or are conformationally masked.

A 9 kD CNBr fragment of α-toxin containing the modified arginine residue was isolated using the boric acid gel and a reverse phase HPLC system. This fragment possessed similar immunological specificities as a 9 kD CNBr fragment of the toxin characterized by Surujballi (1987 Ph. D. Dissertation). Percent composition of this fragment localized the binding site to a region in the C-terminal domain (Trp 175–Met 197) of α-toxin and the functional arginine as Arg 184.

Modification of a single histidine residue in α-toxin with diethylpyrocarbonate also led to complete inhibition of hemolytic activity with no subsequent conformational change. Preincubation of modified toxin with erythrocytes protected them in a dose-dependent manner against native α-toxin. Thus the
histidine residue modified is involved in events that occur after the toxin binds to its receptor. No difference in titre was detected in ELISAs performed on native and diethylpyrocarbonate modified toxin using polyclonal anti α-toxin populations. Using monoclonal antibodies developed in this laboratory against α-toxin (Surujballi et al, in prep) it was determined that the modified toxin differed in titre when compared to the native toxin using MAb-3. A MAb-3 immunoaffinity column was used to isolate a 12 kD fragment from a CNBr digest of the toxin. Percent composition determination localized the fragment to a region near the N-terminus of the native toxin (His 36-Met 112). These results confirm earlier fragmentation studies on the localization of important functional domains in the toxin (Kato and Watanabe, 1980; Watanabe and Kato, 1978; Blomqvist and Thelestad, 1986a; Blomqvist et al, 1987a).
DEDICATION

This thesis is dedicated to my parents and my younger brother who are ultimately responsible for any success I enjoy.
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. H.B. Packrell for his guidance and support during my tenure here and especially for being tolerant of my sometimes misguided irreverence. I am also grateful to the members of my committee, Dr. A.H. Warner and Dr. K.E. Taylor. Dr. Paul Taylor also receives a hearty thank you for much technical and logistical assistance as well as a great deal of almost fatherly advice. I am deeply indebted to a number of former graduate students in this department who helped me in my initial adventures in the world of biology, especially Om Surujballi, John Austin, Lou Passadore and Jamie Pitts. The technical assistance of Mrs. Julie Dosescu was also invaluable. Finally, I would like to thank a great deal of wise friends both in the department and elsewhere who helped strengthen my tenuous grip on reality.
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Chapter 1. Inhibition of staphylococcal α-toxin by covalent modification of an arginine residue

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LIST OF ABBREVIATIONS

AB-Ab  antibinding antibodies
C      degrees centigrade
CHD    1,2-cyclohexanedione
CNBr   cyanogen bromide
DPC    diethylpyrocarbonate
EDTA   ethylenediamine tetraacetic acid
ELISA  enzyme linked immunosorbent assay
g      unit of gravity
HPLC   high performance liquid chromatography
HRP    horseradish peroxidase
HU     hemolytic unit
IgG    immunoglobulin G
IHA-Ab  indirect hemagglutinating antibody
MAb    monoclonal antibody
PAGE   polyacrylamide gel electrophoresis
PBS    phosphate buffered saline
PITC   phenylisothiocyanate
PGO    phenylglyoxal
rpm    revolutions per minute
SDS    sodium dodecyl sulphate
TBS    Tris buffered saline
Introduction
\(\alpha\)-toxin has been demonstrated to be a major virulence factor during infections of \textit{Staphylococcus aureus} (Jansson \textit{et al}, 1985; Kinsman \textit{et al}, 1981; O'Reilly \textit{et al}, 1986). It has a number of biological effects including hemolysis, cytotoxicity, dermonecrosis, and lethality (for review, see Freer and Arbutlnott, 1983; Wiseman, 1975). It has become clear that the common site of action for the toxin is the cell membrane. However, the precise mechanism of action has remained an enigma for almost a century. Fragmentation studies have defined distinct domains within the toxin. The C-terminal domain contains the binding site of the toxin and regions important in hemolytic activity whereas the N-terminal domain contains the cytotoxic and lethal activities of the toxin (Watanabe and Kato, 1978; Kato and Watanabe, 1980; Blomqvist and Thelestam, 1986a; Blomqvist \textit{et al}, 1987a). Surujballi (1987 Ph.D. dissertation) has localized the binding site to a 9000 D CNBr fragment of \(\alpha\)-toxin. However, few studies have been undertaken to determine the functional role of individual amino acids in the activity of \(\alpha\)-toxin. Cassidy and Harshman (1976a,b) demonstrated that a single tyrosine residue was important in hemolysis but did not affect binding of \(\alpha\)-toxin to rabbit erythrocyte membranes.

The objectives of this study were twofold:

1) To characterize the functional importance of amino acid residues other than tyrosine in the activity of staphylococcal \(\alpha\)-toxin.

2) To generate fragments of \(\alpha\)-toxin containing the functional residues and characterize them antigenically as well as structurally.

Arginine residues were chosen for initial modification studies for a number of reasons. First, it had been shown (Maharaj and Fackrell, 1980; Simpson and Fackrell, in prep) that the receptor for \(\alpha\)-toxin in rabbit erythrocytes was the
carbohydrate moiety of Band 3 and that the receptor site itself was negatively charged. Arginine residues have been shown to be important in the anion binding sites of a number of proteins (Riordan, 1979; Patthy and Thesz, 1980). Also, a number of arginine specific reagents are commercially available including phenylglyoxal (PGO) and 1,2-cyclohexanedione (CHD). CHD has the added advantages of being stoichiometrically removable from arginine, quantifiable, and capable of forming complexes with borate ions (Patthy and Smith, 1975a,b). This latter feature enables CHD containing peptides to be isolated with an affinity chromatography system (Patthy et al, 1979).

α-toxin also possesses a limited number of histidine residues (for review, see Wiseman, 1975; Gray and Kehoe, 1984). Histidines have also been shown to be important in the functions of a number of proteins (Atassi, 1977; Miles, 1977; Iglesias and Andreo, 1983). Histidine can be modified specifically using the reagent diethylpyrocarbonate (Miles, 1977).

This study also required the development of a reverse-phase HPLC system to isolate fragments generated by digestion of α-toxin. Early attempts (Surujballi, 1987 Ph.D. dissertation) using acetonitrile as a solvent failed because the toxin and its fragments precipitated out. The conversion of a non-dedicated HPLC system to one capable of performing amino acid analysis according to a method described by Henrickson and Meredith (1984) was also required.
Literature Review
A batch of diphtheria toxoid contaminated with *Staphylococcus aureus* in 1928 brought sharply into focus the relationship between toxin production and bacterial pathogenicity (Elek, 1959). As early as 1929, Burnet showed that the effects of staphylococcal supernatants, i.e., hemolysis, dermonecrosis, and lethality could be neutralized with antisera from rabbits injected with a crude bacterial filtrate. It was later concluded that a single toxin was responsible for all these manifestations (Burnet, 1931).

It has subsequently been shown that *S. aureus* produces four hemolysins each with a distinct mechanism of action which were termed \( \alpha \), \( \beta \), \( \gamma \), and \( \delta \) in order of their discovery. \( \alpha \)-hemolysin or \( \alpha \)-toxin as, it has come to be known, was found to be cytotoxic, hemolytic, dermonecrotic, as well as lethal for a number of animals (Kumar *et al* 1962; Bernheimer and Schwartz, 1963; Wiseman, 1975).

\( \beta \)-hemolysin displayed a greater effect on sheep erythrocytes than \( \alpha \)-toxin which had its most dramatic effect on rabbit erythrocytes (Bigger, 1933). \( \beta \)-hemolysin also demonstrated a "hot-cold" phenomenon, that is, erythrocytes exposed to the hemolysin at 37°C and then incubated at 4°C displayed an intensification of hemolysis (Walbom, 1921; Bigger *et al* 1927). It was subsequently shown to be serologically distinct from \( \alpha \)-toxin (Glenny and Stephens, 1935) and eventually proved to be a \( \text{Mg}^{2+} \) dependent phospholipase C specific for sphingomyelin (Doery *et al* 1963; 1965; Maheswaran *et al* 1967; Wadstrom and Mollby, 1971a,b; Wiseman and Caird, 1967).

Smith and Price (1938) proposed the existence of \( \gamma \)-hemolysin. Until relatively recently it has remained effectively uncharacterized but purification has
become possible (Guyonnet and Plommet, 1970; Packrell, 1974). It is hemolytic toward human, rabbit and sheep erythrocytes (Packrell, 1974). It was shown that the hemolytic activity of \( \gamma \)-hemolysin could be inhibited by phosphatidylinositol, phosphatidylcholine, phosphatidylglycerol, phosphatidylserine, nervonic acid, stearic acid, palmitic acid and cardioloopin all of which possess an esterified 3-\( \beta \)-hydroxy group (Bernheimer, 1974). Release of acid soluble phosphorus and nitrogen from \( \gamma \)-hemolysin treated erythrocytes has been demonstrated (Packrell and Wiseman, 1976b) suggesting it possesses phospholipase activity, but no change in erythrocyte lipids was detected (Taylor and Bernheimer, 1974). The exact mode of action of \( \gamma \)-hemolysin still remains unclear.

\( \delta \)-hemolysin was detected on sheep blood agar infused with antisera against \( \alpha \) and \( \beta \)-hemolysins (Williams and Harper, 1947). This hemolysin does not exhibit the species specificity demonstrated by other staphyloccoccal hemolysins (Kayser and Renaud, 1965) and has a wide spectrum of cytotoxic activity. Again, some evidence suggests that \( \delta \)-hemolysin possesses phospholipase activity (Wiseman and Caird, 1967) but an actual substrate has not been determined. Bernheimer (1974) suggested that \( \delta \)-hemolysin acts via a detergent-like action on susceptible cells. More recent studies (Bhakoo et al., 1982; Freer and Birkbeck, 1982) provide evidence that \( \delta \)-hemolysin is a rod shaped amphipathic molecule with an \( \alpha \) helical conformation that allows it to insert itself in aggregates across the lipid bilayer resulting in the loss of selective ion permeability and leading to osmotic lysis of the cell.

Physiochemical Properties of \( \alpha \)-Toxin.
α-toxin exists as a single polypeptide chain which possesses neither cysteine or carbohydrate (Bernheimer and Schwartz, 1963; Lominski et al. 1963; Arbuthnott, 1970; Six et al. 1973a, b). α-toxin is a positively charged molecule with a pI of 8.5 (Coulter, 1966; Wiseman, 1975). Although the molecular weight of α-toxin has been estimated to be anywhere in the range of 10–45 kD as summarized by Wiseman (1975), Six and Harshman (1973b) reported a molecular weight between 26–31 kD for α-toxin using four independent methods on a single batch of toxin. This is supported by the findings of Gray and Kehoe (1984) who sequenced the structural gene for the toxin and predicted the molecular weight to be 31 kD.

Various groups have performed amino acid analyses on the toxin which are all in reasonable agreement (Bernheimer and Schwartz, 1963; Coulter, 1966; Fackrell and Wiseman, 1976a; Kato and Watanabe, 1980; Six and Harshman, 1973a; Watanabe and Kato, 1978; Wiseman and Caird, 1970). Discrepancies exist in the assignment of a N-terminal amino acid to α-toxin (Coulter, 1966; Six and Harshman, 1973a, b; Fackrell and Wiseman, 1976a; Wiseman and Caird, 1970; Watanabe and Kato, 1978) but this variation may be explained by the presence of different proteases during the purification procedure (Dalen, 1976b). This observation may also account for some of the variation in molecular weight.

Studies aimed at estimating the sedimentation coefficient of α-toxin have revealed that multiple forms of the toxin exist. The main component of α-toxin has a sedimentation coefficient of approximately 3S (Bernheimer and Schwartz, 1963; Cooper et al. 1963, Coulter, 1966; Goode and Baldwin, 1973; Lominski et al. 1963; Six and Harshman, 1973a, b; Fussle et al. 1981). However, in even highly purified preparations of the toxin, a component has been found with a sedimentation coefficient of 10–16S. This is believed to represent an aggregated or polymerized
form of the toxin and is biologically inactive (Arbuthnott et al. 1967). They further showed that treatment of the 12S form with urea resulted in a retrieval of the 3S form and biological activity. Electron microscopic studies (Freer et al. 1968) demonstrated that the 12S toxin consisted of small rings 9–10nm in outer diameter and 2–2.5nm in inner diameter. These rings were a hexagonal array of toxin monomers. In contrast, the 3S form of the toxin was shown to be amorphous in negatively stained preparations (Freer et al. 1968; Dalen, 1975c).

Multiple forms of the toxin have been shown to exist which have similar biological properties (Berheimer and Schwartz, 1963; Goode and Baldwin, 1973; 1974; McNiven et al. 1972; Wadstrom, 1968; Fackrell and Wiseman, 1976b). \( \alpha \)-toxin could be separated into four populations based on isoelectric focussing. However, the main component (>80%) was at pI 8.5. Wadstrom (1968) suggested that the four forms were intraconvertible as refocussing of any of the four isoelectric components generated a pattern consisting of four pIs. Six and Harshman (1973a,b) also demonstrated that the different forms of the toxin were intraconvertible.

More recent studies have measured the secondary structure of \( \alpha \)-toxin using circular dichroism spectroscopy (Tobkes et al. 1985; Ikigai and Nakae, 1985). They determined that \( \alpha \)-toxin consisted of approximately 66% \( \beta \)-sheet structure, 26% random coil or unassigned structure, and approximately 11% \( \alpha \)-helical character. As noted earlier, some of the variation in the N-terminus and in molecular weight determination was as a result of variation used in methods of purification. The various methods used to purify \( \alpha \)-toxin have been extensively reviewed by a number of authors (Arbuthnott, 1970; Freer and Arbuthnott, 1983; Rogolsky, 1979; Wiseman, 1973).

**Genetics and Biosynthesis of \( \alpha \)-Toxin.**
The Wood 46 strain of *S. aureus* has become the most widely studied producer of α-toxin although it is known that considerable variation exists within the strain (Freer and Arbuthnott, 1983). α-toxin produced by different strains, however, has similar biological and chemical properties (Goode and Baldwin, 1974).

Few studies have been initiated regarding the nutritional factors important in the production of α-toxin by *S. aureus*. Concentrations of 10–20% CO₂ were found to stimulate the production of the toxin (Parker *et al.* 1926; Burnet, 1930). Glycine, arginine and proline were also found to be important precursors in the synthesis of α-toxin (Gladstone, 1938). Dalen (1973c) later noted that serine and histidine also increased yields and suggested that CO₂, serine and glycine were important as they were precursors of histidine synthesis. He concluded, however, that the rate of α-toxin production was not directly correlated with free intracellular histidine concentration. It was also noted that maximal production of α-toxin occurs at a glucose concentration of 0.2% (Duncan and Cho, 1972). Starvation of staphylococci surprisingly did not affect the production of the toxin whereas the production of other exoproteins was markedly reduced (Szewczyk and Mikucki, 1981).

The production of α-toxin in liquid batch cultures occurs in a biphasic manner. During exponential growth, low levels of toxin are produced with a marked increase occurring just prior to the stationary phase (Coleman and Abbas-Alil, 1977; Duncan and Cho, 1971). α-toxin can account for up to 2% of the total dry weight of the culture by stationary phase (Duncan and Cho, 1971) and is localized on the inner surface of the cytoplasmic membrane prior to secretion (Coulter and Mukherjee, 1971).
1971). Cultures of *S. aureus* treated with the antibiotic cerulenin showed a marked reduction in both secreted α-toxin and membrane bound toxin as well (Saleh and Freer, 1984). Cerulenin prevents fatty acid biosynthesis by inhibiting β-keto acyl carrier protein synthesis. The authors speculate that since they detected a 50% reduction in membrane lipid levels without a significant effect on growth rate, the proper insertion of a membrane docking protein was prevented thus affecting subsequent secretion of the toxin. It is generally accepted now that α-toxin is secreted from intact cells and autolysis is not involved (Freer and Arbuthnott, 1983).

α-toxin is synthesized as a larger molecule which is subsequently processed to its mature form. Tweten *et al.* (1983) identified and partially characterized two larger precursor proteins of the toxin. Both were present in small quantities in the cell membrane and rapidly converted to the mature toxin. In peptide maps of the precursors, four extra peptides were found and these were believed to represent a signal sequence involved in secretion of the toxin. When the gene for α-toxin was cloned into *E. coli* it was not secreted (Kehoe *et al.* 1983). However, when the gene was cloned into α-toxin negative strains of *S. aureus* or *B. subtilis* it was secreted in its mature form (Fairweather *et al.* 1983).

The structural gene for α-toxin was sequenced (Gray and Kehoe, 1984) and found to possess a 26 amino acid leader peptide which possessed characteristic features of a signal sequence involved in secretion.

The genetic control of α-toxin synthesis has generated considerable interest, especially in recent years. The production of α-toxin among the various strains of *S. aureus* is not ubiquitous and may even vary among strains.
that produce it (Elek, 1959). Early studies implicated a link between toxin production and lysogeny, although a search for a prophage was fruitless (Blair and Cass, 1961; Hendrick and Altembern, 1968). The suggestion that α-toxin activity is controlled by a plasmid at least in some strains came about as a result of the high spontaneous rate of loss of the α-toxin marker in clinical isolates of S. aureus (Witte, 1976).

Studies of α-toxin mutants indicated that they fell into two distinct groups (Dalen; 1976c; McClatchy and Rosenbloom, 1966). In the first group of mutants, the effects were pleiotropic on both α-toxin and fibrinolysin production and probably involved a regulatory locus. The second group of mutants produced α-toxin cross reacting material and involved the structural gene for the toxin itself. Recombination only occurred between mutants in different groups. Other groups have also demonstrated a close association between the production of α-toxin and fibrinolysin (Wheeler, 1975; Brown and Pattee, 1980). These studies indicated that the hla locus once believed to be the α-toxin structural gene was more likely a regulatory gene controlling synthesis of both the toxin and fibrinolysin. Brown and Pattee (1980) found a high degree of instability in the hla gene which suggested that it may have been a transposable element. Hla- derivatives of the Hla+ phenotype in S. aureus strain 8325 did not revert back to Hla+ (Rogolsky, 1979). This data supported the hypothesis that hla is a transposable element. Phillips and Novick (1979) suggested that this may explain earlier observations that placed α-toxin under control of plasmids in some cases and phage conversion in others. This, however, has not been substantiated.

Insertion mutagenesis studies have provided strong evidence that α-toxin
is indeed under the control of the \textit{hla} locus. Mallonee \textit{et al} (1982) and Rescei \textit{et al} (1986) reported that insertion of the transposon Tn551 into the \textit{hla} locus reduced the production of $\alpha$-toxin and subsequently elevated the production of another staphylococcal exoprotein, protein A. Coordinate control of exoprotein synthesis was also noted by Janzon \textit{et al} (1986). In these studies, the defect in $\alpha$-toxin synthesis was at the level of transcription suggesting that a regulatory protein was affected. This locus, \textit{hla} has since become known as \textit{agr} for accessory gene regulator and maps between the \textit{pur B} and \textit{fib} loci on the staphylococcal chromosome. Its product and its mode of action are at present unclear. The structural gene which has recently been cloned and sequenced (Kehoe \textit{et al} 1983; Fairweather \textit{et al} 1983; Gray and Kehoe, 1984) is now known as \textit{hly} and maps to a location distant from \textit{hla} (\textit{agr}) between the \textit{ura B} and the \textit{thr B} loci (O'Reilly \textit{et al} 1985, Pattee, 1986).

**Biological Activities**

$\alpha$-toxin exhibits a diverse array of biological effects on an equally large array of susceptible cells and tissues. It is hemolytic, dermonecrotic, cytotoxic, embryotoxic, and lethal for many small laboratory animals (Freer and Arbuthnott, 1976; 1983; Rogolsky, 1979; Wiseman, 1975; Harshman, 1979; Lesinski \textit{et al} 1967).

$\alpha$-toxin is lethal in minute amounts in rabbits and an LD$_{50}$ of 1.3 $\mu$g/kg body weight has been determined (Lominski \textit{et al} 1963). For mice the LD$_{50}$ was determined to be 40-60 ng/kg body weight i.v. (Lominski \textit{et al} 1963; Bernheimer and Schwartz, 1963). In addition to being lethal for many mammalian
species, \( \alpha \)-toxin was found to be equally lethal for many cold-blooded animals including frogs, toads and fish (Lominski et al. 1965).

The most widely studied effect of \( \alpha \)-toxin has become its hemolytic activity. In addition, the toxin has a wide variety of other effects. \( \alpha \)-toxin damages leucocytes (Gladstone, 1966; Jeljaszewicz, 1972; Maheswaran et al. 1969; McGee et al. 1983), platelets (Siegel and Cohen, 1964), human diploid fibroblasts, HeLa cells, rabbit kidney cells (Artenstein et al. 1963), and Y1 adrenocortical cells (Thelestat and Blomqvist, 1984). Epithelial cells in general were found to be more sensitive to the toxin than fibroblasts (Thelestat, 1983a). At sublytic concentrations \( \alpha \)-toxin was actually found to be mitogenic for rabbit leucocytes \textit{in vivo} and \textit{in vitro} (Szmigelski et al. 1967; Czerski et al. 1967).

\( \alpha \)-toxin was also found to disrupt lysosomes as well as bacterial spheroplasts (Bernheimer and Schwartz, 1964; Bernheimer, 1974).

In addition, \( \alpha \)-toxin causes a contraction and spastic paralysis of mouse skeletal muscle in both \textit{in vivo} and \textit{in vitro} systems (Lominski et al. 1962) although these data are controversial (Thal and Egner, 1961). Vascular smooth muscle is affected in a similar fashion (Cassidy and Harshman, 1974; Wurzel et al. 1966; Gulda et al. 1978). \( \alpha \)-toxin also seems to exert a direct effect on cardiac muscle as well (Adamo, Fackrell, and Taylor, unpublished observations).

Exposure to \( \alpha \)-toxin also has effects on the nervous system of various animals. \( \alpha \)-toxin induces abnormal EEG readings in rabbits prior to death (Edelwejn et al. 1976) as well as in rats (Lipman and Harshman, 1985). In isolated nerve cells, \( \alpha \)-toxin preferentially injures myelin sheaths. Recently,
Chan and Lazarovici (1987) demonstrated that α-toxin can stimulate the phosphorylation of several myelin specific proteins including myelin basic protein which may facilitate its demyelinating effect. This demyelination was seemingly a very specific event since leakage of ions or changes in EEG tracings were not detected although extensive demyelination was evident (Harshman et al. 1981; 1985; Szmygelski and Harshman, 1978; Szmygelski et al. 1979). The nerve cell itself however, may be unaffected as the toxin had no effect on cultured neuroblastoma cells (Harshman, 1979; Thelestam, 1983a) and produced no change in conductivity in isolated Xenopus laevis nerve cells (Blomqvist, personal communication).

α-toxin also has profound effects on the circulatory system. At sublytic concentrations, it is a potent stimulator of prostacyclin release from lung endothelial cells (Suttrop et al. 1985) as well as leukotriene release from polymorphonuclear lymphocytes (Suttrop et al. 1987) both of which cause marked vasoconstriction. The result of this vasoconstriction was a subsequent rise in pulmonary arterial pressure and eventually pulmonary edema (Seeger et al. 1984). A direct action on vascular smooth muscle also results in vasoconstriction (Wurzel et al. 1966; Cassidy and Harshman, 1974). α-toxin also has direct effects on the kidneys, specifically inducing selective renal vasospasm (Thal, 1955) which was not due to effects on the blood clotting system (Nakai and Margareten, 1963; Rodriguez-Erdman, 1966).

α-toxin was shown to induce permeability changes in pheochromocytoma (PC 12) cells (Ahnert-Hilger et al. 1985a,b). Recently, it has been demonstrated that α-toxin reduces the affinity of the epidermal growth factor receptor in PC 12 cells as well (Lazarovici and Chan, 1987).

131I-labelled α-toxin upon injection into rabbits was found to be
distributed in a number of organs including brain (CNS in general), heart, kidneys
and lungs (Jeljaszewicz et al 1969). Blomqvist et al (1987b) found
essentially the same distribution in mice using $^3$H-labelled $\alpha$-toxin with
the exception that none was found in any CNS compartment. This may be due to
differences in the blood brain barrier in rabbits and mice and may reflect the high
sensitivity of rabbits to the toxin. The major lethal effect of $\alpha$-toxin in most
animals is probably on the circulatory system involving mediators of inflammation
(Seeger et al 1984; Suttrop et al 1985; 1987).

The major reservoir for S. aureus is man (Sheagren, 1984) with 20–40% of
the population being healthy carriers. Hospital personnel have an even higher rate of
colonization. $\alpha$-toxin has been shown to be one, if not the most important
virulence factor in staphylococcal infections (Jansson et al 1985; Kinsman
et al 1981; O'Reilly et al 1986). It has profound effects on many
different tissues and cells but it has become clear that the action of the toxin is
on the cell membranes of its target cells. The system of choice for studies on the
mechanism of action of the toxin has become the rabbit erythrocyte because of their
high sensitivity and the ease with which damage is measured.

**Mechanism of Action**

As stated earlier the effect of $\alpha$-toxin on the erythrocyte has become a
well studied model system for the toxin's mode of action. A turbidometric assay was
first used to measure lysis of erythrocytes by $\alpha$-toxin (Mangalo and Renaud,
1959). It was determined that the time to 50% lysis of an erythrocyte suspension was
dependent on toxin concentration. Studies on the rate of lysis showed that it was
dependent on the concentration of the toxin added (Lominski and Arbuthnott, 1962) and
that lysis could be described by a sigmoidal curve (Cooper et al. 1964; 1966; Madoff et al. 1964; Marucci, 1963a, b). Three distinct stages during the time course of hemolysis were identified in these studies: a pre-lytic lag phase, a phase of rapid lysis and a tailing off or slowing down of lysis. In the pre-lytic lag phase, α-toxin is absorbed rapidly onto the surface of the erythrocyte membrane. This bound toxin reaches a maximum when the lytic rate is maximized followed by a gradual decrease after lysis (Klainer et al. 1964).

After binding of the toxin, the first detectable event is a release of K+ ions. Using 86Rb, a K+ analogue, Cassidy and Harshman (1976a, b) demonstrated that prelytic release of K+ closely parallels binding of 125I-α-toxin at 20°C. However, at 0°C the two events were distinct. Lysis itself is represented as a release of hemoglobin from α-toxin treated erythrocytes (Lominski and Arbuthnott, 1962; Cooper et al. 1964; 1966).

Studies using anti-α-toxin identified two steps in the lytic process (Marucci, 1963b). Again the first step involved the interaction of the toxin with erythrocytes and could be inhibited by specific antisera. The second step proceeded without further participation of the toxin and seemed to be an intrinsic reaction of the damaged erythrocyte. This step could not be affected using antisera raised against the toxin. These findings were reiterated by Lo and Fackrell (1979) but they found that both steps could be blocked by specific antisera.

Studies designed at determining the mechanism of action of α-toxin have focussed in three main areas: 1) an enzymatic mode of action, 2) characterization of the surface active properties of α-toxin both morphologically and biochemically, and 3) the search for specific receptors for the toxin on the cell surface.
Enzymatic activity for α-toxin was first suspected by Forssman (1933; 1934a,b). Levine (1938; 1939) however, demonstrated that the three major effects of the toxin (i.e. hemolysis, dermonecrosis, and lethality) could be adsorbed stoichiometrically with concentrated suspensions of erythrocytes implying that α-toxin was not acting as an enzyme. Later studies designed to separate the proteolytic and hemolytic activities of α-toxin failed suggesting that the toxin acts as a protease even though no strict kinetics were determined (Robinson et al. 1960). Lominski and Arbuthnott (1962) recovered all the hemolytic activity in the supernatant of α-toxin lysed erythrocytes and a plot of hemolysis versus toxin concentration was similar to that of an enzyme reaction. Marucci (1963a,b) suggested that the plot may have simply reflected a situation of toxin in excess but also recovered hemolytic activity in supernatants of toxin-treated cells. Bernheimer (1970) compared both enzymatic and non-enzymatic lytic agents by plotting % lysis versus the log of erythrocyte concentration. α-toxin conformed to the enzymatic curve. However, it was difficult to draw a conclusion from this study because β-hemolysin (known to be an enzyme) did not show a similar relationship.

α-toxin treated erythrocytes showed a release of soluble nitrogen indicating that proteolysis was occurring (Wiseman and Caird, 1970; 1972; Wiseman et al. 1975). Further, release of soluble nitrogen was correlated with the hemolytic sensitivity of erythrocytes from various species. It was also demonstrated that immobilized trypsin could activate α-toxin to hydrolyze tosyl arginine methylester (TAME), a property which the native toxin did not exhibit. The activated toxin actually had a higher affinity for TAME than did trypsin itself. Nine percent of the total protein content of the erythrocyte membrane protein was hydrolysed. It was speculated that the native "pro-toxin" is activated to become a protease by
endogenous proteases in the target cell membrane (Wiseman et al. 1975; Wiseman, 1975). The endogenous proteolytic activity of target cell species was correlated with sensitivity to \(\alpha\)-toxin (Morrison and Neurath, 1953; Moore et al. 1970).

The hemolytic activity of \(\alpha\)-toxin can be enhanced by preincubation with live fibroblasts in culture (Szligelski and Harshman, 1978) which tends to support this hypothesis.

Several other investigators have been unable to confirm the proteolytic activity of \(\alpha\)-toxin. The protease inhibitor PMSF did not inhibit the hemolytic activity of the toxin, nor was any major membrane protein in erythrocyte ghosts affected by exposure to \(\alpha\)-toxin (Freer et al. 1973). They further found that the freeze-etch pattern of toxin-treated ghosts was not similar to that obtained by known proteases. Wiseman (1975) speculated that the ghosts prepared by Freer et al. (1973) had lost their endogenous proteolytic activity. In addition, some groups have shown that \(\alpha\)-toxin is consumed in the lytic process (Madoff et al. 1964; Cassidy and Harshman, 1975a,b).

In contrast to an enzymatic mechanism of action for \(\alpha\)-toxin considerable evidence exists demonstrating the surface active properties of the toxin on biological membranes. It has been shown by a number of researchers that \(\alpha\)-toxin disrupted liposomes of varying composition (Weissman et al. 1966; Freer et al. 1973; Cassidy and Harshman, 1974). Disruption by the toxin resulted in the release of marker molecules from the liposomes. This interaction as well as the interaction with erythrocyte ghosts resulted in a loss of hemolytic activity. Liposomes prepared from human erythrocytes (which are one of the least sensitive species) were found to be as sensitive to the toxin as those prepared from rabbit erythrocytes. A lipid component alone cannot account for the difference in
sensitivity between human and rabbit erythrocytes as rabbit erythrocytes are known to be 100 times more sensitive. In these studies 15-30µg/ml α-toxin was used which is much greater than the amount required to lyse rabbit erythrocytes (0.1-0.2µg/ml) (Cassidy and Harshman, 1974).

A hydrophobic interaction between α-toxin and lipids in general was confirmed by studies using mixed lipid monolayers (Buckelew and Colacicco, 1971). In the absence of lipid, α-toxin spreads as a film at the air-water interface. When lipid was present however, the toxin penetrated the lipid monolayers to different degrees. It was also demonstrated that the native 3S toxin polymerizes to the 12S form on surfaces of intact cells and liposomes (Freer et al. 1968).

These 12S oligomers were morphologically indistinguishable from 12S forms found naturally in toxin preparations or resulting from heat denaturation of the toxin. This ring formation was also noted on membranes from rabbit, human, horse, and guinea pig erythrocytes, platelets, rat hepatocyte plasma membranes and lysosomal membranes but not on bacterial spheroplasts (Bernheimer, 1974). The 12S structure was seen to penetrate the lipid region of platelet membranes (Bernheimer et al. 1972).

Significant changes in the hydrophobic fracture plane of rabbit erythrocyte membranes occurred upon exposure to α-toxin as well (Freer et al. 1973). Using Triton X-100 the ringlike structures were isolated from toxin treated human and rabbit erythrocyte membranes (Fussle et al. 1981). These structures appeared as hollow cylinders with an outer diameter of 8-10 nm and an inner diameter of 3-5 nm in the electron microscope. Denaturing SDS-PAGE performed on the 12S oligomers (mw 200 kD) yielded native toxin monomers implying that the 12S form was composed of six monomeric subunits. In the presence of sodium deoxycholate α-toxin monomers oligomerized into active hexamers (Fussle et al. 1981; Bhakdi et al. 1981). These hexamers when extracted would bind lipid and could be incorporated into lipid
vesicles. Tobkes et al (1985) confirmed using cross linking studies that the ultimate form of polymerization of α-toxin resulted in the hexamer.

Using prelabelled resealed erythrocyte ghosts treated with α-toxin hexamers in marker flux studies, Fussle et al (1981) demonstrated that the size of the lesions generated coincided with the ultrastructural data from their study and that of Freer et al (1968) and speculated that the hexameric toxin formed a transmembrane pore. It was suggested that a "repair protein" present in human erythrocytes prevented damage by α-toxin and was absent in rabbit erythrocytes (Fussle et al 1981).

α-toxin has been shown to enhance transbilayer reorientation of phospholipids as well as disrupt the asymmetry of phosphatidylethanolamine and phosphatidylcholine in erythrocyte membranes (Schneider et al 1986). Sheep erythrocytes upon treatment with β-hemolysin (a phospholipase C) become resistant to hemolysis by α-toxin (Elias and Kofer, 1980; Christie and Graydon, 1941). The reason for this antagonism is unknown. Watanabe et al (1987) demonstrated that choline containing phospholipids and cholesterol were important for the interaction of α-toxin with liposomes. A number of other steroids including digitonin and progesterone are known to inhibit the toxin, presumably by binding to surface active, hydrophobic regions of the toxin directly (Schaeeg et al 1985; Yotis and Sakov, 1970). Interestingly, plasma low density lipoprotein was also found to partially inhibit α-toxin and trigger oligomerization (Bhakdi et al 1983). Phimister and Freer (1984) demonstrated that binding of fully hemolytic 125I-α-toxin to erythrocytes was independent of temperature between 0-45°C, pH, cell concentration and toxin concentration implying that the interaction did not require specific receptors and was dependent on membrane lipids alone. They noted
earlier (Ferre et al. 1958) that α-toxin penetrated mixed lipid films of 17 dynes/cm pressure but not 34 dynes/cm. Since the surface pressure in the intact lipid bilayer of human erythrocytes is between 31–34 dynes/cm, they would be less sensitive to the toxin. They speculate that peculiarities of the lipid membrane itself and its stability are responsible for differing sensitivities in different erythrocyte species. Unfortunately, erythrocytes of other species have not been evaluated in terms of their surface pressure. These data imply that a lipid component of the target membrane may be involved in the mechanism of action of the toxin.

There is now evidence that a structural change occurs in the toxin as the monomer is converted to the hexamer. A conformational change occurs with the transformation of the monomer to the hexameric form (Ikigae and Nakae, 1985). The secondary structure of the toxin has been described (Töbkes et al. 1985; Ikigae and Nakae, 1985). Using circular dichroism spectra it was shown that two-thirds of the molecule in both the monomer and the hexamer is either β-sheet or β-turn. In contrast there is little α-helix and some random coil or unassigned structure.

The conformational change must therefore be in the tertiary structure of the toxin molecule. The intramembranous section of the hexamer was suggested to be made up of domains of anti-parallel β-sheet that surround a central pore. A simple working model for the assembly of α-toxin into lipid membranes has been presented (Töbkes et al. 1985). The monomer in its native state consists of two large domains separated by a flexible, centrally located hinge. Once the toxin has contacted the membrane it turns partially inside out about the hinge before it forms the hexamer. Hydrophilic residues initially on the surface of the monomer participate in domain–domain or subunit–subunit interactions and hydrophobic regions occluded in the monomer interact with the membrane of the target cell. Support for this model has
come from a number of researchers. Blomqvist and Thelestam (1986b) have demonstrated that a similar conformational change occurs in both erythrocytes and Y1 adrenocortical cells in culture. The sequence of the α-toxin gene (Gray and Kehoe, 1984) indicates a region centrally located in the toxin (residues 119–143) which is 32% glycine. This may be the postulated hinge (Tobkes et al. 1985).

Ikigae and Nakae (1987a,b) have shown that the toxin-membrane interaction occurs first, followed then by a conformational change resulting in assembly of the hexamer into liposomes.

The transmembrane pore theoretically generated by α-toxin (Bhakdi and Tranum-Jensen, 1986; Bhakdi et al. 1981; Fussle et al. 1981) has also received a great deal of attention. Ultrastructural and marker-flux studies suggest the pore size is roughly 2–3 nm in diameter (Bhakdi and Tranum-Jensen, 1986 for review; Suttrop et al. 1985; 1987). However, single channel conductance studies on voltage-clamped planar lipid membranes treated with toxin have demonstrated an effective pore size of approximately 11 nm (Menestrina, 1986).

Further complications arise as a result of a study by Bashford et al. (1986). It was demonstrated that intoxication with α-toxin had many features in common with a diverse array of membrane damaging agents including melittin, Triton X-100, polylysine and the complement membrane attack complex. All exhibited positive cooperativity in their membrane damaging effects on Lette cells in culture and many acted synergistically. It was speculated that none of these agents generated protein lined channels but rather caused the formation of leaky patches in the membrane itself.

Divalent cations (especially Zn$^{2+}$ and Ca$^{2+}$) could prevent the induction of these leaky patches or effectively close them once generated. Other studies (Menestrina, 1986; Blomqvist and Thelestam, 1986b; Harshman and Sugg, 1985)
have also shown that divalent cations can inhibit the hemolytic and membrane damaging effects of α-toxin. Whether divalent cations actively close toxin generated pores or patches or simply stabilize the membrane is at present unclear. In contrast, at sublytic doses of α-toxin Ca$^{2+}$ has no inhibitory effect and is indeed required for toxin-mediated events (Suttrop et al 1985; 1987).

Blomqvist and Thelestand (personal communication) have demonstrated that $^3$H-toxin bound Y1 cells and subsequently oligomerized. They, like Phimister and Freer (1984), demonstrated binding kinetics indicative of a non-specific interaction with the membrane which was independent of pH. A fragment which did not damage membranes was also noted to form oligomers on the surface of Y1 cells. These oligomers had the same apparent molecular weight as ones formed by the native toxin. Thus, either the fragment oligomers were defective or the relevance of hexamers as the sole component of toxin induced membrane damage should be questioned.

It is becoming clear that the action of α-toxin cannot solely be explained on the basis of its surface active properties and certainly the wide range of sensitivities exhibited by various cells cannot be explained simply on the basis of their lipid content.

Harshman (1979) using low levels of α-toxin (0.1–0.2 μg/ml) did not observe hexamers on rabbit erythrocytes even though lysis was evident. Cassidy and Harshman (1976a,b) and Barei and Fackrell (1979) observed ringlike structures on rabbit erythrocyte membranes but only at high concentrations of α-toxin. These structures were probably unrelated to the binding of the toxin to high affinity receptors at low but still lytic concentrations. Earlier EM studies performed by Freer et al. (1973) involved the use of erythrocyte ghosts rather than intact erythrocytes. About 5% of added toxin binds to intact erythrocytes whereas 60–70% of added α-toxin binds to osmotically prepared ghosts (Arbuthnott et al
1973; Cassidy and Harshman, 1974). It is therefore possible that α-toxin binds non-specifically to ghosts leading to ring formation. Since Cassidy and Harshman (1974) failed to find a species specificity for lipids that exists for erythrocytes and the surface active theory of α-toxin action cannot explain the varying sensitivities of different cells (Bernheimer, 1965; Cooper et al. 1965) the existence of specific receptors was postulated.

Cassidy and Harshman (1976a,b) found that $^{125}$I-α-toxin bound irreversibly to rabbit erythrocytes. Binding was greater for rabbit than human erythrocytes and was correlated with the extent of lysis in both cells. Binding and lysis were again found to be separate events. Scatchard analysis of the binding data indicated that there were approximately 5000 high affinity binding sites on the rabbit erythrocytes and none on human erythrocytes. Treatment of the rabbit erythrocyte with pronase reduced hemolytic sensitivity by 97%.

Barei and Fackrell (1979), also using Scatchard analysis determined the number of receptors to be 125,000/ rabbit erythrocyte. The discrepancy between the two numbers may be due to the fact that the $^{125}$I-α-toxin used by Cassidy and Harshman (1976a,b) retained 10% of its hemolytic activity while Barei and Fackrell (1979) used heat inactivated toxoid which was labelled with fluorescein. The residual activity in the initial study may have caused a release of bound toxin as described by Klainer et al. (1964). The release of this bound toxin would have prevented establishment of any true equilibrium. Barei and Fackrell (1979) established a correlation between receptor number and hemolytic sensitivity for various erythrocyte species. They also noted that hemolytic sensitivity was independent of receptor number when there are less than 37,000 receptors/cell.

Cassidy and Harshman (1979) isolated high molecular weight complexes of
$^{125}$I-$\alpha$-toxin and an erythrocyte membrane protein providing further evidence that a specific receptor existed. These complexes were resistant to SDS (at room temperature) urea or proteolysis however could be broken down by SDS at 100°C to yield a labelled monomer. It was postulated that high affinity receptors increase local concentrations of the toxin on erythrocyte membranes. This would explain why such low concentrations of $\alpha$-toxin are required to lyse rabbit erythrocytes relative to other cells.

Bhakdi et al. (1984) also demonstrated a correlation between the binding of $\alpha$-toxin and its hemolytic activity. A pH dependence for $\alpha$-toxin intoxication was seen in human erythrocytes but not in rabbit erythrocytes. The results obtained by this group indicated that the interaction of $\alpha$-toxin and membranes did not exhibit overall characteristics of a simple receptor-ligand interaction.

Using antigenic determinants as reference points immunologic evidence was presented by Lo and Fackrell (1979) which suggested that the toxin is oriented in a specific manner on the rabbit erythrocyte membrane. Again this is consistent with the concept of a specific receptor. They further found that polyclonal anti-$\alpha$-toxin could be separated into two distinct populations. One population prevented the toxin from binding to erythrocyte membranes, hence antibinding antibodies (AB-Ab). A second population neutralized membrane bound toxin and could be measured by an indirect hemagglutination, hence IHA-Ab. This again reiterated the findings of Cassidy and Harshman (1976a,b) and Madoff et al. (1964) separating hemolysis into two distinct events.
It has been suggested that the receptor is an N-acetyl-glucosaminyl ganglioside since nanogram amounts of this compound inhibited lysis by \( \alpha \)-toxin (Kato and Nakai, 1976). This, however, has not been substantiated by other groups (Bernheimer, personal communication; Fackrell, unpublished observation). Inhibition studies by various groups have shown that flavin mononucleotides (and related flavines) as well as the integral membrane protein, glycophorin inhibited toxin-mediated lysis as well (Kato et al. 1975; Raff et al. 1977; Fackrell, unpublished observation; Bernheimer and Avigad, 1980). It now seems likely that the inhibitory effect of these compounds is probably due to non-specific neutralization of the toxin's surface active properties, charge interactions or hydrophobic interactions (Bernheimer, personal communication; Thelestim, 1983a).

Maharaj and Fackrell (1980) suggested that Band 3, another integral membrane protein (Steck et al. 1971) was a receptor for \( \alpha \)-toxin based on several pieces of evidence. As with other groups (Cassidy and Harshman, 1976a,b; Kato et al. 1975) the receptor was found to be sensitive to pronase. Chymotrypsin also destroyed the putative receptor and it is known that chymotrypsin specifically cleaves Band 3 in the erythrocyte membrane (Steck et al. 1976; Marchesi et al. 1976).

Erythrocytes preincubated with concanavalin A (Con A) are protected from lysis by the toxin. Since Con A binds to Band 3 alone in erythrocyte membranes (Marchesi et al. 1976; Findlay, 1974), Band 3 was implicated as a specific receptor. Purified Band 3 when preincubated with \( \alpha \)-toxin inhibited hemolytic activity. Simpson and Fackrell (in prep) have further shown that the receptor is located in the 35 kD C-terminal chymotryptic fragment of Band 3. Complete destruction of the isolated polypeptide portion of the fragment with hydrazine or pronase has no effect on its ability to inhibit the toxin. However, neuraminidase digestion or sialic acid
modification of the isolated receptor reduces its inhibitory capability substantially. This suggests that the carbohydrate moiety of rabbit erythrocyte Band 3 is a receptor for $\alpha$-toxin. Since Band 3 occurs in human as well as rabbit erythrocytes the data presented by Maharaj and Fackrell (1980) and Simpson and Fackrell (in prep) would only be valid if the receptor in human erythrocytes is somehow masked, or altered. In fact trypsin treated human erythrocytes show a ten-fold increase in hemolytic sensitivity (Fackrell et al 1985). Trypsin is known to specifically cleave glycophorin from the human erythrocyte membrane (Marchesi et al 1976) and may unmask receptors on human erythrocytes. Band 3 isolated from human erythrocytes inhibits $\alpha$-toxin as well as isolated rabbit erythrocyte Band 3 (Simpson and Fackrell, in prep). Indeed, an immunoreactive analogue of Band 3 isolated from mouse kidney also inhibits the hemolytic activity of the toxin (Tang et al 1987).

It seems that Band 3 may play a direct role in lysis at least in erythrocytes. Modification of sulphhydril groups in Band 3 with a number of specific reagents markedly increases the rate of $\alpha$-toxin-induced hemolysis as well as reducing the lag time (Fackrell and Austin, 1985).

A number of researchers have initiated structure-function studies on the toxin itself in order to characterize regions of the toxin responsible for its various effects. Watanabe and Kato (1978) found that trypsin treatment of $\alpha$-toxin generated a 17 kD lethal fragment with no hemolytic activity and a 20 kD fragment which was weakly hemolytic and tended to form aggregates. Blomqvist and Thelestan (1984; personal communication; Blomqvist et al 1987a) similarly noted an 18.5 kD fragment generated spontaneously during purification was hemolytic and formed oligomeric complexes similar to that of the native toxin. This fragment was subsequently localized by partial protein sequencing to be the C-terminal domain of
the toxin (Blomqvist et al, 1987a). Through the use of monoclonal antibodies directed against the 18.5 kD \( \alpha \)-toxin fragment (Sjogren and Blomqvist, in press), the MAbs generated affected the hemolytic, dermonecrotic and lethal activities of the toxin to varying degrees suggesting that distinct regions of \( \alpha \)-toxin are responsible for the different effects. Amend et al (1985) achieved similar results with MAbs directed against the hexameric [12S] form of the toxin. A MAb directed against the C-terminal region of \( \alpha \)-toxin had no effect on either its hemolytic or lethal effects (Harshman et al 1986).

Further examination of two distinct polyclonal anti-\( \alpha \)-toxin populations (Lo and Packrell, 1979) indicated that neutralization of \( \alpha \)-toxin depended on the concentration of antibodies (Lo, 1984 Ph.D. dissertation). There was a linear dependence for IHA-Ab and a complex, non-additive relationship for AB-Ab. The inference from this study was that in the native toxin only a single IHA determinant is exposed and required for neutralization. In contrast, AB-Ab recognized at least two determinants on the native toxin. In addition, the protective effect of IHA-Ab is reduced if added after the prelytic lag phase, implying that at least two separate lytic events occur after binding of the toxin.

At a more specific level, Cassidy and Harshman (1976a,b) demonstrated that a single tyrosine residue was important for the lytic phase but not involved in toxin binding. The tyrosine residue (Tyr 29) has been localized to the N-terminal CNBr fragment of \( \alpha \)-toxin (Harshman, personal communication). A second tyrosine residue was implicated as being important in the binding of the toxin but this residue was not characterized (Cassidy and Harshman, 1976a,b).

Much interest has been generated in the lethal activity of \( \alpha \)-toxin. Early studies noted that the lethal effect of the toxin was two-fold. That is, death could
be rapid, almost immediate or slow and prolonged depending on the dose of toxin used
(for review see Elek, 1959; Berheimer, 1974). Much attention was focussed on the CNS
as the site of action for rapid death and many studies have shown that α-toxin
has marked effects on CNS functions (Edelwejn et al. 1976; Lipinan and Harshman,
1985; Harshman et al. 1986; Szmicelski and Harshman, 1978). However, this effect
has come into question recently (Blomqvist et al. 1987b). This group found using
whole body autoradiography in mice that α-toxin did not distribute to any CNS
compartment, in contrast with a study performed earlier with rabbits (Jeljaszewicz
et al. 1969). It seems that an effect on the CNS by α-toxin may be
dependent on the status of the blood brain barrier or the route of entry of the
infecting staphylococci (Blomqvist et al. 1987b).

Recent work has shown that at sublytic concentrations α-toxin can elicit a
Ca$^{2+}$ dependent release of many mediators of inflammation such as prostacyclins
and leukotrienes (Suttorp et al. 1985; 1987). Through this mechanism the toxin
can cause marked vasoconstriction, hypertension and pulmonary edema which can result
in rapid death in a fashion similar to certain types of hypersensitivity (Svihovec
and Raskova, 1967; Seeger et al. 1984). The immune status of the host may also
affect the outcome of α-toxin intoxication. Lo et al. (1980) demonstrated
that anti-α-toxin antibodies can fix complement. Complement fixation can itself
result in a number of adverse reactions for the affected animal including
hypersensitivity.

Slow death may be due to an accumulation of α-toxin in other organ systems
in which more time is needed for a lethal effect (Jeljaszewicz et al. 1969;
Blomqvist et al. 1987b).

It is also becoming clear that α-toxin is but one of many membrane
damaging toxins that seem to elicit similar effects. Membrane damage by streptolysin O and the membrane attack complex of complement is basically analogous to that induced by $\alpha$-toxin (Bhakdi et al. 1985; Bhakdi and Tranum-Jensen, 1986). In addition, the mechanism of cytotoxic T-cell action on susceptible cells also seems to involve formation of a proteinaceous pore (Simone and Henkart, 1980; Blumenthal et al. 1984). Also, aerolysin (a membrane-damaging toxin produced by Aeromonas hydrophila), Pseudomonas aeruginosa cytotoxin and the E. coli hemolysin all generate functional pores similar to that of $\alpha$-toxin (for review see Bhakdi and Tranum-Jensen, 1986). The cytotoxin from P. aeruginosa also possesses the ability to induce prostacyclin synthesis in cultured endothelial cells in a manner identical to $\alpha$-toxin (Suttrop et al. 1985b).

Although much research has been performed on $\alpha$-toxin and its mode of action, there are still many questions to be answered. It appears that at least two modes of action of the toxin are possible. Austin (1984 M.Sc. thesis) demonstrated two types of kinetics for lysis of rabbit erythrocytes. One involved a single "hit" by an $\alpha$-toxin molecule and the other required a number of $\alpha$-toxin molecules to bind the cell membrane before lysis occurred. It seems that this may reflect in some small way the fact that $\alpha$-toxin acts in different ways on different cells. This observation may help clarify the seemingly conflicting observations about its mode of action.
Materials and Methods
Reagents and Buffers.

CHD, PGO and DPC were purchased from Aldrich Chemicals, Milwaukee, WI. Guanidine HCl, Boric acid gel, Girard T reagent, trypsin (EC 3.4.21.4), soybean trypsin inhibitor (STI) type I–S, were all purchased from Sigma (St. Louis, Missouri). CM–Biogel A, goat anti-rabbit IgG Horseradish peroxidase conjugate (HRP–IgG) and HRP colour development reagent, as well as goat anti–mouse IgG–Alkaline phosphatase and its colour development reagent were purchased from Bio–Rad (Richmond, California). HPLC grade acetonitrile and trifluoroacetic acid was purchased from Fisher Canada. HPLC grade 1–propanol, sequenal grade pyridine, triethylamine, phenylisothiocyanate (FITC), 6N slow boiling HCl, and amino acid standard mixture were all purchased from Pierce Chemical, Rockford, IL. CNBr was from Eastman Kodak, Rochester, NY. All tissue culture reagents were purchased from Gibco, Burlington, Ontario. All other chemicals including individual amino acid standards used were reagent grade. Phosphate–buffered saline (PBS) consisted of 145 mM NaCl and 10 mM sodium phosphate buffer (pH 7.4). Tris–buffered saline (TBS) consisted of 150 mM NaCl and 10 mM Tris base (pH 7.4).

Production and Purification of α-toxin.

*Staphylococcus aureus* Wood 6 strain was inoculated into Dolman–Wilson medium (Dolman and Wilson, 1940) and incubated at 37 °C with shaking under 10% carbon dioxide and 90% air for 36 h. Supernatants of the culture were obtained by centrifugation at 8000 x g for 15 min and were the source of crude α-toxin. Crude toxin was purified according to the method of Wiseman et al (13). Purified toxin was hemolytic and its activity could be neutralized with antisera derived from New Zealand white rabbits immunized with heat—inactivated toxoid. α-toxin was
inactivated by incubation at 60°C, pH 4.0 for 5 min (Dalen, 1976).

Hemolytic Assays.

Blood was obtained from New Zealand white rabbits and the sera and buffy coat were removed by centrifugation. The erythrocytes were washed three times in PBS. Hemolysis was measured by the reduction in turbidity procedure (650 nm) using a 2% suspension of rabbit erythrocytes in PBS (Wiseman and Caird, 1972). Hemolysis was followed and analyzed by coupling a spectrophotometer (Perkin Elmer Double Beam 124) through an analog-to-digital converter to a microcomputer (Packrell and Glasgow, 1984). A BASIC program called KINETICS calculated various parameters such as lag time, time to 50% lysis as well as the rate throughout the course of hemolysis.

Cell Culture.

VERO cells (from African green monkey kidney) were maintained and passaged in Dulbecco’s minimal essential medium (DMEM) with 10% fetal calf serum. Cells were subcultured by treatment for 10 minutes with 0.25% trypsin to remove them from the surface of the flasks.

Treatment of VERO cells with native and modified toxin.

VERO cells were removed from tissue culture flasks either by trypsinization or gentle scraping using a rubber policeman and suspended in PBS. 24–48 hr cultures were used in all experiments. 100 μg of native or CHD modified toxin was incubated with the cells for 30 minutes with shaking at RT. Cell damage was estimated using 1% trypan blue dye exclusion. Viable cells do not take up the stain and were counted with a hemocytometer.
Atrial Perfusion and Treatment with α-toxin.

Hearts were removed under ether anesthesia and perfused by the Langendorff technique at RT for 5 min to wash out residual blood. To arrest contractions the K⁺ concentration in the buffer was raised to 15mM. The initial perfusate was a Krebs-Henseleit buffer containing the following salts in mM concentrations: NaCl, 118; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25; and glucose, 11. The perfusate was oxygenated with 95% O₂ / 5% CO₂ and maintained at RT during the initial washout procedure or at 30°C during the experiments. Left atria were carefully dissected from the hearts and placed in a horizontal muscle chamber. The tissue was secured at one end with a miniature plastic clamp that was mounted directly to the base of the muscle bath. A small stainless steel wire hook passed through the free end of the tissue was attached directly to the arm of a force transducer (Grass FT03). The atria were paced at 1.0 Hz and were allowed to equilibrate for 60 min. During the stabilization period a preload of 1.0 g was applied to the muscle. All data were analyzed from digital records obtained from a microcomputer. Force signals were initially amplified with a Grass preamplifier and the signals converted to digital form with a 12 bit A/D converter. By using the programmable gain during A/D conversion, the signal was further amplified by a factor of six. The data were recorded at a speed of one A/D conversion every 250 µs. A single contractile function curve had at least 250–300 data points. The following mechanical parameters were measured from the data: peak isometric developed tension, maximum rate of tension development, time to peak tension, maximum rate of relaxation, and time to complete relaxation.

Perfused atria were incubated with either native toxin or modified toxin at concentrations of 6–12 HU/ml for 60 min or until activity dropped below measurable limits. α-toxin was modified with PGO as described in Chapter One.
Modification of $\alpha$-toxin with arginine specific reagents.

The procedure for CHD modification was basically that of Pithy and Smith (1975a,b). $\alpha$-toxin was incubated with varying concentrations of CHD in 0.2 M borate buffer, pH 7.6 at 37 °C for times specified. Unbound CHD was removed by dialysis versus borate buffer for 48 h at 4 °C.

Phenyglyoxal modifications were carried out as follows. Varying concentrations of PGO were incubated at 37 °C in the dark in 0.2 M bicarbonate buffer. Unbound PGO was removed by dialysis versus bicarbonate buffer (48 hr, 4 °C).

Modification of $\alpha$-toxin with $\rho$-hydroxyphenylglyoxal was performed according to the method of Yamasaki et al. (1980). Excess reagent was removed by dialysis and the product was quantified by measuring absorbance changes at 340 nm.

Modification of $\alpha$-Toxin with Diethylpyrocarbonate (DPC).

The procedure for DPC modification was essentially that of Miles (1977). $\alpha$-toxin was incubated with varying concentrations of DPC in sodium phosphate buffer, pH 6.5 at RT in the dark for times specified. Excess reagent was removed by dialysis.

Measurement of Conformational Change due to Modification.

The fluorescent tryptophan spectra (emission wavelength 280 nm) of native, and modified $\alpha$-toxin, and toxoid were measured in a Turner Model 430 spectrofluorometer and compared as an indicator of gross conformational change (Chu and Bergdoll, 1969).

Regeneration of Amino Acid Residues from Modified Toxin.
Regeneration of arginine or histidine residues was performed by the procedure of Smith (1977). Modified toxin was incubated with 0.5M hydroxylamine, pH 7.0 at 37°C for 6 h under nitrogen. 1mM EDTA (Ethylenediamine-tetraacetate) was added to prevent copper catalyzed non-specific effects. CHD containing peptides could be precipitated with NiCl₂ as described by Patthy and Smith (1975a,b).

Quantitation of Number of Modified Arginine Residues.

The Girard T reagent (trimethylaminoacetohydrazide chloride) which reacts with the CHD-arginine complex to yield an ultraviolet absorbing product (300 nm) as described by Patthy et al. (1979) was used to determine the number of arginines modified.

Quantitation of Modified Histidine Residues.

Quantitation of the number of DPC modified histidine residues was performed as described by Miles (1977). The reaction between DPC and histidine results in a product which absorbs at 330 nm. Absorbance was also measured at 280 nm to determine if tyrosine residues were modified. Reaction of DPC with tyrosine results in a decrease in absorbance at 280 nm.

Trypsin Hydrolysis of α-toxin.

CHD was initially used to protect arginine during trypsin digests so that isolation of arginine containing fragments was possible (Toi et al., 1967). Samples of both α-toxin and modified α-toxin were digested with trypsin. Denatured samples of native or modified α-toxin were lyophilized, reconstituted, in distilled water, and boiled for three minutes. The protein was then treated with trypsin (5% w/w) in 0.1 M NH₄CO₃ with 1 mM CaCl₂ pH
8.5 for 2 hours at 37 C. The reaction was stopped by adding soybean trypsin inhibitor (1:1 w/w ratio with trypsin).

Peptide Mapping.

Peptide mapping was performed essentially by the method of Cassidy and Harshman (1976a). Trypsin (1% w/w) was added to heat denatured α-toxin (2 mg) in 0.05 M ammonium bicarbonate buffer, pH 8.0. Peptides were then lyophilized. Peptide maps were performed using number 3MM Whatman filter paper (46 X 57cm). Chromatography in direction one for 20 hr in 1-butanol-acetic acid-pyridine-H$_2$O (90:18:72:60) was followed by electrophoresis at 40 V/cm for 3 hr, pH 3.5 in a pyridine/acetic acid buffer. Papers were dried and stained either with a ninhydrin spray or a NiCl$_2$ spray as described by Patthy and Smith (1975b).

CNBr Digestion of α-Toxin.

α-toxin was digested with CNBr according to the method of Gross and Witkop (1961). α-toxin was dialysed extensively against distilled water, denatured in boiling water for three minutes and lyophilized. Samples (>1mg/ml) were reconstituted in 70% formic acid in glass stoppered vials. A 50-100 fold molar excess of CNBr over methionine residues was added and the solution was sealed under N$_2$ and incubated in the dark at RT for 48 hr. Samples were diluted 20 fold in distilled water and lyophilized twice to remove excess reagent. The final product was reconstituted in distilled water.

Affinity Chromatography of CHD Modified Toxin Digests.

The CHD modified fragments were isolated using a boric acid gel system as
described by Patthy et al. (1979). Briefly, a boric acid gel column (20 X 1cm)
was equilibrated in 0.2 M acetate buffer pH 5.8. Samples were added to the column and
washed with five column volumes of both acetate buffer pH 5.8 and the same buffer
with 6 M guanidine-HCl to suppress non-specific binding. Samples were eluted with an
0.1 M acetic acid/0.1 M formic acid buffer pH 1.9.

Electrophoresis.

Samples were desalted on a P-10 (Bio-Rad) gel filtration column (30mm X
50mm) and separated in 12% SDS polyacrylamide slab gels according to the method of
Laemmli (1970). After electrophoresis samples were transblotted to nitrocellulose and
an ELISA was performed on the transblots as described by Surujballi and Packrell
(1985) which is a modification of Towbin et al. (1979). Briefly, 10 μg samples
were electrophoresed and the gels were equilibrated in transfer buffer (25 mM Tris,
192 mM glycine, 20% v/v methanol). Gels were transblotted overnight at 225 mA. After
treating the nitrocellulose with 0.2% v/v glutaraldehyde (Van Eldik and Wolchok,
1984) and blocking with 5% w/v bovine serum albumen the sheets were incubated with
primary antibody overnight at 23 C. Goat Anti-rabbit IgG conjugated to horseradish
peroxidase was added and the membranes were again incubated overnight at 23 C. The
substrate used was 4-chloro-1-napthol. The sheets were washed between incubations
with TBS with and without 0.05% Tween-20 to suppress non-specific binding.

ELISAs

ELISAs were performed as described by Surujballi and Packrell (1984) with
some modifications (Surujballi and Packrell, unpublished). α-toxin or PGO
modified toxin (1:50 dilution of 1 mg/ml stock solution) were used to coat individual
wells in 96 well microtitre plates (Gibco, Toronto, Ontario) instead of antibody.
After blocking with a 1% BSA solution in TBS pH 7.4 the wells were incubated with primary antibody at RT overnight. Goat anti-rabbit IgG-HRP conjugate was added to the wells after washing and again incubated overnight. The substrate used was 2,2'-azinodi-(3-ethyl benzthiazolone sulphonate(5)) (Litton Bionetics, Kensington, MD).

When mouse monoclonal antibodies were used as the primary antibody, the second antibody was goat anti-mouse IgG-Alkaline phosphatase and the substrate used was p-nitrophenyl phosphate.

Separation of Anti-α-toxin Antibody Populations.
Antibinding antibodies and indirect hemagglutinating antibodies were separated from whole rabbit antisera using the method of Lo and Packrell (1979).

Monoclonal Antibodies.
Monoclonal antibodies (MAbs) were produced as described by Surujballi (1987-Ph.D. dissertation).

Preparation of Immunoabsorbent using MAb-3.
Coupling of MAb-3 to CM-BioGel A was performed as described (Affinity Chromatography: Principles and Practice, Pharmacia Fine Chemicals). MAb-3 (10 mg/ml) was dissolved in distilled water, pH 4.5 and mixed with CM-BioGel A in a 2:1 ratio. EDC (carbodiimide, Bio-Rad, Richmond, CA) was added dropwise to a final concentration of 20mg/ml of gel and stirred constantly overnight. Excess reactive groups were blocked with 0.1M Tris-HCl, pH 7.5 at 4 C. The gel was suspended in PBS after centrifugation. 700 μl of MAb-3 gel was combined with 100 μl of a CNBr digest of α-toxin and incubated overnight at 4 C. After removal of unbound material by centrifugation and washing, bound peptides were eluted with 0.1M glycine-
HCl buffer, pH 2.5. The pH of the eluted fragments was raised to 8.5 with solid Tris. The eluted fragments were dialyzed extensively against distilled water and lyophilized. Samples were then applied to the HPLC for isolation and characterization.

Isolation of Peptides Using HPLC.

A Gilson HPLC was used to separate CNBr fragments of α-toxin. The immobile phase was a reverse-phase Alltech 5μ C18 column. The mobile phase consisted of a linear gradient of 12% 1-propanol (+0.1% TFA) to 60% 1-propanol (+0.1% TFA) over a 60 minute period. Flow rate was 1ml/min. Peptides were detected at 214 nm and 2 AUFS. Separations were performed at RT and fractions of interest were collected in Eppendorf centrifuge tubes.

Amino Acid Analysis.

Percent composition of selected fragments was determined using the method of Henrickson and Meredith (1984). Peptide samples were hydrolysed in vacuo for 24 h at 110° C in 6N HCl. The acid was removed by evaporation twice before subsequent derivitization with PITC.

Amino acid standards both singly and as a mixture were derivatized with PITC as follows: A 10 μl sample of the standard mixture (2.5μmol/ml/amino acid) was dried by evaporation (since the sample was in 0.1N HCl) and reconstituted in coupling buffer (acetonitrile: pyridine: triethylamine: water, 10:5:2:3). 5μl of PITC (from 1ml vacuum sealed ampules) was reacted with the amino acids for 60 min at RT in the dark. Samples were evacuated to dryness and reconstituted in distilled water or 50% acetonitrile (+0.1% TFA). A similar procedure was followed for single amino acid standards as well.

Separation of PITC linked amino acids was achieved using a Gilson HPLC reverse
phase system as follows. The immobile phase was an Alltech 5μ C_{18} column.

The mobile phase consisted of a gradient system using 0.1M ammonium acetate, pH 6.8 as solvent A and 0.1M ammonium acetate, pH 6.8 in 50% (v/v) acetonitrile:water as solvent B. The gradient program was as shown in Table M-1. The detector was set at 254 nm and 0.2 AUFS. Flow rate was 1ml/min. Separations were performed at 52°C using a circulating water jacket. Standards were run before and after each unknown sample as absolute position of amino acids varied but relative position did not. At least five determinations were performed on each fragment.

**N-Terminal Amino Acid Determination.**

Manual Edman degradations of isolated fragments were performed as described (Edman, 1970). Peptides were reacted with PITC as for amino acid analysis but without prior acid hydrolysis. Conversion of the phenylthiocarbamyl derivitaves to phenylhydantoins was performed at 80°C in 0.1N HCl for 10 min. The N-terminal amino acid is subsequently released as a PTH- amino acid and analyzed using the HPLC system described earlier.

**Protein Determinations.**

Protein concentrations were determined by the method of Bradford (1976).
Table M.1

Gradient profile for amino acid analysis using HPLC.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% Solvent A</th>
<th>% Solvent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>30</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>34</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>37</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Solvents were as described in Materials and Methods. Running temperature was 52°C and flow rate was 1ml/min.

Solvent A = 0.05 M ammonium acetate buffer, pH 6.8
Solvent B = 0.05 M ammonium acetate buffer, pH 6.8 in 50% acetonitrile (v/v)
Results
Chapter One

Inhibition of Staphylococcal Alpha Toxin by Covalent Modification of an Arginine Residue
Introduction

The strong correlation that exists between binding of staphylococcal $\alpha$-toxin and sensitivity of erythrocytes ([Barei and Fackrell, 1979; Cassidy and Harshman, 1976b]) suggest that a specific receptor exists in the rabbit erythrocyte membrane. We have demonstrated (Maharaj and Fackrell, 1980; Austin and Fackrell, 1984; Fackrell et al, 1985) that band 3, the anion transport protein of the rabbit red blood cell, is a receptor for staphylococcal $\alpha$-toxin.

Receptor binding and subsequent hemolysis of rabbit erythrocytes by $\alpha$-toxin can be separated into different events ([Barei and Fackrell, 1979; Cassidy and Harshman, 1976c; Harshman and Sugg, 1985]). However, few studies have been done on the chemical modification of $\alpha$-toxin in order to characterize these events. Cassidy and Harshman (1976a,b,c) iodinated a single tyrosine residue on $\alpha$-toxin and found that although binding was unaffected lysis was strongly inhibited.

Arginine residues often function as ligand recognition sites in proteins and thus made a good choice for further modification studies. In this paper, we examine the effect of 1,2- cyclohexanedione (CHD) and phenylglyoxal (PGO), two extremely useful arginine specific reagents, on the hemolytic activity of staphylococcal $\alpha$-toxin. CHD can be used as a protective group for arginine in trypsinolysis studies ([Toi et al, 1967]), and can be removed from arginine under mild conditions ([Patthy and Smith, 1975a,b]). It also forms a stable complex with arginine in borate buffer which can be used in chromatographic applications.
Results

Inactivation of α-toxin with arginine-specific reagents.

Incubation of α-toxin with different concentrations of phenylglyoxal resulted in a time dependent loss in hemolytic activity which proceeded in a pseudo-first order fashion (Figure 1.1a). Inactivation was linear up to 95%. Further incubation rendered the toxin completely non-hemolytic but conformational changes became evident (Figure 1.2). This indicated that the toxin could be substantially inactivated and still retain its native conformation, implying a structure-function relationship between arginine residues and hemolytic activity.

The rate of inactivation was temperature dependent (with 10 mM PGO the reaction had a kₐ of 0.044 min⁻¹ at 37 C and 0.018 min⁻¹ at 25 C), although pH had no effect on the rate of inactivation in the range from 7.0 to 10.0 (data not shown). The second order rate constant determined from a plot of kₐ versus PGO concentration was 0.0043 M⁻¹ min⁻¹ and showed clearly that inactivation was dependent on PGO concentration (Figure 1.1b).

A log-log plot of kₐ versus PGO concentration was linear, yielding a slope of 1.06 ± 0.04 (Figure 1.1c). This indicated that the reaction between PGO and α-toxin was first order and suggested that modification of a single arginine residue resulted in a substantial loss in hemolytic activity. A side reaction between PGO and cysteine residues is known to occur (Riordan, 1979), but as there are no such residues in α-toxin only arginine is modified. Direct quantitation using the PGO derivative p-hydroxyphenylglyoxal (Yamasaki et al, 1980) confirmed that a single arginine residue was modified.
A similar pattern was seen for modification of α-toxin using CHD (data not shown). However, the affinity of CHD for the essential arginine residue is considerably lower than that of PGO. Inactivation to 95% with CHD required longer incubation times (6-8 hr) and higher concentrations of inactivator (30-50 mM).

Direct quantitation of the CHD-arginine complex with the Girard-T reagent (Patthy et al., 1979) also indicated that a single arginine was modified ($\bar{X} = 0.90 \pm 0.13$, four separate determinations), as expected. Regeneration of the CHD modified toxin with hydroxylamine resulted in a retrieval of hemolytic activity (Table 1.1). However, attempts to regenerate PGO modified toxin were unsuccessful indicating that the reaction was essentially irreversible (Table 1.1).

Figure 1.2 shows the fluorescent spectra of native and modified α-toxin as well as heat inactivated toxoid. It is clear that the conformation of the toxin is not altered to any great extent by CHD or PGO in the time required to substantially inactivate the hemolytic activity of the toxin. By comparison, the toxoid, which binds to the rabbit erythrocyte as strongly as the toxin (Barei and Packrell, 1979) is quenched considerably. Significant conformational changes are seen upon further incubation of CHD or PGO with α-toxin. Quantitation of CHD-arginine indicated that a second arginine was modified in the conformationally altered toxin (data not shown).

To determine whether arginine modification prevented α-toxin from binding to its receptor or a subsequent event in the lytic pathway, rabbit erythrocytes were preincubated with modified toxin for 30 min. Subsequent addition of native toxin resulted in lysis, as in toxin control samples, even when modified to native ratios were as high as 8:1 (Table 1.1). This indicated that modification of α-toxin with arginine specific reagents specifically affected a residue in the binding site, or at least physically close to it.
Electrophoretic transblot/ELISA of modified and native α-toxin

Lo and Fackrell (1979) demonstrated that two populations of anti-α toxin antibodies exist in immune sera from rabbits and pigs. One population prevented the binding of α-toxin to erythrocyte membranes (AB-Ab). A second population neutralized α-toxin after it was bound to membranes and caused an indirect hemagglutination (IHA-Ab).

Electrophoretic blots were performed using unfractionated rabbit antisera, as well as the separated AB-AB and IHA-Ab populations as primary antibodies in ELISAs. As can be seen in Figure 1.3 CHD α-toxin shows little difference from native α toxin in transblots with all three populations. Harshman and Cassidy (1970b) found that iodination of a single tyrosine residue affected the migration of α-toxin in polyacrylamide gels. This was not found to be the case for CHD modification of α-toxin as CHD was still bound to α-toxin after electrophoresis (data not shown).

In ELISAs performed with IHA-Ab a number of high molecular weight bands were seen for both native and modified α-toxin (Figure 1.3). However, most of these high molecular weight bands were not seen in AB-Ab ELISAs. In contrast, the monomeric toxin, which in our electrophoretic system has a M_r of 37 kD, was not seen in the ELISA with IHA-Ab for either native or CHD modified α-toxin. When, however, the concentration of IHA-Ab was doubled the monomeric form of the toxin was seen in the ELISA (Figure 1.3).

ELISAs were performed using native and PGO modified α-toxin. As can be seen in Table 1.2, no significant difference exists between the two proteins when compared
in ELISAs with whole sera or AB-Ab populations. However, the PGO modified toxin showed a significant decrease in titre compared to native α-toxin in IHA-Ab ELISAs.

Trypsin digestion of denatured α-toxin and CHD toxin

Peptide maps of native and CHD modified α-toxin showed a pattern paralleling that seen by Cassidy and Harshman (1976a) when stained with ninhydrin. When stained with NiCl₂ spray a single reactive spot was obtained (data not shown). Figure 1.4 shows the effect of trypsin digestion on denatured samples of modified and native α-toxin. Native α-toxin is essentially digested completely while trypsin treatment of CHD modified α-toxin yields a Mr 20 kD fragment (Figure 1.4a). This 20 kD fragment was also isolated using a boric acid gel column (Patthy et al., 1979) indicating that the modified arginine is located in this region (Figure 1.4d). The low molecular mass trypsin products (Figure 1.4b,c) were lost upon isolation of the 20 kD fragment with the boric acid gel column. This indicated that these fragments did not contain CHD. In all samples the high molecular mass aggregates (over 50kD) were resistant to trypsin digestion (Figure 1.4a,b, and c). This has been observed by other researchers as well (Tobkes et al., 1985; Fussle et al., 1981). Tobkes et al. (1985) noted a progression of subunits resulting in hexamer formation from the monomer. These 60-90 kD fragments may be intermediate stages in hexamer assembly recognized selectively by different anti-α-toxin populations. When electrophoretic blots were performed on trypsinized samples of CHD modified α-toxin the 20 kD fragment was seen in
an ELISA using IHA–Ab (Figure 1.4b) but not in an AB–Ab ELISA (Figure 1.4c). This indicated that IHA–Ab bind α toxin physically closer to its receptor binding site than does AB–Ab.
Figure 1.1 Hemolysis due to alpha toxin and PGO modified alpha toxin.

A) Effect of PGO concentration on hemolysis by alpha toxin. Alpha toxin was incubated with 0 (×), 3 mM (△), 7 mM (■), and 10 mM (□) PGO in 0.2 M bicarbonate buffer, pH 8.5. At times specified 1.5 µg/ml aliquots were removed and hemolytic activity was determined as described in Materials and Methods. Absorbance was measured at 650 nm. Relative inactivation was determined by comparing maximum rates of lysis.

B) Dependence of $k_a$ on PGO concentration (determined from linear regression of data from Fig 1A).

C) Data from Fig 1B plotted in a log-log fashion.
Figure 1.2 Fluorescent Spectra of native alpha tosin (A), CHD modified alpha tosin (C), PGO modified alpha tosin (B) and tosoid (F).

Prolonged incubation of alpha tosin with CHD (D) or PGO (E).

Proteins were at a concentration of 1 mg/ml in 0.2M borate buffer, pH 7.6.
**TABLE 1.1**

Effect of native toxin on erythrocytes preincubated with modified toxin.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lag Time (min)</th>
<th>Max. Rate of lysis (^2) (O.D. units/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha toxin</td>
<td>1.07</td>
<td>0.595</td>
</tr>
<tr>
<td>Regenerated alpha toxin (CHD)</td>
<td>2.03</td>
<td>0.451</td>
</tr>
<tr>
<td>Alpha toxin (added to erythrocytes preincubated with CHD modified alpha toxin (^3))</td>
<td>1.11</td>
<td>0.550</td>
</tr>
<tr>
<td>Regenerated alpha toxin (PGO)</td>
<td>6.32</td>
<td>0.087</td>
</tr>
<tr>
<td>Alpha toxin (added to erythrocytes preincubated with PGO modified alpha toxin (^3))</td>
<td>1.05</td>
<td>0.540</td>
</tr>
</tbody>
</table>

1. Lag time is defined as time to a 5% change in absorbance.
2. Maximum rate of lysis is defined as the highest rate of change in absorbance as determined by plotting the first derivative of absorbance (lysis).
3. Modified alpha toxin:alpha toxin ratio was 9:1. Modified toxin was 95% inactive.

Rate determinations and lag time were made using KINETICS program described in Materials and Methods. Erythrocytes were treated with 0.31 g/ml alpha toxin or modified alpha toxin.
Figure 1.3 Electrophoretic transblots of native and CHD modified alpha toxin.

Native alpha toxin in lanes 1, 3, and 5. CHD modified alpha toxin in lanes 2, 4, and 5. Numbered arrows (▼) represent molecular weight standards (Bio-Rad) in kD. (▼) represents the alpha toxin monomer which in our electrophoretic system has an apparent molecular weight of 37 kD. Large dark arrow (►) indicates oligomeric forms of alpha toxin. ELISA was performed using whole rabbit antisera (lanes 1 and 2), fractionated AB-Ab (lanes 3 and 4) and fractionated IHA-Ab (lanes 5 and 6) as primary antibodies as described in Materials and Methods. Lanes 7 and 8 are IHA-Ab blots using doubled concentrations of primary antibody.
TABLE 1.2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Whole Sera</th>
<th>AB–Ab</th>
<th>IHA–Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha toxin</td>
<td>0.881±0.023</td>
<td>0.936±0.016</td>
<td>0.903±0.096</td>
</tr>
<tr>
<td>PGO modified alpha toxin</td>
<td>0.835±0.12</td>
<td>0.983±0.027</td>
<td>0.785±0.091</td>
</tr>
</tbody>
</table>

Differences between alpha toxin and PGO modified alpha toxin were determined to be insignificant for whole sera and AB–Ab (n=9, 3 independent ELISAs) using the F distribution (p<0.025). A significant difference (p<0.025) existed between the two proteins in IHA–Ab ELISAs (n=9, 3 independent ELISAs).
Figure 1.4. Trypsin digestion of native and CHD modified alpha toxin.

a) SDS PAGE of native (---) and CHD modified (——) alpha toxin treated with trypsin as described in Materials and Methods. b) Electrophoretic transblots of trypsin treated native (-----) and CHD modified (——) alpha toxin using IHA–Ab as primary antibody in an ELISA. c) Electrophoretic transblots of trypsin treated native (-----) and CHD modified (——) alpha toxin using AB–Ab as primary antibody in ELISA. d) Electrophoretic transblots of trypsin treated CHD modified alpha toxin isolated with boric acid gel chromatography. (---) represents AB–Ab and (——) IHA–Ab as primary antibody in ELISA. Numbered arrows (↑) represent molecular-weight standards (Bio Rad). Densitometry was performed on transblots using a Shimadzu dual-wavelength TLC scanner CS-930. Transblots were performed as described in Materials and Methods.
Discussion

Patthy and Thesz (1980) demonstrated that active site arginine residues react selectively with α-dicarbonyl reagents such as CHD because the pKa of these residues is lower than that of other arginines. Arginine residues, because of their positively charged guanidino group are often found in anion binding sites. In this study we found that one arginine was selectively modified out of ten such residues found in α-toxin (Gray and Kehoe, 1984).

Maharaj and Fackrell (1980) showed that Band 3, the anion transport protein of human and rabbit (Austin and Fackrell, 1984) erythrocytes was a receptor for α-toxin. Fackrell et al. (1985) have demonstrated that the receptor resides in a 35 kDa chymotryptic fragment from the carboxyl terminus of Band 3. Simpson and Fackrell (unpublished observations) further demonstrated that the carbohydrate moiety of this fragment is the site of toxin binding. Treatment of the fragment or rabbit erythrocytes with sialidase greatly reduces their ability to bind α-toxin.

Sialic acid residues are negatively charged and hence a positively charged binding site on the toxin containing the modified arginine residue may be involved in binding to the receptor. Cassidy and Harshman (1976b) showed that modification of a single tyrosine residue in α-toxin with iodine abrogated hemolytic activity without affecting binding. They further showed that modification of a second tyrosine residue prevented the toxin from binding to its receptor. Whether this was due to an effect on the binding site by iodine or to a conformational disturbance was not determined. We are now attempting to determine whether the essential arginine residue for binding and the tyrosine residue (Cassidy and Harshman, 1976b) are located near each other.

The overall conformation of the molecule when a single arginine residue is
modified was similar to that of the native toxin. In contrast, toxoid showed a marked quenching from the fluorescent spectra of native \(\alpha\)-toxin. However, it has been demonstrated that toxoid binds receptors on the erythrocyte membrane equally well as toxin (Barei and Fackrell, 1979) despite the conformational difference. It seems likely, therefore, that the receptor binding site of \(\alpha\)-toxin itself is conformationally stable to heat denaturation.

Native \(\alpha\)-toxin and CHD \(\alpha\)-toxin both migrate at the same rate in SDS polyacrylamide gels. An ELISA with unfractionated rabbit antisera also failed to discriminate between the two proteins since prolonged exposure to Tris buffers removes CHD (Patthy and Smith, 1975a,b). Lo and Fackrell (1979) proposed that two separate sites were recognized by antibinding antibodies. From the trypsin digestion of modified \(\alpha\)-toxin it can be seen that AB–Ab do not recognize regions at/or adjacent to the actual binding site. Harshman et al (1986) have shown that a monoclonal antibody directed against the C-terminal region of \(\alpha\)-toxin had no effect on its activity. Thus, this is not a site for AB–Ab binding. Further binding analyses to elucidate the nature of the AB–Ab – \(\alpha\)-toxin interaction are underway.

IHA–Ab on the other hand recognize the CHD derived fragment, IHA–Ab ELISA with both native and modified \(\alpha\)-toxin show a number of high molecular weight aggregates. The IHA–Ab inhibit \(\alpha\)-toxin after it has become bound to target cell membranes and thus perhaps recognize determinants featured on the hexameric pore formed by \(\alpha\)-toxin. One such candidate is the hinge region exposed by membrane interaction as proposed by Tobkes et al (1985). This hinge region may not be fully exposed until contact with receptors and thus antibodies recognizing determinants near the binding site of \(\alpha\)-toxin may actually be IHA–Ab. Our
results demonstrate that at least one indirect hemagglutination determinant is physically close to the binding site as modification with PGO results in a decrease in titre in IHA ELISAs. Surujballi and Fackrell (unpublished observations) have shown that multiple IHA determinants exist in trypsin and CNBr digested α-toxin while only one determinant is exposed in the whole toxin (Lo and Fackrell, 1979). The AB–Ab may recognize determinants that prevent the hinge from opening and thus prevent interaction with target membranes. Recently, Thelestam and Blomqvist (1986a) have suggested that only one binding site exists on α-toxin and it is centrally located. This is consistent with our findings and those of Tobkes et al (1985). They postulated that the hinge region is an early site of cleavage in the monomeric toxin by proteinase K. If this site is occluded, as in the hexamer, or blocked by modification little cleavage would result as we have demonstrated using trypsinolysis of CHD modified toxin.

Watanabe and Kato (1978) found that trypsin treatment of α-toxin generated a 17 kD lethal fragment with no hemolytic activity and a 20 kD fragment that tended to form aggregates. Thelestam and Blomqvist (1984) also found an 18.5 kD fragment in their toxin preparations, presumably as a spontaneous breakdown product. This fragment had the same specific hemolytic activity as native α-toxin but had lost its toxicity for adrenocortical cells in culture. This 18.5 kD fragment has also been noted in our own toxin preparations (Surujballi and Fackrell, unpublished observation). Further analysis of the 20 kD fragment in conjunction with CNBr digestion should localise the binding site of the toxin and are in progress. Whether CHD modification has effects on the cytotoxicity and lethality of α-toxin have yet to be tested. The relationship of the 20kD fragment seen in our study to fragments derived by other workers (Watanabe and Kato, 1978; Thelestam and Blomqvist, 1984) are unknown at present.
Chapter Two

Isolation and Characterization of the Binding Site of Staphylocoecal Alpha Toxin
Introduction

The mechanism of action of staphylococcal α-toxin on rabbit erythrocytes is believed to involve the following steps: (i) binding of the toxin monomer to receptors on the cell surface, (ii) hexamer formation once a critical number of toxin molecules have bound the surface, (iii) leakage of small ions through 2–3nm diameter transmembrane channels generated by the hexamers and (iv) colloid osmotic lysis of the cell. It is also becoming clear that a conformational change in the toxin is necessary for oligomerization to occur (Tobkes et al., 1985; Blomqvist and Thelestam, 1986a; Igikae and Nakae, 1985).

However, it is not clear whether this a general mechanism of action for the toxin on in vivo target cells as well. Blomqvist and Thelestam (1986a) have shown that a similar pattern of events occurs during the action of α-toxin on adrenocortical Y1 cells in culture. Indirect evidence also suggests that a single region of α-toxin is responsible for binding to the various cell types (Watanabe and Kato, 1978; Blomqvist and Thelestam, 1986b, Blomqvist et al., 1987). On the basis of fragmentation studies these researchers have localized the binding site to the C-terminal domain of the toxin. Surujballi (1987 Ph.D. dissertation) has further localized the binding site to a 9000 D CNBr fragment of α-toxin.

We have shown (Hebert and Fackrell, in press) that a single arginine residue is essential for binding of α-toxin to its erythrocyte receptor. This chapter describes the isolation and characterization of a CNBr fragment containing that essential arginine residue. The effect of modification of this arginine on the ability of α-toxin to damage cells other than the erythrocyte was also investigated in an attempt to determine if the toxin acts through a common binding site in different cells.
Results

Effect of Arginine Modification of α-Toxin on Cytolytic Activity.

α-toxin is known to be cytotoxic for a wide variety of nucleated cells (Thelestam, 1983). However, it is not known if the mechanism of action on these cells proceeds through a common pathway and if that pathway is similar to that in the rabbit erythrocyte. This study addresses these questions.

α-toxin was modified with CHD as described in Chapter 1. 24–48 hr cultures of mitotic VERO cells were incubated with native α-toxin, CHD modified toxin, PBS or toxin + anti-toxin for 30 min with shaking. Trypan blue dye exclusion was used as a measure of cells remaining viable. Trypsinization did not have any effect on the sensitivity of the cells to the toxin as viability counts were similar to cells removed from flasks by scraping (data not shown). Table 2.1 demonstrates that the CHD modified toxin had little effect on viability as compared to control cells treated with PBS or anti-α-toxin. In contrast cells treated with native α-toxin showed a significant reduction in viability. Also, cells preincubated with CHD modified α-toxin did not prevent subsequent intoxication with native toxin suggesting that the binding site was affected (Table 2.1).

α-toxin modified with PGO was used to determine if the arginine residue was important for the intoxication of perfused isolated rat atrial tissue. When treated with native toxin the muscle shows a time dependent reduction in the force of contraction generated (Figure 2.1a, Adamo et al. in prep). In contrast, PGO modified α-toxin does not have a significant effect on the atrial force of contraction (Figure 2.1b). Again preincubation of the isolated atria with modified toxin did not prevent subsequent damage by native α-toxin (Figure 2.1c).
Isolation of a CNBr Fragment Containing the Modified Arginine.

Both native and CHD modified \( \alpha \)-toxin were digested with CNBr. The native CNBr digest upon preincubation with rabbit erythrocytes was non-hemolytic but prevented subsequent intoxication with \( \alpha \)-toxin (Figure 2.2a). In contrast, the modified CNBr digest had no such protective effect against the native toxin (Figure 2.2b). However, upon regeneration of the modified arginine residue with 0.5 M \( \text{NH}_2\text{OH} \) the modified CNBr digest possessed inhibitory activity against the native toxin (Figure 2.2c).

Using the boric acid gel system described by Pathy et al. (1979) it was possible to isolate the modified CNBr fragment. A column profile for the boric acid gel is shown in Figure 2.3. Subsequent western blots of the pre-elution and eluted fragments is shown in Figure 2.4. The CHD containing fragment has an apparent molecular weight of 9000 D (Figure 2.4c) and is the smallest of the five antigenically reactive CNBr fragments of \( \alpha \)-toxin as described by Surujballi and Fackrell (in prep). This fragment is absent from the pre-elution fragments (Figure 2.4b).

Reverse phase HPLC with a linear gradient of 1-propanol was used to isolate the CNBr fragment for further study. However, instead of the expected single peak, four peaks were generated from the single 9000 D fragment (Figure 2.5a). Two of the peaks (1 and 3) could be precipitated with 0.2 M \( \text{NiCl}_2 \) indicating that they still contained the CHD-arginine complex (Pathy and Smith, 1975b). The reaction between CHD and arginine is known to be spontaneously reversible and in older samples the amounts of peaks 1 and 3 were substantially reduced (Figure 2.5b).

Characterization of the Isolated CNBr Fragment.
Lo and Fackrell (1979) demonstrated that two populations of anti-α-toxin exist in immune sera from rabbits and pigs. One population prevented the binding of the toxin to erythrocyte membranes (AB–Ab). A second population neutralized α-toxin after it was bound to membranes and caused an indirect hemagglutination (IHA–Ab). A number of monoclonal antibodies have also been developed in our lab (Surujballi, 1987 Ph.D. dissertation) against α-toxin. These MAbs can also be divided into AB–Ab and IHA–Ab. All five of the MAbs failed to distinguish between PGO modified α-toxin and the native toxin (Table 2.2) indicating that they did not recognize the binding site. We had earlier shown that PGO modification directly affects the binding site of the toxin (Chapter 1).

ELISAs were performed on all four peaks isolated from the HPLC (Table 2.3). It is clear that all four react with the various polyclonal populations (i.e. whole antisera, IHA–Ab, and AB–Ab). The differences are probably due to differing concentrations after isolation from the HPLC. However, none of the monoclonal antibodies (MAbs) react with any of the peaks. Surujballi (Ph.D. dissertation) removed the CNBr fragment containing the binding site by adsorption onto rabbit erythrocyte ghosts. They could detect no differences in the adsorbed fragments versus unadsorbed fragments using any of the MAbs but noted that the fragment was recognized by both polyclonal antisera populations. Also, preadsorption selectively removed the 9000 D fragment which agrees with results presented in this study.

Percent compositions of the four peaks were determined using the method of Henrickson and Meredith (1984). In our HPLC system the standards ran slightly different than described (Figure 2.6). Individual amino acids had retention times and integrator coefficients as listed in Table 2.4.

All four peaks had essentially the same percent composition except that peaks 1 and 2 possessed one homoserine lactone residue (as the C-terminal due to CNBr
digestion) while peaks 3 and 4 possessed a single homoserine residue (Table 2.5). The production of both homoserine and its lactone derivative as the C-terminal amino acid both occur as a result of CNBr digestion in a 40:60 ratio (Armstrong, 1949) and have been shown to markedly effect the retention times of peptides in reverse phase HPLC systems especially with smaller peptides (Hermoson and Mahoney, 1983). Changes in local charge due to CHD modification would also effect retention time resulting in four peaks from a single fragment. The percent composition determined for the fragment consists of a mixture of polar and nonpolar amino acids (59 and 41% respectively) and is unremarkable in this sense. Percent composition of tryptophan could not be determined because of its destruction during acid hydrolysis and results for proline were difficult to interpret because a reagent derived peak appeared at the same time in some samples. A manual Edman degradation on the fragment demonstrated that tryptophan was the N-terminal amino acid.
Table 2:1

Effect of Native and CHD Modified α-Toxin on VERO Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viable Cell Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-toxin</td>
<td>38,000 ± 6,200</td>
</tr>
<tr>
<td>CHD α-toxin</td>
<td>60,700 ± 5,100</td>
</tr>
<tr>
<td>PBS control</td>
<td>64,500 ± 5,600</td>
</tr>
<tr>
<td>Antitoxin control</td>
<td>58,000 ± 4,800</td>
</tr>
<tr>
<td>α-toxin added after preincubation with CHD toxin</td>
<td>36,900 ± 5,700</td>
</tr>
<tr>
<td>for 30 min.</td>
<td></td>
</tr>
</tbody>
</table>

Reduction in viability by α-toxin over controls was determined to be significant using the F distribution (p<0.025) in three separate experiments. Values are recorded as means of 5 replicates/experiment with standard error. Viability was determined with trypan blue dye exclusion as described in Materials and Methods.
Figure 2.1

Effect of native and PGO modified α-toxin on isolated perfused rat atria. Graphs are representative of three separate experiments. 6-12 HU/ml native or modified toxin were incubated with the atria in perfusion buffer. Line A represents the effect of native toxin alone on the force of contraction of the isolated atria. Line B represents the effect of PGO modified α-toxin on the force of contraction. Line C represents the effect of native toxin on isolated atria which were preincubated with the modified toxin for 30 minutes. Line D is a control baseline for the isolated atria.
Effect of 1.5 μg/ml of native α-toxin on cells preincubated with CHD modified CNBr fragments of the toxin (B) at a concentration of 50 μg/ml. Line C represents the effect of native toxin (1.5 μg/ml) on cells preincubated with a similar CNBr digest of native α-toxin. Line D represents lysis by native toxin alone.

Line C represents the effect of native toxin on erythrocytes preincubated with a CHD modified toxin digest treated with NH₂OH.
Affinity chromatography of CHD modified fragments of α-toxin. Arrow represents addition of elution buffer as described in Materials and Methods. Positive fractions were pooled and lyophilized for further study.
Figure 2.4

Densitometric scans of western blots performed using a Shimadzu CS-930 Dual Wavelength TLC Scanner. 5–10 µg protein were added per well to 12% SDS polyacrylamide gels. Subsequent blots were incubated with polyclonal anti-α-toxin.

A) CNBr digest of CHD modified α-toxin.
B) CNBr fragments which did not bind to boric acid gel.
C) CNBr fragment eluted from boric acid gel column.

Arrow represents the position of the 9000 D band.
RELATIVE ABSORBANCE (600nm)

DISTANCE MIGRATED FROM THE TOP OF THE GEL (cm)
Figure 2.5

Reverse phase HPLC of putative binding fragment. Samples were run as described in Materials and Methods.

A) 5μl sample of the CNBr fragment five hours after isolation.

B) 5μl sample of the fragment 48 hr after isolation and subsequent storage at 4°C.

Detector was set at 214 nm with 2 AUFS.
Table 2.2

Results of an ELISA using MAbs with native and PGO modified α-toxin

<table>
<thead>
<tr>
<th>Sample</th>
<th>MAb-1</th>
<th>MAb-3</th>
<th>MAb-4</th>
<th>MAb-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-toxin</td>
<td>0.168±0.066</td>
<td>0.186±0.063</td>
<td>0.201±0.012</td>
<td>0.176±0.065</td>
</tr>
<tr>
<td>PGO toxin</td>
<td>0.179±0.047</td>
<td>0.172±0.052</td>
<td>0.206±0.089</td>
<td>0.172±0.097</td>
</tr>
</tbody>
</table>

Differences between α-toxin and PGO modified toxin were determined to be insignificantly for all of the MAbs tested (n=9, in three independent ELISAs) using the F distribution (p<0.025). MAB-1 and MAb-2 were shown by Surujballi (1987 Ph.D. dissertation) to recognize the same determinant and results for MAb-2 are not shown. Values are means with standard deviation.
### Table 2.3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Whole sera</th>
<th>AB-Ab</th>
<th>IHA-Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>0.091±0.03</td>
<td>0.191±0.01</td>
<td>0.075±0.04</td>
</tr>
<tr>
<td>Peak 2</td>
<td>0.094±0.04</td>
<td>0.192±0.01</td>
<td>0.102±0.06</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.192±0.04</td>
<td>0.201±0.02</td>
<td>0.151±0.03</td>
</tr>
<tr>
<td>Peak 4</td>
<td>0.274±0.07</td>
<td>0.204±0.02</td>
<td>0.156±0.07</td>
</tr>
</tbody>
</table>

Values are means with standard deviations and are the results of 3 separate experiments (n=9). All of the peaks showed significant absorbances of the control wells. Differences in absorbance between the peaks are likely due to collection technique. Peaks were isolated using HPLC and collected as described in Materials and Methods.
### Table 2.4

PITC-Amino Acid Standards separated in the HPLC

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Retention Time (min)</th>
<th>Integrator Coefficient (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLU/GLN</td>
<td>3.08</td>
<td>4.38x10$^6$</td>
</tr>
<tr>
<td>ASP</td>
<td>3.53</td>
<td>2.30x10$^4$</td>
</tr>
<tr>
<td>SER/ASN</td>
<td>3.91</td>
<td>5.77x10$^5$</td>
</tr>
<tr>
<td>GLY</td>
<td>4.18</td>
<td>1.16x10$^6$</td>
</tr>
<tr>
<td>THR</td>
<td>5.07</td>
<td>5.11x10$^6$</td>
</tr>
<tr>
<td>ALA</td>
<td>6.35</td>
<td>5.74x10$^6$</td>
</tr>
<tr>
<td>PRO</td>
<td>6.59</td>
<td>2.77x10$^6$</td>
</tr>
<tr>
<td>HIS</td>
<td>7.16</td>
<td>1.01x10$^6$</td>
</tr>
<tr>
<td>ARG</td>
<td>9.13</td>
<td>3.68x10$^5$</td>
</tr>
<tr>
<td>MET</td>
<td>11.32</td>
<td>2.59x10$^6$</td>
</tr>
<tr>
<td>TYR</td>
<td>11.99</td>
<td>3.09x10$^6$</td>
</tr>
<tr>
<td>VAL</td>
<td>12.46</td>
<td>2.65x10$^6$</td>
</tr>
<tr>
<td>LEU</td>
<td>22.60</td>
<td>5.80x10$^6$</td>
</tr>
<tr>
<td>ILE</td>
<td>23.07</td>
<td>1.11x10$^6$</td>
</tr>
<tr>
<td>PHE</td>
<td>27.05</td>
<td>9.5x10$^5$</td>
</tr>
<tr>
<td>LYS</td>
<td>32.44</td>
<td>1.23x10$^6$</td>
</tr>
</tbody>
</table>

Integrator coefficients are determined using area which is calculated by the Data Master module of the Gilson HPLC system. Concentrations of the amino acids were 25 μM. Integrator coefficients are expressed as relative area/concentration in units of M$^{-1}$. Amino acids were coupled with PITC and separated in the HPLC as described in Materials and Methods.
Amino acid standards, 2.5 \( \mu \text{M} \) of each listed amino acid was injected in a 10 \( \mu \text{l} \) sample. Amino acids were separated using the solvent and gradient profiles outlined in *Materials and Methods*. Detector was set at 254 nm and 0.2 AUFS.

Amino acids are marked with standard one letter abbreviations.

Homoserine (HS) and homoserine lactone (HSL) eluted at times marked by dark arrows.
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peak 2</th>
<th>Peak 4</th>
<th>Predicted from sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLU/GLN</td>
<td>1.94(2)</td>
<td>1.87(2)</td>
<td>2</td>
</tr>
<tr>
<td>ASP</td>
<td>1.84(2)</td>
<td>1.94(2)</td>
<td>2</td>
</tr>
<tr>
<td>SER/ASN</td>
<td>4.59(5)</td>
<td>4.87(5)</td>
<td>-5</td>
</tr>
<tr>
<td>GLY</td>
<td>1.40(1)</td>
<td>1.67(2)</td>
<td>2</td>
</tr>
<tr>
<td>THR</td>
<td>——-(0)</td>
<td>——-(0)</td>
<td>0</td>
</tr>
<tr>
<td>ALA</td>
<td>——-(0)</td>
<td>——-(0)</td>
<td>0</td>
</tr>
<tr>
<td>PRO</td>
<td>——-(0)</td>
<td>——-(0)</td>
<td>2</td>
</tr>
<tr>
<td>HIS</td>
<td>——-(0)</td>
<td>——-(0)</td>
<td>0</td>
</tr>
<tr>
<td>ARG</td>
<td>0.82(1)</td>
<td>0.87(1)</td>
<td>1</td>
</tr>
<tr>
<td>MET/HSER</td>
<td>0.75(1)</td>
<td>0.84(1)</td>
<td>1</td>
</tr>
<tr>
<td>TYR</td>
<td>1.03(1)</td>
<td>1.12(1)</td>
<td>2</td>
</tr>
<tr>
<td>VAL</td>
<td>1.00(1)*</td>
<td>1.00(1)</td>
<td>1</td>
</tr>
<tr>
<td>LEU</td>
<td>0.52(1)</td>
<td>0.98(1)</td>
<td>1</td>
</tr>
<tr>
<td>ILE</td>
<td>——-(0)</td>
<td>——-(0)</td>
<td>0</td>
</tr>
<tr>
<td>PHE</td>
<td>0.56(1)</td>
<td>0.79(1)</td>
<td>1</td>
</tr>
<tr>
<td>LYS</td>
<td>——-(0)</td>
<td>——-(0)</td>
<td>0</td>
</tr>
<tr>
<td>TRP</td>
<td>——-(0)</td>
<td>——-(0)</td>
<td>3**</td>
</tr>
</tbody>
</table>

Peaks were isolated, hydrolyzed and percent compositions were determined as described in *Materials and Methods*. Results are averages of five separate determinations. Calculations of amino acid content were performed using integrator coefficients determined from amino acid standards. The percent compositions of peaks 1 and 3 were similar to peaks 2 and 4 but were missing an arginine residue. This was expected since in these peaks arginine was modified with CHD.

*The value for valine was set arbitrarily to 1.00.

**Tryptophan was destroyed by acid hydrolysis and was not included in the amino acid standard kit. However, using reagent grade tryptophan we were able to determine a retention time of 24.6 min which enabled us to perform a manual Edman degradation on the fragment. Trp was determined to be the N-terminal amino acid and thus the number of Trp in the fragment is at least one.
Discussion

Gray and Kehoe (1984) sequenced the gene for \(\alpha\)-toxin. The fragment isolated in this study matches up closely with a theoretical 22 residue CNBr fragment (Trp 175–Met 197) in the C-terminal region of the predicted amino acid sequence (see Table 2.4) including the N-terminal amino acid. The isolated fragment possesses a single tyrosine residue whereas two are predicted from the gene sequence. However, the match was confirmed using a BASIC program (developed by G. Dombi, BCTIC Computer Code Collection, Vanderbilt University) called ANALYSIS which will locate a peptide in a sequence of known amino acids on the basis of its percent composition. Chou and Fasman (1978) analysis of the predicted fragment assigns a relatively high hydrophilicity to this region (Gray and Kehoe, 1984). The interaction between the polar and nonpolar amino acids in this region of the toxin must somehow favor a hydrophilic character.

Maharaj and Fackrell (1980) identified the receptor for \(\alpha\)-toxin in rabbit erythrocytes as Band 3, the anion transport protein. Simpson and Fackrell (in prep) further localized the receptor as a charged region of the carbohydrate moiety of Band 3. It follows, therefore that a region of the toxin involved in binding to the receptor should also be charged. We have shown that a single arginine residue (positively charged) is involved in binding to the receptor and the fragment isolated possesses a single arginine residue (Arg 184). Cassidy and Harshman (1976a) demonstrated that a tyrosine residue may also be important in binding and the fragment also possesses a tyrosine residue. This residue is distinct from Tyr 29 which has been shown to be involved in post binding events (Cassidy and Harshman, 1976a,b; Harshman, personal communication).

Blomqvist et al. (1987) have isolated a naturally occurring 18.5 kD fragment
of α-toxin that possess the binding domain but does not damage membranes. Partial protein sequencing of this fragment determined that it was the C-terminal domain of α-toxin. Tobkes el al. (1985) have postulated that α-toxin consists of two B-sheet domains separated by a flexible hinge. Support for this model has also come from Blomqvist and Thelestan (1986a) and Ikigai and Nakae (1985). The theoretical hinge is centrally located in a region of high glycine content (32%). This hinge is believed to be involved in the transition of α-toxin from a highly hydrophilic to an amphiphilic molecule. This site is also an initial site for the action of proteolytic enzymes on the toxin and is thus at least initially exposed on the surface of the toxin. The binding site, being in the C-terminal domain remains intact after initial cleavage with either naturally occurring or added proteases (Blomqvist et al., 1987) and thus may be partially occluded in the native toxin molecule or perhaps located in a groove between the two domains. This feature would be one shared with a number of carbohydrate binding proteins whose binding site is located in a cleft between two domains of predominately β-sheet structure (Quiocio, 1986). In fact the toxin itself is predominately β-sheet in structure (Tobkes et al., 1985; Ikigai and Nakae, 1985). An initial interaction with the membrane itself may be required to allow a full interaction between the receptor and the binding site on the toxin. Erythrocytes treated with phospholipase C have a reduced sensitivity to α-toxin (Christie and Graydon, 1941; Elias and Kofer, 1980) and thus an initial interaction with a phospholipid component of the membrane may be necessary for complete intoxication.

Recently, Tang and Fackrell (in prep) have shown that the receptor for α-toxin in various tissues including brain, heart, kidney, and lung is also Band 3. Jeljaszewicz et al. (1969) and Blomqvist et al. (1987b) have shown that radiolabelled α-toxin distributes in all these tissues in vivo in rabbits or
mice.

In this study we have shown that a single common binding site exists on α-toxin for rabbit erythrocytes, VERO kidney cells, and isolated rat atrial cells. In VERO cells an anion transport system similar to Band 3 at least kinetically is operating (Sandvig and Olsnes, 1986b) and we are in the process of determining if this protein is immunologically similar to Band 3.

This evidence implies that α-toxin acts via a similar mechanism of action in cells as diverse as erythrocytes and neurons.
Chapter Three

Inhibition of Staphylococcal Alpha Toxin by Covalent Modification of a Histidine Residue and Isolation of a Site Involved in Post-Binding Events
Introduction

Events that occur after the binding of staphylococcal α-toxin to its receptor on rabbit erythrocytes remain unclear. Cassidy and Harshman (1976a,b) demonstrated that the release of small ions directly follows binding of the toxin to susceptible cells. Other researchers have demonstrated that a conformational change involving the tertiary structure of the toxin occurs after binding (Ikigae and Nakae, 1985; Tobkes et al, 1985; Blomqvist and Thelestam, 1986b). This change probably facilitates the transition of the markedly hydrophilic monomer to a amphiphilic hexameric structure which results in the generation of functional transmembrane pores (Bhakdi et al, 1981; Fussie et al, 1981; Ikigae and Nakae, 1987a,b).

However, the exact nature of this conformational change or regions of the toxin involved in post binding events remains largely unknown.

Blomqvist et al (1987a) have shown that regions in the C-terminal domain are involved in hemolysis but not in membrane damage to other types of cells. Indeed, they as well as others demonstrated that the N-terminal domain is critical for the cytotoxic and lethal effects of the toxin (Blomqvist et al, 1987a; Blomqvist and Thelestam, 1986a; Watanabe and Kato, 1978; Kato and Watanabe, 1980).

We report here a functional characterization of an essential histidine residue which is important in post binding events during the action of α-toxin. We have isolated and characterized a CNBr fragment of the toxin containing the histidine residue.
Results

Chemical Modification of $\alpha$-Toxin with Diethylpyrocarbonate (DPC).

Incubation of $\alpha$-toxin with various concentrations of DPC resulted in a time dependent loss of hemolytic activity, which proceeded in a pseudo-first order fashion (Figure 3.1a). Inactivation was linear up to 90%. Incubation with DPC for longer times resulted in complete inactivation but conformational changes in the toxin became evident (Figure 3.2). This implied that a structure-function relationship existed between histidine residues and hemolytic activity.

The rate of inactivation again showed a temperature dependence similar to that of arginine residues (Chapter 1) with a general increase in rate with increasing temperature (data not shown). The overall rate of reaction of the histidine residues was much greater than arginine (Figure 3.1a). The second order rate constant determined from a plot of $k_2$ versus DPC concentration was 0.132 mM$^{-1}$ s$^{-1}$ and showed clearly that inactivation was dependent on DPC concentration (Figure 3.1b). The intercept of this line passed through the origin indicating that a reversible complex of $\alpha$-toxin and DPC did not form prior to covalent modification (Comis and Easterbrook-Smith, 1985; Emanuel et al. 1982).

A log-log plot of $k_2$ versus DPC concentration was linear yielding a slope of 0.831±0.063 (Figure 3.1c). This indicated that the reaction between DPC and $\alpha$-toxin was first order and suggested that modification of a single residue was sufficient to substantially inhibit hemolysis. In the conditions used DPC is fairly specific for histidine residues but side reactions with cysteine, tyrosine and lysine are also possible (Miles, 1977). Since no cysteine residues are found in $\alpha$-toxin this possibility is ruled out. Regeneration of the modified toxin with
NH₂OH resulted in a recovery of hemolytic activity approaching that of controls (data not shown). The side reaction with lysine is not reversible with NH₂OH and thus is not occurring. Reaction of DPC with tyrosine residues results in a decrease in absorbance at 280 nm. This was not seen in any experiment with DPC and thus it was concluded that the reaction between α-toxin and DPC was specific for histidine. Direct quantitation of the reaction as described in Materials and Methods indicated that after 30 min in 0.05M DPC a single histidine residue was modified. Reaction for 60 min led to modification of a second histidine residue but this also led to conformational changes. Figure 3.2 shows the fluorescent spectra of native and modified α-toxin. Incubation times sufficient to inactivate the toxin by 90% do not substantially alter conformation. However, upon further incubation (>30 min) conformational changes become evident.

Preincubation of erythrocytes with DPC modified α-toxin results in a concentration dependent protection against the native toxin (Figure 3.3). This indicated that although the binding site of the toxin was unaffected a subsequent event in the lytic pathway was blocked by modification of a single histidine residue.

Electrophoretic Transblot/ELISA of Modified and Native α-Toxin.

Lo and Fackrell (1979) demonstrated that two populations of anti-α-toxin antibodies exist in immune sera from pigs and rabbits. One population prevented the binding of the toxin to erythrocyte membranes (AB-Ab). A second population neutralized α-toxin after it was bound to membranes and caused an indirect hemagglutination (IHA-Ab).

Western blots of native and DPC modified α-toxin using any polyclonal
antibody population failed to detect any differences between the two proteins and the rate of migration in SDS polyacrylamide gels was unaffected (data not shown). As can be seen in Table 3.1 no difference exists between the native and modified toxin when compared in ELISAs using any of the polyclonal populations as well. A number of monoclonal antibodies (MAbs) have been developed against the toxin in this laboratory (Surujballi, 1987 Ph. D. dissertation) which recognize different determinants in the native toxin molecule. In ELISAs performed using the various MAbs the following results were obtained. MAb-3 showed a significant decrease in titre in the modified toxin (30 min modification) as compared to the native toxin (Table 3.2). After longer modification times (60 min) a decrease in the titre of MAb-1 and MAb-2 also became evident as compared to the native toxin. These MAbs were shown by Surujballi (1987, Ph.D. dissertation) to recognize the same determinant. However, as conformational changes were noted in the modified toxin after 30 minutes of modification with DPC results from MAb-1 and MAb-2 are difficult to interpret.

Isolation of a CNBr Fragment Containing the Essential Histidine Residue

MAb-3 was used to construct immunoadsorbant beads using CM Biogel A as described in Materials and Methods. Since MAb-3 recognized differences in the native and modified toxin it was used to isolate a CNBr fragment containing that histidine residue. Native toxin was digested with CNBr and applied to the immunoadsorbent. A western blot of the absorbed digest demonstrated that the isolated fragment was the largest (12000 D) of the five antigenic fragments of α-toxin (Figure 3.4).

A reverse phase HPLC system using a linear gradient of 12% 1-propanol (+0.1% TFA) to 60% 1-propanol (+0.1% TFA) was used to isolate the fragment for further study. As can be seen (Figure 3.5) two peaks were obtained from the isolated
Characterization of the Isolated Histidine Containing CNBr Fragment.

ELISA's were performed on the two peaks using whole anti-α-toxin as well as MAb-3. Both peaks were recognized by the polyclonal Ab and MAb-3 (Table 3.3) indicating that both peaks were probably identical. Percent composition of the two peaks was determined according to the method of Henrickson and Meredith (1984). Both had the same percent composition and differed only by the presence of a single homoserine in peak 1 and a single homoserine lactone in peak 2 (Table 3.4). The production of both homoserine and its lactone derivative as the C-terminal amino acid both occur as a result of CNBr digestion in a 40:60 ratio (Armstrong, 1949) and have been shown to markedly affect the retention times of peptides in reverse phase HPLC systems (Hermodson and Mahoney, 1983). This fragment is unremarkable in terms of its amino acid content. Percent composition of tryptophan could not be determined because it was destroyed by acid hydrolysis. A manual Edman degradation performed on the fragment determined that histidine was the N-terminal amino acid.
Figure 3.1

A) Effect of DPC concentration on hemolysis by α-toxin. α-toxin was incubated with 0 (○), 5mM (●), 2.5mM (▼), and 0.5mM (▲) DPC in 0.1M phosphate buffer, pH 6.5. At times specified 1.5 μg/ml aliquots were removed and the hemolytic activity was determined as described in Materials and Methods. Absorbance was measured at 650 nm. Relative inactivation was determined by comparing maximum rates of lysis.

B) Dependence of kₐ on DPC concentration (determined from linear regression of data from Figure 3.1a).

C) Data from Figure 3.1b plotted in a log-log fashion.
Figure 3.2

Fluorescent spectra of native $\alpha$-toxin (A), DPC modified toxin (B), heat inactivated toxoid (D), and $\alpha$-toxin incubated with 5mM DPC for 60 min (C). Proteins were at a concentration of 1 mg/ml in 0.1 M phosphate buffer, pH 6.5.
Figure 3.3

Protection of erythrocytes against native $\alpha$-toxin by DPC modified toxin.

Line A represents lysis due to $\alpha$-toxin alone (1.5 $\mu$g/ml). Lines B–E represent lysis by $\alpha$-toxin of erythrocytes preincubated with increasing amounts of DPC modified toxin (up to 1.5 $\mu$g/ml).
Table 3.1

Results of an ELISA using polyclonal antisera with native and DPC modified toxin

<table>
<thead>
<tr>
<th>Sample</th>
<th>Whole Sera</th>
<th>AB-Ab</th>
<th>IHA-Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-toxin</td>
<td>0.514±0.12</td>
<td>1.135±0.036</td>
<td>0.321±0.15</td>
</tr>
<tr>
<td>DPC toxin</td>
<td>0.463±0.058</td>
<td>1.083±0.022</td>
<td>0.315±0.20</td>
</tr>
</tbody>
</table>

Differences between α-toxin and DPC modified toxin were determined to be insignificant for all three polyclonal populations tested (n=9, in three independent ELISAs) using the F distribution (p<0.025). Values are means with standard deviation.
Table 3.2

Results of an ELISA using MAbs with native and DPC modified α-toxin

<table>
<thead>
<tr>
<th>Sample</th>
<th>MAb-1</th>
<th>MAb-3</th>
<th>MAb-4</th>
<th>MAb-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-toxin</td>
<td>0.289±0.085</td>
<td>0.125±0.045</td>
<td>0.007±0.004</td>
<td>0.042±0.01</td>
</tr>
<tr>
<td>DPC-toxin</td>
<td>0.276±0.025</td>
<td>0.085±0.031</td>
<td>0.005±0.004</td>
<td>0.045±0.006</td>
</tr>
</tbody>
</table>

Differences between α-toxin and DPC modified toxin were determined to be insignificant for all of the MAbs tested (n=9, in three independent ELISAs) using the F distribution (p<0.025) except MAb-3. MAb-1 and MAb-2 were shown by Surujballi (1987 Ph.D. dissertation) to recognize the same determinant and results for MAb-2 are not shown. Values are means with standard deviation and are measurements of absorbance at 405 nm.
Figure 3.4

Densitometric scarts of western blots performed using a Shimadzu CS-930 Dual Wavelength TLC Scanner. 5–10 \( \mu g \) protein were added per well to 12% SDS polyacrylamide gels. Subsequent blots were incubated with polyclonal anti-\( \alpha \)-toxin.

A) CNBr digest of \( \alpha \)-toxin.

B) CNBr fragments which did not bind to MAb-3 immunoadsorbent.

C) CNBr fragment eluted from MAb-3 immunoadsorbent.

Arrow represents the position of the 12000 D band.
DISTANCE MIGRATED FROM THE TOP OF THE GEL (cm)

RELATIVE ABSORPTION (600nm)
Reverse phase HPLC of MAb-3 fragment. Samples were run as described in *Materials and Methods.*

5 µl sample of the CNBr fragment.

Detector was set at 214 nm with 2 AUFS.
Table 3.3

ELISA on peaks isolated from the HPLC.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Whole Sera</th>
<th>MAb-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1</td>
<td>0.095±0.08</td>
<td>0.057±0.012</td>
</tr>
<tr>
<td>Peak 2</td>
<td>0.062±0.06</td>
<td>0.036±0.006</td>
</tr>
</tbody>
</table>

Absorbances for both peaks were determined to be significant compared to controls (n=5, 2 separate ELISAs) using the F distribution (p<0.025). Values are means with standard deviations.
Table 3.4

Percent Composition of fragment isolated using MAb-3.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Predicted from sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLU/GLN</td>
<td>7.54(8)</td>
<td>6.95(7)</td>
<td>8</td>
</tr>
<tr>
<td>ASP</td>
<td>4.55(5)</td>
<td>5.08(5)</td>
<td>5</td>
</tr>
<tr>
<td>SER/ASN</td>
<td>9.77(10)</td>
<td>8.56(9)</td>
<td>10</td>
</tr>
<tr>
<td>GLY</td>
<td>3.63(4)</td>
<td>3.77(4)</td>
<td>4</td>
</tr>
<tr>
<td>THR</td>
<td>2.77(3)</td>
<td>3.08(3)</td>
<td>3</td>
</tr>
<tr>
<td>ALA</td>
<td>5.88(6)</td>
<td>5.57(6)</td>
<td>0</td>
</tr>
<tr>
<td>PRO</td>
<td>3.75(4)</td>
<td>2.88(3)</td>
<td>3</td>
</tr>
<tr>
<td>HIS</td>
<td>1.0(1)*</td>
<td>1.0(1)</td>
<td>1</td>
</tr>
<tr>
<td>ARG</td>
<td>3.55(4)</td>
<td>2.56(3)</td>
<td>3</td>
</tr>
<tr>
<td>MET/HSER</td>
<td>0.81(1)</td>
<td>0.79(1)</td>
<td>1</td>
</tr>
<tr>
<td>TYR</td>
<td>6.43(6)</td>
<td>5.38(5)</td>
<td>6</td>
</tr>
<tr>
<td>VAL</td>
<td>5.42(5)</td>
<td>5.00(5)</td>
<td>5</td>
</tr>
<tr>
<td>LEU</td>
<td>3.73(4)</td>
<td>3.61(4)</td>
<td>4</td>
</tr>
<tr>
<td>ILE</td>
<td>4.52(5)</td>
<td>4.03(4)</td>
<td>6</td>
</tr>
<tr>
<td>PHE</td>
<td>2.71(3)</td>
<td>2.89(3)</td>
<td>3</td>
</tr>
<tr>
<td>LYS</td>
<td>5.93(6)</td>
<td>6.57(7)</td>
<td>7</td>
</tr>
<tr>
<td>TRP</td>
<td>---(0)</td>
<td>---(0)</td>
<td>1**</td>
</tr>
</tbody>
</table>

Peaks were isolated, hydrolyzed and percent compositions were determined as described in Materials and Methods. Results are averages of five separate determinations. Calculations of amino acid content were performed using integrator coefficients determined from amino acid standards.

*The value for histidine was set arbitrarily to 1.00.
**Tryptophan was destroyed by acid hydrolysis.
Diethylpyrocarbonate (DPC) ostensibly reacts specifically with histidine residues at pH values less than 6.5 (Miles, 1977). However, side reactions are known to occur with cysteine, tyrosine, and lysine as well (Miles, 1977; Atassi, 1977). As there are no cysteiny1 residues in α-toxin this possibility does not exist. The reaction between DPC and lysine is not reversible by treatment with NH$_2$OH, NH$_2$OH restored greater than 75% of the activity of the DPC modified toxin therefore lysyl residues are not modified to any great extent. It has been shown (Miles, 1977; Iglesias and Andreo, 1983) that reaction of DPC with tyrosine results in a decrease in absorbance of the protein at 280 nm. No change in absorbance at 280 nm was detected during DPC modification of α-toxin. A dramatic change in absorbance around 230 nm indicated that histidine was modified and provided the basis for quantitation of the number of modified residues (Miles, 1977).

Active site histidines are found in a number of proteins (Atassi, 1977; Miles, 1977; Iglesias and Andreo, 1983). Modification of a single histidine residue in staphylococcal α-toxin rendered it essentially nonhemolytic. It still possessed the ability to bind to receptors as it protected erythrocytes against the native toxin. Thus, the histidine residue must be involved in events that occur after the toxin binds to target membranes. The events that occur after binding of toxin at present remain unclear. An early event after binding seems to be a conformational change which probably facilitates the shift from monomer to hexamer (Tobkes et al., 1985; Ikigae and Nakae, 1985; Blomqvist and Thelelam, 1986b; Bhakdi and Tranum-Jensen, 1986). Leakage of K$^+$ ions also occurs after the binding step (Madoff et al., 1964; Cassidy and Harshman, 1976a,b). Austin and Fackrell (1984) demonstrated that α-toxin doubled the rate of SO$_4^{2-}$ influx whereas
heat inactivated toxoid had no effect. Thus the binding event itself does not cause changes in ion permeability. At present it is not clear which of these events is affected by histidine modification and this is an area where further study is needed.

A number of monoclonal antibodies developed in this lab (Surujball, 1987 Ph.D. dissertation) were used to compare the native and DPC modified toxin. In samples of the DPC modified toxin containing a single modified histidine residue the only significant difference from the native toxin was seen using MAb-3. Surujball (1987, Ph.D. dissertation) demonstrated that MAb-3 fell into the AB-Ab-class since it failed to demonstrate an IHA titre yet still neutralized the toxin. He also demonstrated that as the toxin molecule is opened up using sequential CNBr digestion the number of both IHA and AB determinants increases from those found in the native toxin. Theoretically, these cryptic determinants could represent determinants which become exposed when α-toxin interacts with target cells and undergoes changes in conformation and tertiary structure. A limitation of the IHA test is that it uses toxoid coated erythrocytes in order to bind antibodies which recognize membrane bound toxin. The toxoid itself does not undergo the necessary conformational change that results in conversion of the monomer to the hexamer as it fails to induce any post binding events (Austin and Fackrell, 1984). It seems probable therefore that some AB-Ab actually recognize determinants which are exposed after the toxin is bound to target cell membranes and successfully undergoes conformational changes which allow its full effect on the cell membrane. A redefinition of the terms AB-Ab and IHA-Ab may become necessary as their respective effects on the toxin become more clearly understood.

The fact that MAb-3 could differentiate between the native and DPC modified toxin made its use as the basis of an immunoassorbent for isolation of the histidine containing fragment feasible.
The gene for α-toxin was sequenced by Gray and Kehoe (1984). The fragment isolated in this study matches up relatively well with a large theoretical CNBr fragment located near the N-terminus of the predicted amino acid sequence (His36-Met112; see Table 3.4). There are some discrepancies but these are well within the norm for α-toxin as there has always been some differences in percent composition (for review see Wiseman, 1975; Gray and Kehoe, 1984). The N-terminal amino acid in both the predicted fragment and the isolated fragment was histidine. Thus the essential histidine residue is histidine 36. This match was confirmed using a BASIC program called ANALYSIS (developed by G. Dombi, BCTIC Computer Code Collection, Vanderbilt University). This program will locate a peptide in a known sequence of amino acids on the basis of its percent composition.

Choy and Fasman (1978) analysis of the predicted fragment assigns a high hydrophobic character to this region of the toxin (Gray and Kehoe, 1984). Thus the fragment is probably located on the inner fold of the toxin as described by Tobkes et al (1985) and probably forms part of the outside of the hydrophobic channel generated by the hexamer when the toxin is flipped inside-out.

Cassidy and Harshman (1976a,b) have described a tyrosine residue which is essential for membrane damage but is not involved in binding. This tyrosine has been localized as tyrosine 29 in a region in the N-terminal CNBr fragment of the toxin (Harshman, personal communication). Digestion with CNBr therefore splits an important active region of the toxin which is intimately involved in membrane damaging events which occur after binding to target cell receptors. Other groups (Kato and Watanabe, 1980; Watanabe and Kato, 1978; Blomqvist et al, 1987a) have also demonstrated that the membrane damaging region of α-toxin is located in the N-terminal domain.

Studies designed to elucidate the precise effect of modification of histidine 36
on α-toxin mediated hemolysis are in progress.
Overview
The main objective of this study was to relate structure to the function of staphylococcal α-toxin at the level of individual amino acids. Arginine residues were chosen initially because of their positive charge in light of the fact that the receptor for α-toxin is negatively charged (Simpson and Packrell, in prep). Modification of a single arginine residue with two independent arginine-specific reagents, 1,2-cyclohexanediol and phenylglyoxal, resulted in complete inhibition of the hemolytic and cytotoxic activities of α-toxin. Further, the modified toxin did not protect erythrocytes or cultured cells against the effects of the native toxin indicating that the binding site was affected by arginine modification.

Arginine residues have been found in ligand recognition sites, and especially anion binding sites of a large number of proteins (Riordan, 1979; Patthy and Thesz, 1980).

Trypsin digestion of CHD modified α-toxin led to the generation of a 20 kDa fragment which could be isolated using a CHD-arginine specific affinity system (Patthy et al, 1979). This fragment reacted with IHA-Ab but not with AB-Ab, in contrast to a 20 kDa fragment generated by limited trypsin digestion of the native toxin which is recognized by both polyclonal populations (Surujballi, 1987 Ph.D. dissertation). This implies that the fragments are not identical but do overlap. It is possible that the CHD masks an early site of cleavage by trypsin or somehow alters the site selectivity of the enzyme for α-toxin.

Subsequent CNBr digestion of the CHD modified α-toxin localized the modified arginine to a 9000 D fragment. This confirms earlier work by Surujballi (1987, Ph.D. dissertation) in which a 9000 D fragment was adsorbed from a digest
using erythrocyte ghosts implying that the binding site was located on this fragment. That study as well as this one determined that the CNBr fragment reacted with both polyclonal Ab populations but not with any of the monoclonal antibodies developed against α-toxin in this lab. It thus also overlaps with the 20 kD fragment generated by trypsin treatment of the CHD modified α-toxin.

Amino acid analysis of the 9000 D fragment localized the binding site to a short region in the C-terminal domain of the toxin (Trp175-Met197). This confirms previous studies (Watanabe and Kato, 1978; Kato and Watanabe, 1980; Blomqvist et al 1987a) which have localized the binding site to the C-terminal domain of the toxin. The functional arginine residue is Arg 184.

Only a small number of histidine residues (for review see Wiseman, 1975; Gray and Kehoe, 1984) are found in α-toxin. They have been known to play functional roles in a number of proteins (Atassi, 1977; Miles, 1977; Iglesias and Andreo, 1983) and thus became a good choice for further modification studies. Modification of a single histidine residue with diethylypyrocarbonate (DPC) resulted again in complete inhibition of the hemolytic activity of the toxin. However, in this case the modified toxin protected erythrocytes against subsequent exposure to the native toxin. Thus the histidine residue was functionally important in events that occur after the toxin binds to its membrane receptor. Precisely which events are affected by modification are at present unclear and are certainly worthy of further study. In particular the effects of DPC modification on K⁺ release and swelling of α-toxin treated erythrocytes could be easily measured. Swelling of erythrocytes by α-toxin has been shown to occur following the initial lag phase in which the toxin is binding to target membranes (Austin and Fackrell, unpublished observation). In addition it would be interesting to determine if modification of the histidine residue abrogates the stimulatory effect of specific Band 3 modifying sulfhydryl reagents such as PCMBS
(Fackrell and Austin, 1985; Austin et al., in prep) on hemolysis. Another initial event in α-toxin induced membrane damage is the collapse of the transmembrane potential gradient in nucleated cells (Bashford et al., 1985). It would be interesting to see if DPC modification affects the ability of the toxin to disrupt the transmembrane potential.

The modification of α-toxin by DPC did not impair recognition by either polyclonal population of anti-α-toxin (i.e., IHA-Ab or AB-Ab). However, one of the monoclonal antibodies (MAb-3) had a lower titre for the modified toxin as compared to the native toxin. This led to the construction of an immunoaffinity system and subsequent isolation of a 12 kDa CNBr fragment containing the essential histidine residue.

Amino acid analysis of the isolated fragment localized it to a region near the N-terminus of α-toxin (His36-Met112) with the essential histidine as the N-terminal amino acid. This confirms data presented by other researchers (Harshman, personal communication; Blomqvist and Thelestam, 1986a; Blomqvist et al., 1987a) who also demonstrated that the membrane damaging region of α-toxin is located in the N-terminal domain.

α-toxin has been shown to bind a carbohydrate receptor in at least two distinct cell types (Simpson and Fackrell, in prep; Tang et al., 1987) and interestingly α-toxin bears an overall gross structural homology to a number of carbohydrate binding proteins. These proteins consist of two domains of β-sheet structure surrounding a central groove (Quiocho, 1986). α-toxin also consists of two domains of antiparallel β-sheet structure separated by a flexible hinge region (Tobkes et al., 1985). A search of a sequence database
(MicroGenie) finds little sequence homology between α-toxin and known carbohydrate binding proteins. However, a more thorough search seems necessary as well as comparison at the amino acid level rather than DNA bases as known carbohydrate binding proteins often differ in sequence but show a conserved domain structure and similar percent compositions (Quirocho, 1986). α-toxin did bear sequence homology to four envelope proteins from the AIDS virus but what this means is completely unclear.

An interesting observation noted during the course of this project was that certain treatments designed to elucidate the nature of the α-toxin receptor (Maharaj and Fackrell, 1980) also affected the as yet uncharacterized diptheriae toxin receptor. The diptheriae toxin receptor is sensitive to pronase as is the α-toxin receptor, Band 3. Treatment of susceptible cells with phospholipase C reduces the effects of both α-toxin and diptheriae toxin (Elias and Kofer, 1980, Olsnes et al, 1984). Also an anion transport protein in VERO cells (which are susceptible to both toxins) has been shown to be important in the binding and subsequent internalization of diptheriae toxin (Sandvig and Olsnes, 1986a). This anion transport protein is kinetically similar to Band 3 and susceptible to the same inhibitors (Sandvig and Olsnes, 1986b). Anion transport inhibitors do not inhibit α-toxin but do prevent internalization of the A fragment of diptheriae toxin.

Preliminary experiments in our lab using diptheriae toxoid have shown that preincubation of rabbit erythrocytes with diptheriae protects them against α-toxin (Hebert, unpublished observations). It would be intriguing to continue this project in both VERO cells and rabbit erythrocytes. Preliminary evidence as reviewed by Eidels et al (1983) has shown that the receptor for diptheriae toxin is a glycoprotein but may reside in the protein moiety rather than the carbohydrate which
is the case for \(\alpha\)-toxin (Simpson and Fackrell, in prep).

Finally, on the basis of structure-function studies performed in this lab as well as many others, a preliminary structural map of important functional and antigenic regions of \(\alpha\)-toxin is presented (Figure 4.1). The N-terminal region contains the membrane-damaging (cytotoxic and lethal) regions of the toxin (this study; Blomqvist et al., 1987a; Cassidy and Harshman, 1976a,b; Harshman, personal communication).

Blomqvist and Thelestam (1986a) have isolated an 18.5 kD fragment of \(\alpha\)-toxin corresponding to the C-terminal domain which possessed the full hemolytic activity of the intact toxin. This study has demonstrated that a histidine residue (His 36) located near the N-terminus of the toxin is required for hemolytic activity in the native toxin. Cassidy and Harshman (1976a,b; Harshman, personal communication) have also shown the N-terminal region (specifically Tyr 29 in the N-terminal CNBr fragment) to be involved in hemolytic activity. This leads to the conclusion that perhaps the N-terminal region of the toxin is required for the full hemolytic activity of the toxin or that \(\alpha\)-toxin is acting through more than one mechanism on the rabbit erythrocyte.

In the central region of the toxin is the proposed hinge region about which the toxin undergoes a conformational change during its interaction with target cell membranes (Tobkes et al., 1985). In the C-terminal region is the binding site including an essential arginine residue (Arg 184) as well as regions necessary for full hemolytic activity (this study, Blomqvist and Thelestam, 1986a). Regions reacting with various polyclonal and monoclonal antibody preparations used in structure-function studies are also noted (this study; Surujballi, 1987 Ph.D. dissertation; Harshman et al., 1986; Blomqvist et al., personal communication).

An obvious next step toward a final characterization of the mechanism of action
of $\alpha$-toxin is to apply the techniques of modern molecular biology to the problem. Specific site directed mutagenesis now that the gene has been cloned and sequenced (Kehoe et al., 1983; Fairweather et al., 1983; Gray and Kehoe, 1984; O'Reilly et al., 1986) should allow a more complete picture of how alpha toxin damages cell membranes.
Figure 4.1

Structure-Function map of Staphylococcal α-toxin. Regions known to be recognized by IHA-Ab are represented by lines from hollow arrows (♂). Regions known to be recognized by AB-Ab are represented by lines from solid arrows (♀).
Staphylococcal Alpha Toxin

$M_r = 33,182$

**Nucleotide Number**

410 510 610 710 810 910 1010 1110 1210

**CNBr Peptide**

IV  III  I  VI  V  II  VII

**N-Terminal Region - Cytotoxic and Lethal Domain**

(Watanabe and Kato, 1980; Blomqvist et al, 1987)

CNBr III also Involved in Hemolytic Activity (This Study)

Recognized by Monoclonal Antibody Developed by Harshman et al (1988)

**C-Terminal Region - Binding Site and Hemolytic Domain**

(Watanabe and Kato, 1980; Blomqvist et al, 1987) (Residues 175-197) Binding Site of the Toxin (This Study)

Proposed Hinge Region (Residues 119-143) (Tobkes et al, 1986)
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1981–1985
BSc. (Biological Sciences), University of Windsor

1976–1981
Honours High School Diploma
W.F. Herman C.I., Windsor, Ontario

1967–1976
Attended Holy Family Elementary School, Windsor, Ontario

ACADEMIC AWARDS

1987–1988 NSERC Postgraduate Scholarship

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1984 NSERC Undergraduate Scholarship

1981–1985 University of Windsor Entrance Scholarship

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UNIVERSITY SERVICE

1984–1985 4th Year Representative to Departmental Council (Biology); Student Representative on Promotion and Tenure Committee (Biology)

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EXPERIENCE

1984 – Present Teaching Assistant in the Department of Biological Sciences for the following courses:
Basic Ecology
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