IDENTIFYING DOMAINS OF SHIGA-LIKE TOXIN I THAT ARE RESPONSIBLE FOR ITS MEMBRANE TRANSLOCATION

by

Mazen T. Saleh

A Thesis Submitted in Conformity with the Requirements for the Degree of Doctor of Philosophy, Graduate Department of Medical Biophysics, in the University of Toronto

©copyright by Mazen T. Saleh, 1997
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.
ABSTRACT

Identifying domains of shiga-like toxin I that are responsible for its membrane translocation

Mazen T. Saleh
Doctor of Philosophy, 1997, Graduate Department of Medical Biophysics, in the University of Toronto

Shiga-like toxin I (SLT-I), produced by pathogenic strains of Escherichia coli, is a representative member of a superfamily of multimeric protein toxins known as the A/B family of toxins. SLT-I is internalized by cells via receptor-mediated endocytosis and localizes in various intracellular compartments. However, targets of these toxins are invariably located in the cytoplasm, thus internalized SLT-I must translocate across a vesicular membrane in order to deliver its cytotoxic function.

Earlier studies have revealed that toxins may exploit conditions in intracellular environments to facilitate translocation. For example, acidic pH of endocytic vesicles might allow exposure of hydrophobic domains that associate with membranes, a necessary initial step in membrane translocation. SLT-I was used to determine if this proposed mechanism was a general feature of all members of this family of toxins in terms of their mode of membrane insertion and translocation.

Fluorescence spectroscopy has revealed that Trp-34 in SLT-I B subunit is perturbed at pH levels normally found in endocytic and lysosomal vesicles. This effect was further characterized using circular dichroism, solid-phase receptor binding assays and gel permeation chromatography. The results support the conclusion that the conformational
change observed with fluorescence studies is a local structural effect and that the secondary and tertiary structures of the B subunit remain unaltered during a pH transition between pH 7 and pH 4. Moreover, studies carried out with the holotoxin indicated that the structure of the toxin remains largely unaffected by acidic pH in relation to neutral pH.

The effects of other intracellular microenvironments on SLT-I translocation were investigated. Hydropathy profiles of the A subunits of SLT-I revealed the presence of a hydrophobic domain between residues 220 and 246 flanked by positively charged amino acids that resembles the composition of signal sequences and transmembrane domains of integral membrane proteins. Studies with synthetic vesicles containing acidic lipids, such as phosphatidyl glycerol, have shown that synthetic peptides corresponding to this region of the A1 chain, can spontaneously insert into membrane vesicles. Our hypothesis is that this region may function in a similar way within the intact A1 chain once localized in the ER.

It is therefore concluded that acidic pH alone is not capable of eliciting a structural change in internalized SLT-I. Rather, other intracellular processes, such as proteolytic processing and disulfide reduction, may be involved in exposing the hydrophobic C-terminus of the A1 chain. The exposed domain may then insert into the lumenal side of the endoplasmic reticulum membrane as an initial step in membrane translocation. Furthermore, factors endogenous to the ER, such as those involved in signal-sequence processing, protein folding and degradation, may play a role in the translocation of the A chain of SLT-I across the ER membrane.
ACKNOWLEDGMENTS

This body of work is the result of the collective efforts of many individuals. I am grateful for the efforts and contributions of my supervisor, Dr. Jean Gariépy. The present frame of my scientific thinking was moulded by his relentless pursuit of excellence in research. His patience provided me with the independence in the laboratory that I needed and his guidance allowed me to successfully complete my work.

I also wish to thank members of my student supervisory committee, Dr. David Rose and Dr. Cheryl Arrowsmith for their concise encouragement, support and helpful discussions; my past and present partners in the laboratory for making my stay stimulating and pleasant both at work and during evening discussions in bars. Without their efforts, I would have probably saved more money and my liver would be in a better state of health. They are: Beth Boyd, Bruce Carpick, Jim Ferguson, Francesca Bahr, Rajiv Ayra, Deming Liu, Kate Sheldon, Eric LaCasse, Mark Bray, Devender Singh, Reza Kiarasch, Wai-May Lim, Stuart Bisland, Kim Kawamura, Ray Reilly and Nancy Stokoe, and a special thanks to John Simard.

I also take this opportunity to thank our collaborators, Dr. Joan Boggs and Godha Rangaraj (University of Toronto), for the EPR work presented in chapter 4; Dr. Robert Hodges and Mr. Lorne Burke (Department of Biochemistry, University of Alberta) for performing mass spectrometry on the purified peptides and, Dr. Avi Chakrabartty for his help and access to his circular dichroism spectrometer and fluorometer. Finally, I would like to acknowledge Ms. Tanuja Chitnis and Dr. David Isenman for their help in the early stages of the work presented in chapter 1 as well as Drs. Penelope Stein and Randall Read for providing us with the crystal coordinates for the B subunit of Shiga-like toxin I.
TABLE OF CONTENTS

List of Tables .................................................. x
List of Figures .................................................. xi
List of Abbreviations .......................................... xv

CHAPTER 1 .............................................................................. 1
Introduction ........................................................................... 2

1.1 The Role of Shiga-like Toxins in Disease .................................................. 4

1.2 The Structure of Shiga-like Toxin I .................................................. 7

1.2.1 The Structure and Function of Shiga-like Toxin I A subunit ....... 8

1.2.2 The Structure of Shiga-like Toxin I B subunit .................................. 10

1.3 The Receptor of Shiga-like Toxin I .................................................. 15

1.4 Receptor-Mediated Endocytosis of Bound Toxin .................................. 18

1.5 Structural and Functional Homologies with other Toxins ....................... 20

1.6 Goals and Objectives .................................................. 23

CHAPTER 2 .............................................................................. 25

Experimental Methods

2.1 Biochemical Methods

2.1.1 Purification of SLT-I ............................................................. 26

2.1.2 Purification of SLT-I B subunit ............................................. 27

2.1.3 Purification of the synthetic peptides ......................................... 30

2.1.4 Electroelution of SLT-I A subunit ............................................ 31

2.1.5 Radiolabeling of SLT-I and SLT-I B subunit ................................ 37

2.1.6 Gb3 solid phase binding assay ............................................. 37
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.7</td>
<td>Spin-labeling of ShTA(220-246)</td>
<td>38</td>
</tr>
<tr>
<td>2.1.8</td>
<td>Preparation of lipid vesicles</td>
<td>38</td>
</tr>
<tr>
<td>2.1.9</td>
<td>Binding of radiolabeled peptide to lipid vesicles</td>
<td>39</td>
</tr>
<tr>
<td>2.1.10</td>
<td>Total Phosphorus Assay</td>
<td>39</td>
</tr>
<tr>
<td>2.1.11</td>
<td>Amino acid analysis</td>
<td>40</td>
</tr>
<tr>
<td>2.1.1</td>
<td>Amino acid analysis</td>
<td>41</td>
</tr>
<tr>
<td>2.2</td>
<td>Characterization of Purified SLT-I and its B subunit</td>
<td>42</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Polyacrylamide gel electrophoresis</td>
<td>42</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Western blot analysis</td>
<td>42</td>
</tr>
<tr>
<td>2.2.3</td>
<td>Cytotoxicity assay</td>
<td>45</td>
</tr>
<tr>
<td>2.2.4</td>
<td>Cell-free protein synthesis inhibition assay</td>
<td>49</td>
</tr>
<tr>
<td>2.2.5</td>
<td>Gel permeation chromatography</td>
<td>50</td>
</tr>
<tr>
<td>2.2.6</td>
<td>Calculation of the molar absorption coefficient of SLT-I</td>
<td>50</td>
</tr>
<tr>
<td>2.3</td>
<td>Immunological Methods</td>
<td>51</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Generation of rabbit Anti-SLT-I A subunit antisera</td>
<td>51</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Detection of rabbit anti-SLT-I antibodies by ELISA</td>
<td>51</td>
</tr>
<tr>
<td>2.4</td>
<td>Biophysical Methods</td>
<td>53</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Fluorescence Spectroscopy</td>
<td>54</td>
</tr>
<tr>
<td>2.4.2</td>
<td>Circular Dichroism</td>
<td>55</td>
</tr>
<tr>
<td>2.4.3</td>
<td>EPR measurements</td>
<td>55</td>
</tr>
</tbody>
</table>

**CHAPTER 3**

Local Conformational Change in the B subunit of Shiga-like Toxin I at Endosomal pH

3.1 Introduction ................................................................. 58

3.2 Results and Discussion .................................................. 59
3.2.1 The fluorescence of tryptophan-34 is perturbed at endosomal pH ..................................................... 59
3.2.2 The structure of the B-subunit is gradually altered by pH conditions ranging from pH 2 to 4.5 ......................... 61
3.2.3 The tertiary and quaternary structures of the B subunit pentamer are stable between pH 2 and 10 ...................... 63

CHAPTER 4 69
Effect of Endosomal pH on the Conformation of the Holotoxin
4.1 Introduction .......................................................................................................................... 70
4.2 Results and Discussion
  4.2.1 Acidic pH alters the microenvironment of tryptophan(s).............. 73
  4.2.2 Acidic pH does not alter the exposure of tryptophans in SLT-I ................................................................. 76
  4.2.3 pH does not alter the secondary structure of SLT-I .................. 78

CHAPTER 5 82
Insertion and Orientation of a Synthetic Peptide Representing the Shiga Toxin A\textsubscript{1} C-terminus into Phospholipid Membranes
5.1 Introduction .......................................................................................................................... 83
5.2 Objectives ............................................................................................................................ 84
5.3 Results and Discussion
  5.3.1 ShTA(220-246) adopts a partially helical structure in the presence of negatively charged lipid vesicles .............. 87

vii
5.3.2 Changes in the fluorescence spectrum of W232A242ShT(220-246) in the presence of lipid vesicles suggest that this hydrophobic region of the A₁ domain partitions readily into a lipid bilayer ....... 90

5.3.3 Binding of radiolabeled W232A242ShT(220-246) to lipid vesicles ................................................................. 94

5.3.4 Monitoring the interaction of ShTA(220-246) with membranes by EPR ................................................................. 94

5.3.5 ShTA(220-246) causes leakage of calcein trapped inside DMPG vesicles ................................................................. 100

5.3.6 A model summarizing the interaction of ShTA(220-246) with a negatively charged lipid bilayer ........................................ 102

CHAPTER 6

Concluding Remarks

6.1 Does the B subunit play a role in A chain translocation? ................. 107

6.2 Domains analogous to ShTA(220-246) are present on other proteins ...... 108

6.3 Does ShTA(220-246) and the A₁ chain interact with component(s) of a protein transporter located in the ER membrane? ................. 109

6.4 The involvement of the ubiquitin-proteosome pathway ...................... 109

6.5 A hypothetical model describing membrane insertion and translocation of the SLT-1 A₁ fragment ........................................ 110

6.6 Future Directions ..................................................................... 112

REFERENCES .............................................................................. 114

viii
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Relationship between members of the family of Shiga toxins</td>
<td>5</td>
</tr>
<tr>
<td>2.1</td>
<td>Amino acid analysis of ShTA(220-246) and W^{232A242}ShT(220-246)</td>
<td>36</td>
</tr>
<tr>
<td>2.2</td>
<td>Amino acid analysis of SLT-I and its B subunit</td>
<td>48</td>
</tr>
<tr>
<td>4.1</td>
<td>Effect of endosomal pH on the gel permeation properties of SLT-I and its B subunit</td>
<td>80</td>
</tr>
<tr>
<td>5.1</td>
<td>EPR parameters derived from the spectra of spin-labelled ShTA(220-246)</td>
<td>97</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

CHAPTER 1

Figure 1.1 Distribution of 12.9 million deaths among children under five years old in all developing countries .................................. 3

Figure 1.2 Sequence alignment of A subunits from SLT-I and related toxins... 9

Figure 1.3 Proposed mechanism of the enzymatic A subunit of SLT-I ....... 11

Figure 1.4 Structure of Shiga-like toxin I B subunit ............................ 13

Figure 1.5 Various representations of the X-ray crystallography structure of Shiga toxin .................................................. 14

Figure 1.6 A model of the structures of Gb$_3$ and Gb$_4$ ........................ 16

Figure 1.7 Schematic representation of the receptor-mediated endocytosis of bound SLT-I ................................................. 19

CHAPTER 2

Figure 2.1 Typical chromatography profiles observed during the purification of SLT-I .................................................................. 28

Figure 2.2 Typical chromatography profiles observed during the purification of SLT-I B subunit .............................................. 29

Figure 2.3 Reverse-phase purification profile of ShTA(220-246) ............. 32

Figure 2.4 Reverse-phase purification profile of W$^{232}$A$^{242}$ShTA(220-246) ................................................................. 33

Figure 2.5 Mass spectrum of ShTA(220-246) ..................................... 34

Figure 2.6 Mass spectrum of W$^{232}$A$^{242}$ShTA(220-246) ................... 35

Figure 2.7 Electrophoretic mobility of purified SLT-I .......................... 43
| Figure 2.8 | Electrophoretic mobility of purified SLT-I B subunit | 44 |
| Figure 2.9 | A typical curve obtained in the Vero cell cytotoxicity assay | 46 |
| Figure 2.10 | A typical curve obtained in the cell-free protein synthesis inhibition assay | 47 |
| Figure 2.11 | Calibration of the gel permeation column | 49 |
| Figure 2.12 | The absorption gradient of SLT-I | 50 |

**CHAPTER 3**

| Figure 3.1 | Effect of pH on the tryptophan fluorescence intensity of the B subunit of Shiga-like toxin I | 60 |
| Figure 3.2 | Stereoprotjections of the B subunit pentamer of Shiga-like toxin I | 62 |
| Figure 3.3 | Effect of pH on the circular dichroism spectrum of the B subunit of Shiga-like toxin I | 64 |
| Figure 3.4 | Effect of pH on ellipticity values at 222 nm for the SLT-I B subunit | 65 |
| Figure 3.5 | Histogram depicting the effect of pH on the specific binding of radioiodinated Shiga-like toxin I B subunit to its natural receptor Gb3 | 68 |

**CHAPTER 4**

| Figure 4.1 | Various representations of the structure of SLT-I highlighting the location of tryptophans | 72 |
| Figure 4.2 | Tryptophan fluorescence emission spectra of SLT-I | xi |
and its B subunit ........................................................................ 74

**Figure 4.3** Change in tryptophan fluorescence of SLT-I as a function of pH ................................................................. 75

**Figure 4.4** Acrylamide quenching of Trp fluorescence in SLT-I and its B subunit at neutral and at acidic pH ....................... 77

**Figure 4.5** CD spectra of SLT-I in various pH conditions ............ 79

**CHAPTER 5**

**Figure 5.1** Hydrophobicity profile of the A subunit of Shiga toxin .......... 86

**Figure 5.2** Circular dichroism spectrum of the synthetic peptide ShTA(220-246) ................................................................. 88

**Figure 5.3** Circular dichroism spectra of the peptide ShTA(220-246) in the presence of small unilamellar vesicles composed of DMPG lipids ......................................................... 89

**Figure 5.4** Circular dichroism spectra of the peptide ShTA(220-246) in the presence of small unilamellar vesicles composed of DMPC lipids ......................................................... 91

**Figure 5.5** Effect of lipid concentration and composition on the tryptophan fluorescence emission spectra of the peptide W232A242ShTA(220-246) ................................................................. 93

**Figure 5.6** Effect of DMPG vesicles and ascorbic acid on the EPR spectrum of proxyl-labeled ShTA(220-246) ...................... 96

**Figure 5.7** Reduction of only the immobilized EPR spectral component by entrapped ascorbic acid ................................. 98
Figure 5.8 Peptide-induced release of calcein from DMPG vesicles as a function of time and peptide-to-lipid ratio .......... 101

Figure 5.9 A schematic model depicting possible modes of interaction of peptide ShTA(220-246) with the lipid bilayer of DMPG vesicles .......................................................... 103

CHAPTER 6

Figure 6.1 A schematic diagram depicting the proposed mechanism of membrane translocation by SLT-I ........................................... 111
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARI</td>
<td>Acute Respiratory Infection</td>
</tr>
<tr>
<td>Bip/GRP94/HSP47</td>
<td>Members of the family of heat shock proteins</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism spectroscopy</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera Toxin</td>
</tr>
<tr>
<td>DEAE-sepharose</td>
<td>Diethyl aminoethyl-sepharose, anion exchange resin</td>
</tr>
<tr>
<td>DMPG</td>
<td>Dimyristoyl-L-α-phosphatidyl-dl-glycerol</td>
</tr>
<tr>
<td>DMPC</td>
<td>Dimyristoyl-L-α-phosphatidyl choline</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria Toxin</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid, cation chelating agent</td>
</tr>
<tr>
<td>EHEC</td>
<td>Enterohemorrhagic Escherichia coli</td>
</tr>
<tr>
<td>EIEC</td>
<td>Enteroinvasive Escherichia coli</td>
</tr>
<tr>
<td>EPEC</td>
<td>Enteropathogenic Escherichia coli</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance spectroscopy</td>
</tr>
<tr>
<td>ETEC</td>
<td>Enterotoxigenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>Gb₃</td>
<td>Globotriasyl ceramide</td>
</tr>
<tr>
<td>Gb₄</td>
<td>Globotetraasyl ceramide</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GO</td>
<td>Golgi Apparatus</td>
</tr>
<tr>
<td>HC</td>
<td>Hemorrhagic Colitis</td>
</tr>
</tbody>
</table>
HPLC  High performance liquid chromatography
HUS  Hemorrhagic Uremic Syndrome
K_{s,\text{(eff)}}  Effective Stern-Volmer quenching coefficient
LT  *E. coli* heat-labile enterotoxin
MRE  Mean residue ellipticity
PMSF  Phenyl methyl sulfonyl fluoride, protease inhibitor
PT  Pertussis Toxin
PVDF  Polyvinylidene difluoride
RT  Ricin Toxin
SDS-PAGE  Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sec61p  Subunit of the ER protein translocation channel
ShT  Shiga Toxin produced by *Shigella dysenteriae* type 1
ShTA(220-246)  Residues 220-246 of the A subunit of Shiga toxin
SLT  Shiga-like Toxin(s) produced by *E. coli*
SUV  Small unilamellar vesicles
T_{max}  Hyperfine splitting parameter
\tau_0  Motional parameter
TFE  Trifluoroethanol
VT  Verotoxin, synonornous with SLT
VTEC  Verotoxin-producing *Escherichia coli*
W^{23}_{24}A^{24}_{24}ShTA(220-246)  Residues 220-246 of the A subunit of Shiga toxin with substitutions, I232W and C242A
WHO  World Health Organization
CHAPTER 1

INTRODUCTION
CHAPTER 1

INTRODUCTION

The World Health Organization (WHO) has recently reported that 25% of infant deaths in third world countries is a consequence of diarrhea (Fig. 1.1; WHO, 1995). Diarrheagenic strains of *E. coli* represent a major culprit in the incidence of such diseases. These strains can be classified into four main groups; enterotoxigenic, enteroinvasive, enteropathogenic and enterohemorrhagic (Karmali et al., 1989). Enterotoxigenic *E. coli* (ETEC) strains represent the major cause of diarrhea, including travellers’ diarrhea, in third world countries. Members of this group produce one or both of a cholera toxin-like heat-labile enterotoxin (LT) and a heat-stable enterotoxin (ST). Enteroinvasive *E. coli* (EIEC) strains cause severe diarrhea that resembles dysentery caused by *Shigella dysentery* type 1. Enteropathogenic *E. coli* (EPEC) strains include specific *E. coli* serotypes that are associated with infantile diarrhea and can be shown to adhere to Hep-2 cells *in vitro* (Cravioto et al., 1979), a property not seen with non-pathogenic *E. coli* or other classes of pathogenic *E. coli* (Karmali et al., 1989). Enterohemorrhagic *E. coli* (EHEC) strains include verotoxin-producing *E. coli* (VTEC) and is characterized by the production of one or more verotoxins or Shiga-like toxins. EHEC strains are associated with episodes of hemorrhagic colitis and the production of characteristic intestinal mucosal lesions (Tzipori et al., 1988; Karmali et al., 1989; Gyles, C. L., 1992).

Shiga-like toxin I (SLT-I) represents one example of a large family of bacterial and plant toxins that share common structural and functional properties, including the targeting of specific surface receptors of eukaryotic cells. Members of this family of toxins are multi-subunit polypeptides ranging in size from 70 kDa to 170 kDa. Cytotoxicity occurs as a result of the internalization of the receptor-bound toxin and the subsequent translocation of
**Figure 1.1** Distribution of 12.9 million deaths among children under five years old in all developing countries. ARI; acute respiratory infection. Reproduced from the World Health Organization (WHO) statistics annual, 1996.
the catalytic subunit of the toxin to the cytosol. My objective was to investigate the mechanisms by which SLT-I enters into cells and translocates across intracellular membranes in order to inactivate its intracellular target, the ribosome, located in the cytoplasm.

1.1 The role of Shiga-like toxins in disease

Patients that developed hemorrhagic colitis during outbreaks of this disease in the early 1980's in North America were found to have consumed contaminated beef patties or meat products from fast food restaurants. The disease was termed “hamburger disease” (Riley et al., 1983; Wells et al., 1983). Clinical isolates collected from these patients revealed the presence of a rare toxin-producing *E. coli* serotype known as 0157:H7 (Riley et al., 1983; Wells et al., 1983). Various laboratories later reported the isolation of related *E. coli* serotypes that produce a family of closely-related toxins (Table 1.1).

Patients infected with toxin-producing *Shigella* or *E. coli* strains may exhibit life threatening symptoms such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) resulting from damage to the colon and kidney, respectively (Riley et al., 1983; Karmali et al., 1983; for a review see Karmali, M. A., 1989). Toxin-producing *E. coli* strains normally express more than one type of shiga-like toxins (SLT-I and SLT-II). The majority of patients exhibit immunological responses (primarily production of antibodies of class IgA) against SLT-I and not against SLT-II (Ashkenazi et al., 1988). Although a mechanism by which active toxin gains entry into the blood circulation has not been identified, the involvement of the toxins in such afflictions is supported by the presence of neutralizing antibodies in infected patients (Karmali et al., 1985). Tesh et al. (1993) have recently demonstrated that a 50% lethal dose (LD₅₀) for SLT-II in mice was 400 times less than that for SLT-I when injected intravenously or intraperitoneally. The authors did not detect any differences in the enzymatic activity of SLT-I versus SLT-II and proposed that the
difference in lethality could be due to differences in toxin stability and/or receptor affinity.

**Table 1.1** The relationship between members of the family of Shiga toxins. The data was compiled and presented according to the proposal of O'Brien et al. (1994) for rationalizing the nomenclature of the *E. coli* cytotoxins. The toxin classification is based on (a) serological specificity (b) host organism and (c) biological activity (cytotoxicity). As shown in the table, SLT-I and SLT-II are serologically different from other related toxins. SLT-IIc and SLT-IIe differ in terms of receptor binding (SLT-IIe binds to Gb₄ instead of Gb₃) and in the nature of the host organism (O’Brien et al., 1994; Jackson, M. P., 1990; Jackson & O’Brien, 1994).

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Source/Strain</th>
<th>Host</th>
<th>Cytotoxicity</th>
<th>Neutralization by Antisera against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vero</td>
<td>Hela</td>
</tr>
<tr>
<td>ShT</td>
<td><em>S. dysenteriae</em></td>
<td>human</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SLT-I</td>
<td><em>E. coli/C600</em> (H19,H7,H30)</td>
<td>human</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SLT-II</td>
<td><em>E.coli/C600</em> (933W)</td>
<td>human</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SLT-IIc</td>
<td><em>E.coli</em> B2F1, E32511</td>
<td>human</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>SLT-IIe</td>
<td><em>E. coli</em> S1191, 412</td>
<td>Pig</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

The involvement of toxin in disease was further supported by the findings of Fontaine et al. (1988), where infection of macaque monkeys with a toxin-deficient strain of *Shigella* was compared with that of a wild type *Shigella* strain. The study showed that toxin production was associated with bloody stools, colonic vascular damage and intestinal inflammatory response. Other evidence indicate that the primary target of the toxins is the vascular endothelium (Kavi et al., 1987; Obrig et al., 1988) and colonic epithelial cells.
(Moyer et al., 1987). Later studies (Wadolkowski et al., 1990a; Wadolkowski et al., 1990b) have demonstrated the presence of toxin receptors in mouse kidneys and that renal damage is the ultimate cause of death in mice challenged with purified toxin. Toxin receptors have now been identified in several tissues including endothelial cells lining blood vessels and red blood cells (Richardson et al., 1988; Bitzan et al., 1994). In rabbits, a challenge with purified SLT-I resulted in anorexia, lethargia and limp paralysis with no effect on renal function (Zoja et al., 1992). The authors have shown that the lesions observed correlated with the tissue distribution of Gb₃, a glycolipid identified as the receptor for ShT and SLT-I. There was no Gb₃ present in the heart, liver or the kidney of rabbits. Tissues having the highest Gb₃ content were the tissues of the central nervous system, gastrointestinal tract, lung and spleen.

Once in the circulatory system, the toxin may target cells of the immune system. Activated B-cells, in particular IgG-committed subsets, are sensitive to SLT-I in vitro (Cohen et al., 1990). The deletion of B cells subsets may explain the relative lack of antitoxin IgG responses in patients infected with toxin-producing E. coli strains (Lingwood et al., 1994). The role of SLT-I in disease was also investigated in rabbits using the pathogenic E. coli strain, RDEC-1. An isogenic form of this strain called RDEC-H19A, was produced by infecting RDEC-1 with an SLT-I-converting bacteriophage. The resulting strain produces a disease in rabbits with symptoms similar to those seen in humans infected with toxin-producing E. coli strains (Sjogren et al., 1994). The intravenous injection of purified SLT-IIe into pigs can reproduce the symptoms of pig edema disease, an illness arising as a result of infections with SLT-IIe-producing E. coli strains (Macleod et al., 1991). Investigation of toxicity in pigs caused by SLT-IIe revealed damage to the stomach, the eyelids, the cerebellum and the colon (Gyles, C. L., 1992). SLT-producing E. coli strains have also been isolated from cats with aggressive diarrhea (Abaas et al., 1989).
1.2 Structure of SLT-I

Shiga and Shiga-like toxins (verotoxins) are closely related oligomeric protein toxins. Shiga toxin, produced by *Shigella dysenteriae* (Shiga et al., 1897) has been known for a century. In the late seventies, a new toxin was isolated from pathogenic strains of *E. coli* that exhibited cross reactivity with antisera raised against Shiga toxin (O'Brien et al., 1978). This new toxin was termed Shiga-like toxin (SLT). Independent studies by Konowalchuk and his coworkers also identified a cytotoxin produced by certain strains of *E. coli* which was able to kill Vero cells (Konowalchuk et al., 1977). This toxin was accordingly called verotoxin (VT). It was later shown that SLT and VT were the same toxin. The terms SLT and VT were subsequently used interchangeably.

With the discovery of new members of this family, SLT was named SLT-I (VT-1) to distinguish it from the new toxins isolated from *E. coli*. Two variants of SLT-I were later identified; SLT-IIc, from *E. coli* isolates recovered from humans (Scotland et al., 1985; Strockbine et al., 1988) and SLT-IIe, from *E. coli* isolates from pigs (Marques et al., 1987). The inter-relationship of these toxins is illustrated in Table 1.1.

The primary structure of SLT-I is identical to that of the ShT with the exception of a serine to threonine substitution at position 45 in the A chain. SLT-I subunit structure is a common design found in a larger group of bacterial and plant toxins now referred to as the A/B family of protein toxins (for reviews see, Merritt & Hol, 1995; Burnette, W. N., 1994; and Read & Stein, 1993). This family of toxins includes cholera toxin (CT), pertussis toxin (PT), diphtheria toxin (DT) and the plant toxin, ricin (RT). Members of this family of toxins are made up of an enzymatic A subunit and a receptor binding B subunit.

SLT-I is made up of one A subunit and five identical copies of a B subunit (Olsnes et al., 1981). The gene for the toxin was cloned and sequenced from *E. coli* O157:H7 (Takao et al., 1988) and from bacteriophage H-19B (De Grandis et al., 1987), isolated from the *E.*
coli isolate 026:H19 (Smith et al., 1983). The association between the A and B subunits involves non covalent interactions. The complex is very stable, as harsh conditions (8M urea, 0.1% propionic acid) are required to dissociate the subunits from each other (Yutsudo et al., 1987; Head et al., 1991).

1.2.1 The Structure and Function of Shiga-like Toxin I A subunit

The A subunit is made up of 293 amino acids (Fig. 1.2) and has an isoelectric point of 8.2 (Yutsudo et al., 1987), whereas the isoelectric point of the holotoxin is 6.72 to 7.02 (O’Brien & LaVeck, 1983; Petric et al., 1987). These pI’s are different from those of 5.2 (Downes et al., 1988) for SLT-IIc and 9.0 (MacLeod et al., 1991) for SLT-IIe. The A subunit possesses N-glycosidase activity which cleaves an adenine located at position 4324 in 28S rRNA (Fig. 1.3; Endo et al., 1988) and resulting in the inhibition of protein synthesis. In the crystal structure of Shiga toxin (Fraser et al., 1994), the A subunit was shown to be lying on one face of the pentamer (referred to as the A-face; see Fig. 1.4), opposite to the face containing the receptor binding site(s), the R-face. The C-terminus of the A subunit (residues 282-287) adopts a partially helical conformation and is inserted into the central pore created by the pentamer. The C-terminus is relatively hydrophobic, a feature compatible with the hydrophobic pore of the pentamer.

The two cysteines at positions 241 and 261 in the A chain form a disulphide bond. The resulting loop contains residues 242 to 260 (Fig. 1.2) and is the region of the A chain most susceptible to proteolytic attack. Interestingly, the side chain of Met-260 is positioned into the pocket corresponding to the putative N-glycosidase active site of the A chain (Fig. 1.3). The location of this residue may explain why the cleaved toxin is more active than its uncleaved counterpart (Garred et al., 1995a; Burgess & Roberts, 1993), since cleavage in the protease sensitive loop could potentially move this residue away from, thus increase the
Figure 1.2 Sequence alignment of A subunits from SLT-I and related toxins. Conserved domains are boxed in green lines and residues in the active site are boxed in red. RTA, A chain of ricin.
accessibility to the active site. The majority of the contacts between the A chain and the B pentamer are due to the A2 fragment (minimal domain; residues 262-293) and a small contribution from a β-hairpin in the A1 fragment (residues 218-226).

Trp-203 in the A1 fragment has been identified as an active site residue by mutagenesis (Yamasaki et al., 1991; Jackson et al., 1990). Other residues implicated in the catalytic action of the toxin include Glu-167, Tyr-77, Tyr-114 and Arg-170 (Hovde et al., 1988; Schlossman et al., 1989; Deresiewicz et al., 1992; Yamasaki et al., 1991; see Fig. 1.3). Using deletion analysis, Haddad and coworkers (1993) defined a C-terminal boundary of the enzymatic A1 fragment at Arg-268. In that study, deletion mutants were screened using an in vitro protein synthesis inhibition assay. N-terminal deletions were also used to map the minimal N-terminus site to Asn-75 (Haddad et al., 1993). The A chain is thus typically processed into an A1 fragment (27kDa; N-terminus) and an A2 domain (5kDa; C-terminus) while associated with the B pentamer.

1.2.2 The Structure of SLT-I B subunit

The B subunit of SLT-I is made up of 69 amino acids (Fig. 1.4) and has an isoelectric point of 5.8, as determined by polyacrylamide gel isoelectric focusing (Yutsudo et al., 1987). The sequence contains cysteine residues at positions 4 and 57 involved in a disulfide bond in the functional B subunit and a single tryptophan residue at position 34 (Newland et al., 1985; Huang et al., 1986). The crystal structure of SLT-I B subunit revealed structural homology with the E. coli heat-labile enterotoxin (Stein et al., 1992). The B subunit exists as a pentamer with each monomer composed of 2 three-stranded antiparallel β-sheets and a 10-residue long α-helix (Stein et al., 1992). The monomers assemble together to form a ring-like structure with a central pore. The diameter of this orifice ranges in values
Figure 1.3 Action of the enzymatic A subunit of SLT-I. (A) Cleavage site in the adenosine nucleotide (position 4324) that results in the liberation of the adenine moiety (Olsnes et al., 1990). (B) The conserved nucleotide loop of 28S rRNA recognized by ricin and SLT’s. It mediates the binding of tRNA’s during polypeptide synthesis. (C) Location of the nucleotide loop within the ribosome. It is located within the cleft in which peptide bond formation and polypeptide elongation takes place. Removal of adenine 4235 inhibits the binding of tRNA’s and thereby disabling protein synthesis and ultimately causing cell death.
from 11Å on the A-face down to 8Å on the R-face (Fig. 1.4 and 1.5). The 5 helices line the pore and each contains four leucine residues which creates an internal surface harbouring an overall hydrophobic character. The pentamer of the *E. coli* LT toxin, on the other hand, displays a hydrophillic central pore.

The *E. coli* LT B subunit (103 amino acids) and the SLT-I B subunit (69 amino acids) share little sequence identity but have a surprisingly similar secondary and tertiary structures. A comparative study by Sixma and coworkers (1993) have shown that both subunits can be superimposed with seven out of eight secondary structure elements retained in both subunits. Using sequence alignment with other members of the SLT family of toxins, the authors also attempted to locate the Gb3-binding site. The most likely site was identified in a cleft between monomers and contains residues Asp-18 and Phe-30 (Stein et al., 1992; Nyholm et al., 1996). Mutagenesis studies have also identified Phe-30 as an important residue for receptor binding (Bast et al., 1994). This cleft also includes the side chains of Asn-15, Thr-19 and Thr-21, all of which are capable of hydrogen bonding (Stein et al., 1992; Nyholm et al., 1996). Lys-53 has indirectly been implicated in receptor binding, since the biotinylation of its side chain was shown to reduce the binding of the B subunit to Gb3 (Khine & Lingwood, 1994). The central helix in the B subunit is capped at the N-terminus end by Trp-34, which places this residue in the same face of the pentamer as the putative Gb3 binding domain (referred to as the R-face of the pentamer; Fig. 1.4). Formylation of its indole ring has been shown to inhibit the binding of SLT-I B subunit to Gb3 (Boyd, B., 1991).

Finally, the reduced quenching of the B subunit tryptophan fluorescence by acrylamide in the presence of DMPC/Gb3 vesicles suggests the proximity of the Trp-34 to the receptor (Surewicz et al., 1989). The monomeric B subunit does not bind to Gb3, since the monomers are not observed in solutions and dissociation of the pentamer
**Figure 1.4** Structure of Shiga-like toxin I B subunit. (A) Sequence alignment of SLT-I B subunit with SLT-II and SLT-IIv B subunits. Identical residues are boxed in green lines and residues implicated in the binding of SLT-I to Gb₃ are boxed in red. (B) Space-filling model of the X-ray structure of the B subunit pentamer (Stein et al., 1992). The red colour shows the location of the side chains of the residues involved in receptor binding (Asp-18 & Phe-30). This top face of the pentamer is the R-face (as defined in the text) where the single tryptophan of the B subunit (purple colour) is located.
Figure 1.5 Various representations of the X-ray crystallography structure of Shiga toxin (ShT) as determined by Fraser et al. (1994). (A) A stick model of the structure of ShT highlighting the $A_1$ fragment (yellow), a hydrophobic domain between residues 220 and 246 in the A subunit (purple), the $A_2$ fragment (magenta) and the active site residues (black ball and stick models) including Tyr-77, Tyr-114, Glu-167 and Arg-170. The red dots in this region represent the oxygens of Glu 167. The B subunit pentamer is shown in green. (B) The same colour scheme as in (A) but in a space filling model to show the level of exposure of the domains to the solvent. (C) The back face of the model shown in (B).
requires denaturing conditions and the presence of reducing agents. This is also true for other toxins containing homopentameric B subunit such as CT and LT. In contrast, the separated monomers in pertussis toxin, which consists of a heteropentameric B subunit, (S1 and S2) are properly folded and can interact, in vitro, with receptor moieties (Saukkonen et al., 1992). The primary structure of the B subunit has been shown to be similar (up to 40% sequence similarity) to the N-terminal 50 residues of the human interferon γ (IFN-γ) receptor (Lingwood & Yiu, 1992) and the N-terminus extracellular domain of CD19 (Maloney & Lingwood, 1994). These findings have implications for the role that Gb₃ may play in IFN-γ- and CD19-mediated signalling and suggests a role for the toxin as a signalling mimic by which the bacterium evades the hosts’ immune system to gain an advantage in colonizing the hosts’ digestive tract.

1.3 The Receptor of Shiga-like Toxin I

SLT-I binds to cells expressing the receptor globotriosyl ceramide (Gb₃) on their surface membrane (Fig. 1.6). Gb₃, also known as CD77, is a human B cell differentiation antigen and can be found on the surface of epithelial cells lining the intestine and colon, on renal endothelial cells, as well as on red blood cells and human platelets (Tedder & Isaacs, 1989; Obrig et al., 1993; Lingwood, C. A., 1993; Moyer et al., 1987). Binding specificity to Gb₃, and to a lesser extent to pentosyl ceramide and galabiosyl ceramide (Cohen et al., 1987; Lindberg et al., 1987) was described in several laboratories using membrane lipid extracts resolved by thin-layer chromatography (TLC) and overlay detection methods based on the use of radiolabeled toxins and antitoxin antibodies (Jacewicz et al., 1986; Lindberg et al., 1987; Lingwood et al., 1987). All of those glycolipids have terminal Galα1-4Gal group and a ceramide lipid tail anchoring it in the outer leaflet of the membrane. Free saccharide
Figure 1.6 A model of the structures of Gb₃ (A) and Gb₄ (B). The terminal disaccharide in Gb₃ is internal in Gb₄ but lectins that bind to Gb₃ do not recognize Gb₄. The ceramide moiety of the molecule is normally heterogeneous showing different chain lengths and variable unsaturation of the fatty acid chains (Kiarash et al., 1994).
shows very weak interaction with the toxin (St. Hilaire et al., 1994), which implicates the ceramide in the interaction between the toxin and the receptor, even though it is not directly involved in the binding. There is one more line of evidence available that suggests a role for the ceramide tail in binding; digalactosyl diglyceride, a plant glycolipid that has the same carbohydrate sequence but has glycerol instead of ceramide, is not recognized by SLT-I. The ceramide group may play a role in maintaining the carbohydrate in a specific orientation by hydrogen bonding between the sphingosine amide and the oxygen linking the first glucose molecule to the other lipid tail (Strömberg et al., 1991; Karlsson, K.-A., 1989).

Certain cell lines, such as A431, express Gb\textsubscript{3} on their cell surface but are not sensitive to SLT-I (Sandvig et al., 1992). On the other hand, sensitivity to the toxin can be induced in some resistant cell lines by treatment with agents that increase the expression of Gb\textsubscript{3} on the cell surface, such as hydrocortisone or butyric acid (Mobassaleh et al., 1994; Sandvig et al., 1991). The reason for the resistance of Gb\textsubscript{3}-positive cells is not clear. These cells are equally resistant to cleaved toxin, eliminating the possibility that resistant cells lack the ability to cleave and activate the toxin (Sandvig et al., 1992). It has been hypothesized that the resistance could be due to different intracellular routing of internalized toxin molecules, such that membrane translocation is not possible (Sandvig et al., 1994). It is not clear how butyric acid sensitizes cells but it appears to be related to changes in the structure of Gb\textsubscript{3}. It has been shown that the lipid composition of glycolipids is important for the positioning of such molecules in the cell membrane and for the conformation of the carbohydrate moiety (Pellizari et al., 1992; Strömberg et al., 1991; Karlsson, K.-A., 1989; and Crook et al., 1986). The physiological role of Gb\textsubscript{3} is not well understood. It may represent a second messenger involved in the regulation of cell cycle and cellular differentiation. For example, tumor necrosis factor-\textalpha{} (TNF-\textalpha{}) activates a neutral sphingomyelinase in leukemia HL-60 cells (Bielawska et al., 1992; Dressler et al., 1992).
The activation of this enzyme leads to an increase in free ceramide which in turn regulates a membrane-associated protein kinase that has been shown to mediate the action of TNF-α. Using Burkitt's lymphoma cell lines, cytotoxicity by SLT-I was shown to be due not only to inhibition of protein synthesis but also due to apoptosis (Mangeney et al., 1993), a regulatory process that can be mediated by ceramide.

1.4 Receptor-Mediated Endocytosis of Bound Toxin

Following Gb3 binding, SLT-I is internalized via receptor-mediated endocytosis from coated pits (Sandvig et al., 1989). The formation of coated pits was observed in electron micrographs and the internalization of the toxin could be inhibited by incubating cells at 4°C or by treating them with dansyl cadaverine, an inhibitor of cytoplasmic trans-glutaminase (Khine & Lingwood, 1993; Davies et al., 1980). The internalization of the toxin via clathrin-coated pits was further supported by the inhibition of toxin uptake as a result of acidifying the cytosol, which blocks the formation of coated pits (Sandvig et al., 1987). Clathrin-coated pits can also be inhibited by potassium depletion from the cytosol, which has been shown to protect cells from the toxin (Sandvig et al., 1994). Cells can also be protected from lysis by the toxin by treating cells with 3-methyladenine and cycloheximide (Sandvig & van Deurs, 1992). Other factors that may influence cell sensitivity to SLT-I include Ca²⁺ transport and certain divalent cations (Sandvig and Brown, 1987). Vero cells were found to be most sensitive in the presence of CaCl₂ and SrCl₂ and were resistant to the toxin in the presence of MnCl₂, BaCl₂, CoCl₂ and inhibitors of Ca²⁺ transport (Sandvig & Brown, 1987). The intracellular trafficking of SLT-I (Fig. 1.7) proceeds via endosomes to the Golgi (GO) and through retrograde transport to the endoplasmic reticulum (ER) and even the nuclear membrane (Sandvig et al., 1992; 1994). Brefeldin A, a drug that disrupts the GO apparatus, protects cells from the toxin (Sandvig et
Figure 1.7 Schematic representation of the receptor-mediated endocytosis of bound SLT-I.

The toxin is composed of an $A_1$ fragment (red rectangle), an $A_2$ fragment (yellow rectangle) and $B$ subunits (blue rectangles). $Gb_3$ is represented on the cell surface by the green symbols. The initial clathrin (brown-green highlight)-coated vesicle is formed from the cell membrane and localizes the toxin in the Golgi, as well as other compartments but are not shown here for clarity. The toxin is then transported via a retrograde mechanism to the endoplasmic reticulum from which, the $A_1$ fragment could potentially cross the membrane and enzymatically modify the 28S rRNA loop located on the ribosomes (shown as irregular purple globules).
Toxin & Extracellular Matrix

Retrograde Lysosome Transport + Ribosomes
al., 1991). It has been estimated that ~4% of bound toxin per hour reach vesicular compartments beyond endosomes (Sandvig et al., 1991). However, before the toxin can reach and inactivate ribosomes, it is required to translocate across vesicular membranes, regardless of which compartment it occupies. Passing through the various compartments of the cell, the internalized toxin is exposed to environments with varying pH. In the endosome, where acidification of the endocytic vesicles begins before fusion with lysosomes, pH drops to ~5 (Tycko & Maxfield, 1982; Ohkuma et al., 1982). The lumen of the lysosomes is more acidic in nature, reaching a pH of 4.6 (Tycko & Maxfield; Geisow & Evens, 1984). In the Golgi network, pH is maintained slightly acidic at pH 6.4 (Kim et al., 1996) and goes back up to neutrality (7 to 7.2) in the ER (Bartido et al., 1995).

The blockage of retrograde transport with brefeldin A protects cells sensitive to the action of ShT and suggests that the toxin must reach the luminal compartments of the Golgi network and the ER before its A₁ subunit can be effectively translocated near ribosomal subunits (Garred et al., 1995b). Since the target site for the N-glycosidase activity of the A₁ chain is a single adenine base of 28S rRNA, the A₁ subunit must possess a mechanism to translocate to the cytoplasmic side of the ER membrane (Endo et al., 1988; Saxena et al., 1989). In the case of diphtheria toxin, there is evidence that the toxin exploits the acidification of the endosome to undergo a conformational change and exposes a hydrophobic domain that enables it to translocate across membranes (Donovan et al., 1981; Hu et al., 1984; Blewitt et al., 1985; and Rolf et al., 1993). A similar effect has been observed in the case of Pseudomonas exotoxin A where low pH alters its structure to reveal a membrane binding domain (Farahbakhsh & Wisnieski, 1989).

1.5 Structural and Functional Homologies With Other Toxins

The structure and cellular mechanism of the A/B family of toxins has been the focus
of several reviews (Middlebrook et al., 1984; Olsnes et al., 1990; Read & Stein, 1993; and Merritt & Hol, 1995). Members of this family of toxins are characterized by coding the enzymatic activity and the receptor binding activity on separate polypeptide chains, which subsequently associate to form biologically active toxin molecules. The A subunit of SLT-I contains distinct homologous regions with ricin A subunit and it has been shown that both Cα traces are superimposable (Sixma et al., 1993; Read & Stein, 1993). Residues in the putative active site are distinctly conserved. The B subunit shows sequence homology only to members of the SLT toxin family. However, its crystal structure revealed a surprising homology in terms of its folding and assembly with the E. coli LT toxin, cholera toxin and Staphylococcus aureus nuclease (Arnone et al., 1971).

In addition to structural similarities, members of the A/B family of toxins also share functional similarities. Although the cellular targets of these toxins are different, they are all located in the cytoplasmic pool of target cells. For this reason, the toxins must overcome the limiting membrane of vesicular compartments during endocytosis. Early work on diphtheria toxin using planar lipid bilayers and lipid vesicles showed that the toxin interacts with membranes, and in some cases, forms transmembrane channels (Donovan et al., 1981; Hu & Holmes, 1984; and Zalman & Wisnieski, 1984). Furthermore, those studies showed that membrane interactions of diphtheria toxin were pH dependent, being highest at pH 4.0 to pH 5.0, comparable to those conditions found in late endosomes and lysosomes. The increased lipid interaction properties of a toxin at endosomal pH requires that the protein undergoes a conformational change to expose hydrophobic domains. Indeed, endosomal pH was found to induce a change in the conformation of diphtheria toxin (Rolf & Eidels, 1993; Blewitt et al., 1985; and Zhao & London, 1988). Dissection of the structure of DT led to the identification of a membrane translocation domain located between residues 202-378 (Zhan et al., 1995; Montich et al., 1995) and was recently shown to insert into model membranes (Zhan et al., 1995; Montich et al., 1995). Similar features were also identified in
*Pseudomonas* exotoxin A (PE). The toxin molecule contains a distinct enzymatic, receptor binding and membrane translocation domains (Allured et al., 1986; Collier, R. J., 1988; and Hwang et al., 1987). As it was observed in the case of diphtheria toxin, PE required proteolytic cleavage for cytotoxicity (Jinno et al., 1989; Ogata et al., 1990). One additional requirement for PE cytotoxicity is the tetrapeptide motif KDEL or REDL(K) at its C-terminus, a necessary signal for the proper intracellular trafficking of this toxin (Chaudhary et al., 1990).

In contrast to these toxins, however, SLT's and the closely related plant toxin, ricin, do not contain distinct translocation domains nor do they contain the KDEL motif. In spite of this missing feature, SLT-I is transported to the GO and the ER and en route, is exposed to a variety of proteases including, among possible others, trypsin, furin and calpain (Garred et al., 1995a; 1995b). The primary cleavage region in SLT-I is located in the disulfide loop, residues 242-261, in the A subunit (Fig. 1.2; residues 265 to 284 in the alignment nomenclature). Cleavage in this region has been shown to increase the potency of the toxin, both *in vitro* and *in vivo* (Garred et al., 1995a; 1995b). Although results vary between different cell lines and incubation periods, deletions in the trypsin and furin sensitive motif (RXXR) resulted in reduced cytotoxicity by as much as 1000 fold in T47D cells following a 1 hour incubation period (Garred et al., 1995a). Longer incubation periods seem to reduce the effects of the deletions. One explanation for this latter observation is that longer incubation periods result in the toxin being cleaved and activated by RXXR-independent protease(s). At the present time, no one has been able to demonstrate SLT-I cytotoxicity without intracellular processing. In the case of ricin, the A and B subunits are already separated from each other in the native toxin, but are associated through a disulfide bond and therefore, cleavage is not required. Interestingly, the proteolytic processing of internalized ricin has been demonstrated experimentally (Blum et al., 1991).
1.6 Goals and Objectives

In an effort to understand intracellular mechanisms that may alter the structure and localization of SLT toxins, I have used purified SLT-I and its individual subunits. The objectives of my thesis were as follows:

1) Previous observations have suggested that the B subunit may be able to unfold in acidified compartments causing the release of the A2 fragment and/or contribute to the translocation of the A1 fragment. I decided to investigate the effects of pH on the structure of the SLT-I B subunit (chapter 3). It was found that endosomal pH causes a local conformational change in the B subunit. The observed change is not large enough in scale and would not occur in the right compartment to support a mechanism for the translocation of the enzymatic subunit across the ER membrane. This change in conformation, however, may contribute to other events that occur in the endosome, such as the proteolytic processing of the toxin A chain en route to the ER, which is required for the translocation to occur.

2) As observed in the case of diphtheria toxin and exotoxin A, both the A and B subunits may participate in membrane insertion and translocation of the enzymatic fragment to the cytosol. To test for the occurrence of similar effects in SLT-I, the influence of pH on the structure of the holotoxin has been investigated (chapter 4). It was observed that endosomal pH induces local conformational changes in the holotoxin but does not alter its secondary or tertiary structures. As mentioned in point (1) above, such local changes in conformation may facilitate proteolytic processing of the A subunit and disulfide reduction prior to its translocation in the ER compartment.

3) The reduction of the single disulfide bond of the A chain of SLT-I and its limited
proteolysis may facilitate the release of the A₁ fragment from the holotoxin and/or expose its hydrophobic C-terminus. These cellular events may favour its interaction with membranes and suggests a mechanism for its translocation across the ER membrane. To test this hypothesis, we have investigated the interaction of the hydrophobic domain of the A₁ fragment with membranes (chapter 5). It was found that a synthetic peptide representing this region associates with membranes in a pH-dependent manner. At neutral pH, as in the lumen of the ER, this peptide inserts across the vesicle membrane. In the context of the translocation of the A₁ fragment, this peptide may mediate the insertion of the A₁ fragment into the inner leaflet of the ER membrane and may constitute the first step in its translocation.

My thesis work has produced two publications in *Biochemistry* (see chapters 3 and 5). In addition, I have contributed to two other bodies of work not discussed in the thesis. One project was led by Dr. Eric LaCasse and published in *Blood* under the title “Shiga-like toxin purges human lymphoma from bone marrow of severe combined immunodeficient mice. Vol. 88, pp1561-1567”. The other piece of work is being conducted by Dr. A. Menikh in collaboration with Dr. J. Boggs at the Hospital for Sick Children. This latter work focuses on the use of ATR-FTIR to investigate the interaction between a synthetic peptide (W²³²A²⁴²ShTA (220-246), see chapter 5) and lipid membranes. The manuscript that describes this work is presently being prepared.
CHAPTER 2

EXPERIMENTAL METHODS
CHAPTER 2

2.1 Biochemical Methods

2.1.1 Purification of SLT-I. Several LB agar plates containing carbenicillin (100 μg/ml) were streaked using a steel loop with an aliquot of a culture of the bacterial strain JB 28 previously stored at -70°C. The plates were incubated at 37°C overnight. A single colony from such plates is then used to inoculate a 100ml LB broth containing 100μg/ml carbenicillin and 0.1% (W/V) glucose. The cultures were grown overnight with mixing (250 rpm) at 37°C. Up to 6L of LB broth were prepared in the same manner and each 6L flask (containing up to 2L of broth) was inoculated with 5ml of the stock culture grown overnight. After an incubation period of 18h, the culture was centrifuged at 10,000 rpm for 15 minutes and the pellet was either used immediately for subsequent toxin purification steps or stored at -70°C for future processing. (1) Protein extraction was performed by suspending the pellet in 400 ml of PBS containing 0.1 mg/ml polymyxin B, 0.4 mg/ml EDTA and 0.1 mg/ml PMSF and mixed vigorously using a blender. The bacterial suspension was subsequently incubated at 37°C with continuous stirring for 1h. Cellular debris were removed by centrifugation (10,000 rpm for 15 min.) and the supernatant was collected and kept on ice for subsequent steps. The pellet was re-extracted two more times in the same way and the supernatants from the three extractions were pooled. The final supernatant was filtered through a glass-fibre filter paper (0.45 μm pore size) and concentrated using an Amicon ultrafiltration cell (500 ml capacity) connected to a nitrogen gas cylinder (≤ 60 psi). Ultrafiltration membrane (Amicon YM10) with a 10,000MW cut off was used. The supernatant was concentrated to 80-100 ml at 4°C and dialyzed (using dialysis tubing with a 3,500MW cut off) against 2L of 10 mM sodium phosphate buffer, pH 7.4 overnight followed by another dialysis in the same buffer.
to bring the final NaCl concentration below 5 mM. (2) The dialysate was loaded onto a hydroxyapatite column (2.5 cm x 30 cm) pre-equilibrated into the dialysis buffer. The column was then washed with dialysis buffer until the absorbance of eluant was < 0.1 (280 nm). The bound protein was eluted in a batch method with two column volumes (300 ml) of 100 mM sodium phosphate buffer, pH 7.4. The column flow rate was 1 ml/min. and 4 ml fractions were collected. The eluate was monitored spectrophotometrically at 280 nm.

Fractions 36-50 were pooled and dialysed (using the same dialysis tubing described above) against 2L of 25 mM imidazole-HCl buffer, pH 7.4 overnight at 4°C. (3) The pooled fractions were loaded onto an anion exchange column (2.5 cm x 20 cm; polybuffer exchanger, Pharmacia Biotech) pre-equilibrated with the imidazole dialysis buffer. The column was then washed with 2-3 column volumes and the pH gradient developed (at a flow rate of 1 ml/min.) with Polybuffer 74 adjusted to pH 5.0. The elution profile was monitored at 280 nm and fractions (33-41) containing a single peak and centred at pH 6.9 were pooled and dialyzed (10,000MW cut-off) against 10 mM sodium phosphate buffer, pH 7.4 overnight. (4) The pooled fractions were subsequently loaded onto a Cibachron blue F3GA column (2.5 cm x 10 cm; Affi-Gel blue gel, Bio-Rad Laboratories) pre-equilibrated with dialysis buffer followed by washing with 60 ml of the same dialysis buffer. Bound toxin was eluted (flow rate of 1.8 ml/min.) using 200 ml of the dialysis buffer containing 0.5M NaCl. The purified toxin (fractions 18-21) was dialysed against 4L of distilled water and lyophilized.

2.1.2 Purification of SLT-I B subunit. Clones expressing the SLT-I B subunit (pJB 122) were grown in a total culture volume of 6L and the extractions were performed as described in section 2.1.1. The final supernatant was concentrated to 80-100 ml at 4°C and dialysed (using dialysis tubing with a 3,500 MW cut off) against 2L of 10 mM Tris-HCl buffer, pH
Figure 2.1 Typical chromatography profiles observed during a purification of SLT-I. (A) Hydroxyapatite chromatography of a concentrated extract of the bacterial strain JB28 treated with PBS containing polymyxin B. (B) Chromatofocusing of hydroxyapatite fractions containing the toxin using a pH gradient of 7.4-5.0. (C) Elution of bound SLT-I from Cibacron blue gel using 0.5M NaCl in phosphate buffer, pH 7.4. Toxin-containing fractions were identified using anti-A subunit antisera. Solid bar indicates the fractions that were pooled and prepared for the next purification step.
Figure 2.2 Typical chromatography profiles observed during the purification of SLT-I B subunit. (A) Ion exchange (DEAE-Sephacel) chromatography of a concentrated extract of the bacterial strain pJB122 treated with PBS containing polymyxin B. (B) Chromatofocusing of DEAE fractions containing the B subunit. The column was developed with a pH gradient going from pH 7.4 to pH 4.0. (C) Gel filtration of the B subunit collected from the chromatofocusing step using Sephadex G-50 (Pharmacia Biotech.). Fractions containing the B subunit were identified using anti-B subunit antisera prepared in our laboratory by Beth Boyd (1991). Solid bar indicates the fractions that were pooled and prepared for the next purification step.
7.4 overnight followed by another dialysis in the same buffer to bring the final NaCl concentration below 5 mM. (1) The dialysate was loaded onto a DEAE-Sephacel column (2.5 cm x 30 cm) pre-equilibrated with the dialysis buffer. The column was then washed with dialysis buffer until the absorbance of eluant was < 0.1 (280 nm). The bound protein was then eluted with a linear gradient (total volume of 600 ml) going from 0 to 0.6 M NaCl prepared in dialysis buffer. The column flow rate was 1 ml/min. and 5 ml fractions were collected. Fractions 73-87 were pooled and dialyzed (using the same dialysis tubing stated above) against 2L of 25 mM imidazole-HCl buffer, pH 7.4 overnight at 4°C. (2) The pooled fractions were loaded onto an anion exchange column (2.5 cm x 20 cm; polybuffer exchanger, Pharmacia Biotech) pre-equilibrated with dialysis buffer. The column was then washed with 2-3 column volumes and the pH gradient developed with Polybuffer 74 adjusted to pH 5.0. The column flow rate was 1 ml/min. Fractions (105-120) containing a single peak and centred at pH 5.8 were pooled and dialysed against 50 mM ammonium bicarbonate buffer, pH 7.4 overnight. (3) The pooled fractions were concentrated by ultrafiltration to 5 ml or less and loaded onto a G50 gel filtration column (2.5 cm x 50 cm; Pharmacia Biotech). The column flow rate was 1.4 ml/min. and 4 ml fractions were collected. The purified B-subunit (contained in fractions 36-47) was dialysed against 4L of distilled water and lyophilized.

2.1.3 Peptide Synthesis and Purification. The peptides were assembled by solid phase synthesis on an Applied Biosystems 431 peptide synthesizer using FMOC chemistry. The following peptides were synthesized: ShTA(220-246), (14C)Ac-RVGRISFGSINAILGS-VAlILNCHHHA, which represents residues 220 to 246 in the native toxin and W232A242ShTA(220-246), (14C)Ac-RVGRISFGSINAWLGsVALILNAHHA, which contains two substitutions, namely I232W and C242A. The N-terminus of each peptide was
acetylated by reacting 100 mg of peptide-resin suspended in dichloromethane with 250 µCi of [1-14C]acetic anhydride overnight at room temperature. The acetylation step was completed by further treating the sample with 10% (v/v) cold acetic anhydride in dichloromethane for 30 minutes prior to cleaving the peptides from the resin. The peptides were purified by reverse-phase HPLC on a C18 column (Beckman ODS; 1 cm O.D. x 25 cm; column was pre-equilibrated in water: 0.1% (v/v) TFA) and eluted with a linear gradient of acetonitrile (AcN): 0.08% (v/v) TFA. The steepness of the elution gradient was 2% AcN/min. Both peptides typically eluted from the column when the composition of the mobile phase reached 60% acetonitrile. The column flow rate was 1 ml/min. The composition of each purified peptide was confirmed by amino acid analysis and mass spectrometry. The molar absorption coefficient of W232A242ShTA(220-246) at 280 nm was calculated to be 5700 M-1 cm-1. The concentration of the melittin stock solution was calculated using the Trp molar absorption coefficient of 5570 M-1 cm-1 at 280 nm (Wetlaufer, 1962). The specific activity of the 14C-labelled peptides was calculated to be 8.6 x 10² cpm/µg for W232A242ShTA(220-246) and 9.8 x 10² cpm/µg for ShTA(220-246). The HPLC elution profiles of both peptides are shown in Figures 2.3 and 2.4. The purified peptides were characterized by Mass Spectrometry (Figures 2.5 and 2.6) and amino acid analysis (Table 2.1).

2.1.4 Electroelution of SLT-I A subunit. For this procedure, normally Tris/Glycine gels (16 cm x 16 cm) were prepared and sample wells either contained 30µl (for the molecular weight markers and controls) or 400µl (for the purified SLT-I). The gels were run as mentioned in section 2.1.4. Immediately following the run, the gel lane containing the toxin was cut into 2
Figure 2.3 Purification of ShTA(220-246). **Panel A** Reverse-phase HPLC profile on a semi-prep C\textsubscript{18} column. Arrow marks the peak containing the peptide. **Panel B** Reverse-phase HPLC profile of the peak collected from the semi-prep elution on an analytical C\textsubscript{18} column.
Figure 2.4 Purification of W232A242ShTA(220-246). **Panel A** Reverse-phase HPLC profile on a semi-prep C18 column. Arrow marks the peak containing the peptide. **Panel B** Reverse-phase HPLC profile of the peak collected from the semi-prep elution on an analytical C18 column.
Figure 2.5 Mass spectrum of ShTA(220-246). System: ion-spray (SCIEX). Each of the peaks shown represents a detected peptide fragment and is expressed as the ratio of mass/charge (m/z). For example, the peak identified in the spectrum by M+4 (at m/z of 725.4) reflects the presence of a peptide in the sample that has four positive charges (4 H+). Therefore, the mass of the peptide can be calculated from the following formula:

\[
\text{peptide mass} = \frac{(m/z \times z) - z}{x}
\]

From the peak at 725.4, the mass of the peptide is \((725.4 \times 4) - 4\), or 2897.6 daltons. The expected mass of this peptide is 2898 daltons.
Figure 2.6 Mass spectrum of $W^{232}A^{242}ShTA(220-246)$. System: ion-spray (SCIEX). The mass of the peptide can be extracted from the data as shown in Figure 2.5.
mm sections. The section containing the A subunit, as identified by staining a corresponding control lane, was further cut into smaller sections (2 mm x 10 mm) and the protein within was eluted out using model 422 Electro-eluter (Bio-Rad laboratories, Hercules, CA). The eluted A subunit was used to generate rabbit anti sera as described below in section 2.3.1.

Table 2.1
Amino acid analysis of purified synthetic peptides.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>ShTA(220-246)</th>
<th></th>
<th>W232A242ShTA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number Expected</td>
<td>Number Present</td>
<td>Number Expected</td>
<td>Number Present</td>
</tr>
<tr>
<td>Ala</td>
<td>3</td>
<td>3.2 ± 0.3</td>
<td>4</td>
<td>4.4 ± 0.1</td>
</tr>
<tr>
<td>Arg</td>
<td>2</td>
<td>1.9 ± 0.6</td>
<td>2</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Asx</td>
<td>2</td>
<td>2.2 ± 0.1</td>
<td>2</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Gly</td>
<td>3</td>
<td>3.3 ± 0.1</td>
<td>3</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>His</td>
<td>3</td>
<td>3.7 ± 0.4</td>
<td>3</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>Leu</td>
<td>3</td>
<td>3.4 ± 0.4</td>
<td>3</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>Ile</td>
<td>4</td>
<td>4.0 ± 0.2</td>
<td>3</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>Phe</td>
<td>1</td>
<td>1.1 ± 0.2</td>
<td>1</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>Trp</td>
<td></td>
<td>--</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>Ser</td>
<td>3</td>
<td>2.0 ± 0.1</td>
<td>3</td>
<td>2.0 ± 0.0</td>
</tr>
<tr>
<td>Val</td>
<td>2</td>
<td>2.2 ± 0.3</td>
<td>2</td>
<td>2.0 ± 0.0</td>
</tr>
<tr>
<td>Cys</td>
<td>1</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


2.1.5 Radiolabeling of SLT-I and SLT-I B subunit. A mixture containing 180 µl of 0.1 M sodium phosphate, pH 7.4, 1 mCi of Na$^{125}$I and two iodobeads (Pierce Chemical Co.) was deposited into a polypropylene tube and incubated at room temperature for 5 minutes. A 20 µl aliquot of SLT-I or SLT-I B subunit (1 mg/ml in water) was added to the reaction tube and the resulting mixture was further incubated at room temperature for 10 minutes. The liquid was removed from the reaction vial and loaded onto a Sephadex G25 column (1 cm x 20 cm) previously equilibrated in 10 mM sodium phosphate, pH 7.4. The column flow rate was 0.4 ml/min and fractions of 1.0 ml were collected and monitored by counting the radioactivity in a γ counter. The labelled protein eluted in the void volume and was well resolved from the remaining free $^{125}$I. The preparations typically had a specific activity of approximately 3 x 10$^6$ cpm/µg of protein.

2.1.6 Gb$_3$ Solid Phase Binding Assay. The binding assay was performed following the method of Ramotar et al. (1990). Briefly, wells of flexible polyvinylchloride 96-well plates were coated with 10 ng of Gb$_3$ (Gb$_3$: PC: Cholesterol, 2:10:5) in methanol:chloroform (9:1) and the solvents were allowed to evaporate overnight at room temperature. Wells were then blocked with 200 µl of 2% (w/v) BSA in 10 mM PBS for 2 hours at room temperature, washed 6 times with PBS (at the given pH) before $^{125}$I-labelled B subunit (3 x 10$^4$ cpm/well) was added either in the presence (50 µg; 1000-fold excess) or absence of unlabeled B-subunit. After incubation at room temperature for 2 hours, the wells were washed 6 times with PBS (at the given pH), cut and the bound radioactivity quantified in a γ counter. To confirm that the denatured protein did not bind to its receptor in this assay, a sample of the B subunit was boiled for 10 minutes and the assay subsequently performed at
neutral pH. Error bars represent the standard deviation associated with experiments performed in triplicate.

2.1.7 Proxy labeling of ShTA(220-246) Peptide. Purified peptide (1.0 mg) dissolved in 160 μL of 8M urea and 40 μL of 3-maleimido proxyl (10 mg/ml in methanol) were mixed together to give a 5 molar excess of the maleimido-proxyl group over the reactive thiol side chain (Cys-242) of ShTA(220-246). Fifty μL of 0.25M MES buffer, pH 6.2 was then added to the mixture and the reaction left to proceed overnight at room temperature. Excess proxyl label was removed by reverse phase HPLC using a semi-preparative C₁₈ column (Beckman ODS; 1 cm O.D. X 25cm). The column was developed with a linear gradient from 0 to 100% acetonitrile/0.08% TFA. The steepness of the gradient was 1% AcN per minute and the proxyl-labelled peptide eluted from the column when the composition of the mobile phase reached 60% acetonitrile. The column flow rate was 1mL/min. The concentration of proxyl-ShTA(220-246) samples was determined by integrating the EPR spectra and comparing the spectral signals to that of a free maleimido proxyl standard dissolved in 5 mM phosphate, pH 7.0. From this analysis, the molar ratio of proxyl group coupled to peptide was determined to be 0.83.

2.1.8 Preparation of Lipid Vesicles. DMPG or DMPC were dispensed from a lipid stock solution (500 μL of a 20 mg/ml solution) prepared in chloroform:methanol (1:1) into a 16 x 125 mm borosilicate test tube and dried under a stream of nitrogen. Residual solvents were removed under vacuum for three hours. The lipid films were then resuspended and hydrated in 2 mL of either 5 mM phosphate, pH 7.0 or in 5 mM acetate, pH 5.0 using 10 cycles of fast freeze-thaw (Mayer et al., 1985). The large multilamellar vesicles formed were then subjected to sonication in a bath sonicator to visual clarity. The small unilamellar vesicles
(SUV) produced by this procedure were used directly in the experiments. For calcein leakage experiments, the vesicles were formed in the presence of 20 mM calcein in either phosphate or citrate buffer. Free calcein was removed by gel filtration on a Sephadex G15 column (1.5 cm x 40 cm) and eluted using the same buffer in which the vesicles were prepared.

2.1.9 Binding of Radiolabeled Peptide to Lipid Vesicles. W232A242ShTA(220-246) samples (42 µg dissolved in either 5 mM phosphate, pH 7.0 or in 5 mM acetate, pH 5.0; specific activity, 8.6x10^2 cpm/µg) were dispensed into centrifuge tubes (13 x 51 mm; Beckman, polyallomer). Increasing amounts of preformed DMPG or DMPC vesicles suspended in the same buffer were then added to the tubes to reach a final volume of 4.0 ml. The tubes were then centrifuged at 175,000 x g for 5 hrs and the radioactivity of the supernatant was measured (Wang et al., 1993). Lipid concentrations were determined by the phosphorus assay (Rouser et al., 1975). The fraction of peptide bound to vesicles was calculated as 1 - [S_{lipid}/S_{total}], where S_{lipid} represents the radioactivity (in cpm) associated with the supernatant recovered from centrifuging samples containing the 14C-labelled peptide in the presence of lipid vesicles at a peptide-to-lipid molar ratio of 1:50. The term S_{total} represents the radioactivity (in cpm) recorded for control supernatants (radiolabeled peptide in the absence of lipid vesicles).

2.1.10 Total Phosphorus Assay. The amounts of lipids used in the experiments were determined by quantifying the amount of inorganic phosphorus produced from complete hydrolysis of the phospholipids according to the method of Rouser et al. (1970). Following preparation of lipid vesicles (see section 2.1.8), aliquots from the vesicles stock solution were dispensed into glass tubes (8 mm o.d. x 120 mm) and dried by lyophilization. Lipids in the tubes were then extracted with a chloroform:methanol (2:1, v/v) mixture and Dispensed
into a new set of tubes (same type as above). Lipids were hydrolyzed by addition of 0.65 ml perchloric acid per tube and heated at 150° C in a heated metal block for 30 minutes. Approximately 2/3 of the tubes extended outside of the surface of the block which served as a condenser. Tubes were not allowed to dry at all times. After cooling, to each tube were added: 3.0 ml water, 0.5 ml of 2.5% ammonium molybdate and 0.5 ml of 10% ascorbic acid. Colour was developed by heating in a boiling water bath for 5 minutes and the intensity measured at 797 nm. A standard curve was constructed from the same lipids used in the experiments.

2.1.1 Amino Acid Analysis. Protein and peptide samples were subjected to amino acid analysis to establish their composition and to calculate the concentrations of stock solutions (Henrikson & Meredith, 1984). Samples (containing approximately 50 nmol of the most common amino acid in that particular sample) dissolved in water were mixed with 30 nmol of norleucine (acting as an internal standard), and dried in acid-washed tubes (5 mm x50 mm). The tubes were placed in a vial containing 200μl of 1% (v/v) phenol in 6N constant boiling HCl. The vials were flushed with nitrogen and sealed under vacuum. Vapour-phase acid hydrolysis was carried out for 48-72h at 108°C at 100 mTorr. The vials were then allowed to cool to room temperature and dried under vacuum. The samples were dissolved in 10 μl of a solution of ethanol:H2O:triethanolamine, 2:2:1 (v/v/v), vortexed and then redried under vacuum. The samples were resuspended in 20 μl of derivatization solution (ethanol:H2O:triethanolamine:PIPC, 7:1:1:1 (v/v/v/v)) and allowed to stand at RT for 20 minutes. The samples were then dried under vacuum. The resulting phenylthio- carbamyl derivatives were separated and quantitated by reverse-phase HPLC (Beckman System Gold, Beckman Instruments Inc., San Ramon, CA). Samples were dissolved in 100 μl of sample
buffer (5 mM sodium phosphate, pH 7.2 containing 5% (v/v) acetonitrile). 20 μl aliquots of each sample were injected onto a silica-based C18 column equilibrated in buffer A (19 g sodium acetate trihydrate, 0.5 ml triethanolamine and 0.06% acetonitrile in 1L of H₂O, pH 6.4 adjusted with acetic acid). The column was developed with a linear gradient (0-60%) acetonitrile in water. The elution profile was recorded at 254 nm and each amino acid was quantitated by integrating peak areas using Beckman System Gold software. A correction factor for each amino acid was obtained from reference chromatograms of a standard mixture of amino acids (standard H, Pierce Chemical Co.) containing 30 nmol of norleucine to account for variation in the degree of derivatization with PITC and the differential stabilities of amino acids and peptide bonds during the hydrolysis process.

2.2 Characterization of purified SLT-I and its B subunit.

2.2.1 Polyacrylamide Gel Electrophoresis. Tris-tricine gels for discontinuous SDS-PAGE analysis of low molecular weight proteins were prepared as described by Schagger and von Jagow (1987). In some cases, ready-made tricine gels were used (Bio-Rad laboratories, Hercules, CA.; catalog # 161-0922). Protein samples (1-10 μg) were dissolved in Laemmli sample buffer (Laemmli, U. K., 1970) consisting of 50mM Tris, pH 6.8 and containing 2% (w/v) SDS, 4% (v/v) β-mercaptoethanol and 5% (v/v) glycerol and heated for 3 min. at 100°C. Separating gels contained 10-15% (w/v) acrylamide, 3% (w/v) bis-acrylamide, 0.3M Tris-HCl, pH 8.45, 10% (v/v) glycerol, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.05% (v/v) TEMED. Stacking gels (1 cm) contained 5% (w/v) acrylamide, 3% (w/v) bis-acrylamide, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.1% (v/v) TEMED. Proteins were separated under a constant current of 30mA/gel and visualized
by staining in 0.2% (w/v) Coomassie Brilliant Blue in 50% (v/v) ethanol and 10% (v/v) acetic acid. The mobility of SLT-I and its subunits are shown in Figures 2.7 and 2.8.

2.2.2 Western Blot Analysis. Following SDS-PAGE, proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore Corp., Bedford, MA) using a semi-dry transfer system (American Bionetics, Hayward, CA), based on the method of Towbin et al. (1979). The transfer time was 15 min. at 250 mA. The membranes were then incubated in a blocking buffer [2% (w/v) Carnation powdered milk in 0.1M Tris-HCl, 0.15M NaCl, pH 7.4] for 2h at room temperature (RT) or overnight at 4°C to minimize non-specific binding. The membranes were then washed in Tris-buffered saline (TBS) several times before incubating them in TBS containing 0.2% (w/v) powdered milk and dilutions of either anti-B or anti-A subunit rabbit antisera or murine anti-B subunit mAb. Following incubation at RT for 2h, the membranes were washed extensively with TBS and incubated in TBS containing horse radish peroxidase (HRP) conjugated anti-rabbit or anti-mouse antibodies at a dilution of 1:5000 at RT for 1.5h. After washing the membranes with TBS, bound antibodies were detected by the method of Young (1989). Briefly, 30 mg of 3,3’-diaminobenzidine tetrahydrochloride and 10 mg of 4-chloro-1-naphthol were dissolved in 5 ml of methanol and combined with 40 ml of PBS and 10μl of 30% (v/v) hydrogen peroxide. Colour development was stopped by washing the membranes in distilled water.

2.2.3 Cytotoxicity Assay.

The cytotoxicity assay was performed using Vero cells essentially as described by Gentry and Dalrymple (1980) for HeLa cells. Vero cells were suspended in tissue culture microwell plates at a density of 6000 to 8000 cells per well in a total volume of 100μl. The
Figure 2.7 Electrophoretic mobility of SLT-I. Fractions collected during the purification protocol were resolved on SDS-PAGE (Tricine, 16.5% C) and stained with coomassie blue. Lane 1, crude JB28 extract; lane 2, pooled hydroxyapatite fractions; lane 3, pooled chromatofocusing fractions; lane 4, purified SLT-I eluted from Cibachron blue gel and dialysed against water.
Figure 2.8 (A) Electrophoretic mobility of purified SLT-I B subunit. SDS-PAGE (Tricine, 16.5% C) of purified SLT-I B subunit stained with coomassie blue. Lane 1, low molecular weight markers (Pharmacia Biotech.); lane 2, purified B-subunit (6 μg); lane 3, 12 μg of B subunit. (B) Western blot of SLT-I and its B subunit. The proteins were separated on SDS-PAGE and the gel processed as described in section 2.1.5. Lanes 1 and 4; Kaleidoscope molecular weight markers (Bio-Rad laboratories, Mississauga, Ont.). Lane 2; purified B subunit as identified by anti-B subunit antisera (Boyd et al., 1991). Lane 3; purified SLT-I as identified by anti-A subunit antisera (see section 2.2.1).
plates were incubated in a CO₂ incubator overnight at 37°C. The following day, SLT-I was added (in a volume of 10μl of media) to each well at various dilutions. The plates were incubated at 37°C for a further period of 18 hours. The media from the plates was removed by aspiration and the wells were rinsed once with fresh media. Finally, 100μl aliquots of media were added to each well followed by 10μl of the cell proliferation reagent WST-1 (Boehringer Mannheim, Germany). The plates were returned to the 37°C incubator for 1.5 to 2 hours and the colour developed by the reagent was measured at 450 nm and 690 nm. Results are expressed as a ratio of the two wavelengths and are converted to percent survival by taking the value of the colour intensity in the control wells (cells with no toxin added) as 100% survival. A representative assay is shown in Figure 2.10.

2.2.4 Cell-free Protein Synthesis Inhibition Assay. The protein synthesis inhibition assay was performed using the TNT® coupled reticulocyte lysate system (Promega Corp., Madison WI) as described by the manufacturer in the technical bulletin number 126. Briefly, a mixture of TNT reaction mix (24μl), TNT SP6 polymerase (24μl), amino acid mix (24μl, lacking Leu and Met), Rnasin (12μl) and SP6 (12μl) was prepared. In a typical assay, 4μl of this mixture was added to 12.5μl rabbit reticulocyte lysate in a reaction tube. The toxin, in PBS, was added to this reaction mixture to give a final reaction volume of 25μl. Tubes were incubated at 30°C for 1.5 hours. Subsequently, 5μl fractions of a 10x dilution of each reaction mixture was used to measure the luciferase activity in a luminometer. A typical result of this assay is shown in Figure 2.10.
Figure 2.9 A typical titration curve obtained in the Vero cell cytotoxicity assay.
Figure 2.10 A typical curve obtained in the cell-free protein synthesis inhibition assay.
Table 2.2
Amino acid analysis of purified SLT-I and its B subunit

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>SLT-I</th>
<th>SLT-I B subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number Expected</td>
<td>Number Present</td>
</tr>
<tr>
<td>Ala</td>
<td>28</td>
<td>31.3 ± 0.5</td>
</tr>
<tr>
<td>Arg</td>
<td>36</td>
<td>37.4 ± 1.5</td>
</tr>
<tr>
<td>Asx</td>
<td>82</td>
<td>65.3 ± 1.5</td>
</tr>
<tr>
<td>Cys</td>
<td>12</td>
<td>ND</td>
</tr>
<tr>
<td>Glx</td>
<td>39</td>
<td>38.7 ± 0.2</td>
</tr>
<tr>
<td>Gly</td>
<td>53</td>
<td>60.5 ± 0.4</td>
</tr>
<tr>
<td>His</td>
<td>13</td>
<td>14.6 ± 0.1</td>
</tr>
<tr>
<td>Leu</td>
<td>54</td>
<td>55.6 ± 1.1</td>
</tr>
<tr>
<td>Ile</td>
<td>32</td>
<td>30.1 ± 0.3</td>
</tr>
<tr>
<td>Lys</td>
<td>28</td>
<td>36.1 ± 2.1</td>
</tr>
<tr>
<td>Met</td>
<td>13</td>
<td>16.9 ± 0.4</td>
</tr>
<tr>
<td>Phe</td>
<td>34</td>
<td>33.9 ± 1.0</td>
</tr>
<tr>
<td>Pro</td>
<td>11</td>
<td>12.8 ± 0.2</td>
</tr>
<tr>
<td>Tyr</td>
<td>17</td>
<td>20.1 ± 0.2</td>
</tr>
<tr>
<td>Trp</td>
<td>7</td>
<td>ND</td>
</tr>
<tr>
<td>Ser</td>
<td>49</td>
<td>45.3 ± 0.6</td>
</tr>
<tr>
<td>Val</td>
<td>52</td>
<td>52.3 ± 0.3</td>
</tr>
<tr>
<td>Thr</td>
<td>78</td>
<td>71.0 ± 6.3</td>
</tr>
</tbody>
</table>
2.2.5 Gel Permeation Chromatography. The column (superdex 75, pharmacia biotech.) was calibrated at room temperature and the values of $V_e/V_o$ for SLT-I and its B subunit were determined under the same conditions. Experiments were run either in phosphate-buffered saline, pH 7.2 or in citrate-buffered saline, pH 5.0. Standards used were as follows: Blue dextran (2 MDa), Bovine serum albumin (66 kDa), Carbonic anhydrase (29 kDa), Ribonuclease A (18.8 kDa) and Myoglobin (14.5 kDa). The retention time of blue dextran was used for the value of $V_o$.

![Figure 2.11 Calibration of the gel permeation column.](image)

Figure 2.11 Calibration of the gel permeation column.

$V_o$ is the void volume of the column as determined by the retention time of blue dextran. $V_e$ is the elution volume of standard proteins.
2.2.6 Calculation of The Molar Absorption Coefficient (ε) of SLT-I. Three stock solutions of SLT-I (that differed in concentrations) were prepared such that their absorbance at 280 nm was between 0.1 and 0.7. Fractions of each solution were used for quantitative amino acid analysis as described in section 2.1.11, page 40. Based on the number of moles of Phe (see Table 4.2), the concentrations of the stock solution were calculated and the values were used to construct the curve shown below in Figure 2.12.

![Figure 2.12 The absorption gradient of SLT-I.](image)

According to Beer-Lambert law for absorption, \[ A = \varepsilon \cdot c \cdot l \]

where A is absorbance, c is the concentration, l is the path length (1.0 cm) and \( \varepsilon \) (slope of the curve in Fig. 2.12) is the absorption coefficient. It was determined to be \( 8.4 \times 10^4 \) L Mol\(^{-1}\) cm\(^{-1}\).
2.3 Immunological Methods

2.3.1 Generation of Rabbit Anti-SLT-I A subunit Antisera. The antisera were generated in New Zealand white rabbits using a primary injection and two booster injections. The primary injection was prepared by emulsifying 1.0 ml of SLT-I A subunit (0.1 mg/ml in PBS) with 1.0 ml of Freunds complete adjuvant. The 2.0 mls of this mixture were used to inject two rabbits (1.0 ml each). The booster injections were prepared by emulsifying 1.0 ml of SLT-I A subunit (0.05 mg/ml in PBS) with 1.0 ml of Freunds incomplete adjuvant. The first booster injection was delivered to the rabbits four weeks after the primary injection followed by the second booster injection four weeks after. The rabbits were bled 10 days after the second booster injection and the blood was centrifuged at 3000 rpm for 10 minutes. The antisera (supernatant) was collected in fractions of 0.2 ml each and immediately stored at -70 ℃.

2.3.2 Detection of Rabbit Anti-SLT-I A subunit Antibodies by ELISA. Purified SLT-I was dissolved in PBS at a concentration of 10 μg/ml. The toxin was coated on 96-well ELISA plates (1 μg/well) at RT for 1.5 hours. The uncoated surface in the wells was then blocked by incubation in PBS containing 1% (w/v) powdered skim milk at RT for 1.5 hours. Serial dilutions of the antisera were made in PBS/ 0.05% TWEEN 20 and added to the wells, in triplicate, in a volume of 100 μl/well. Following an incubation period of 1.5 hrs at RT, the plates were washed several times with PBS and peroxidase-conjugated goat anti-rabbit antibody (at a dilution of 1:2000) was added and incubated at RT for a further 1.5 hours. Finally, plates were washed and bound antibodies were detected with the peroxidase substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) as described by the manufacturer (Sigma Chemical Co., ST. Louis, MO). The bound
Figure 2.13 Detection of immobilized SLT-I with ELISA. Four rabbit antisera were generated: STA1 and STA2 (from two rabbits injected with the electroeluted SLT-I A subunit) and STA3, STA4 (from two rabbits injected with pre-boiled electroeluted SLT-I A subunit). Other details are as described in section 2.3.2, page 51.
peroxidase converts the ELISA reagent ABTS to a product that absorbs at 405 nm. This absorbance is thus a measure of the amount of bound anti-A subunit antibodies. A typical ELISA assay is shown below in Figure 2.14. The antisera were used consistently to identify SLT-I and its A subunit fragments on dot blots and western blots following SDS-PAGE. The antisera dilution used in these protocols was usually 1/1000.

2.4 Biophysical Methods

2.4.1 Fluorescence Spectroscopy. Fluorescence spectra were recorded on either a Perkin Elmer LS-3 fluorescence spectrometer (cell path length, 1.0 cm) or on a Photon Technology International (PTI) fluorometer. The B subunit was dissolved in 0.15 M NaCl to a concentration of 80 μg/ml. Titrations were performed by adding known volumes of dilute HCl and NaOH solutions to the cuvette containing the protein sample. The measurements were obtained at 25°C. The excitation wavelength was set at 298 nm (tryptophan indole group) with the maximal emission signal occurring at 350 nm. Fluorescence emission spectra were recorded from 320 to 410 nm. In experiments with peptide W232A242ShTA(220-246) and lipid vesicles, titrations were performed by adding 15 μL aliquots of preformed vesicles (5 mg/ml) suspended in either 5 mM phosphate, pH 7.0 or 5 mM acetate, pH 5.0 to a 1 cm pathlength cuvette containing the peptide W232A242ShTA(220-246) (3 μM) dissolved in 2.8 ml of the same buffer. The resulting solution was continuously stirred while spectra were acquired. The excitation wavelength was set at 280 nm and tryptophan emission spectra were recorded from 300 to 420 nm. In the case of experiments involving calcein leakage from lipid vesicles, the excitation wavelength used was 490 nm and the emission signal was monitored at 520 nm. In acrylamide quenching experiments, SLT-I (0.18 mg/ml) or its B subunit (0.137 mg/ml) were prepared in either 20
mM phosphate buffer, pH 7.4 or 20 mM acetic acid, pH 3.0 (adjusted with NaOH). The samples (3.0 ml) in the cuvette (1.0 cm x 1.0 cm) were under continuous mixing at RT. Acrylamide, at a concentration of 15 M, was added to the cuvette in 10 μl aliquots and the spectrum was recorded after each addition. The relative change in fluorescence was expressed in some figures as the ratio F_0/F, where F_0 represents the initial sample fluorescence in the absence of either lipids of acrylamide and F represents the fluorescence following the addition of aliquots of acrylamide and acrylamide.

2.4.2 Circular Dichroism. Circular dichroism spectra were recorded at 23°C on either a JASCO spectropolarimeter Model J-720 (cell path length, 0.1 cm) or a AVIV CD spectrometer model 62A DS. The B subunit concentration was 140-160 μg/ml dissolved in 0.15 M NaCl. Individual samples were prepared for each pH condition tested and the protein concentration was spectroscopically determined prior to and after spectrum collection using the extinction coefficient value at 278 nm for the B subunit of 9,500 M^{-1}cm^{-1} (monomer; Boyd, B., 1992). The pH titration experiments were performed in triplicate. Each spectrum represents the average of 12 accumulated spectra. The B subunit has an isoelectric point of 5.7 (Boyd, B., 1992). No aggregation was observed at pH levels near this value as confirmed by the recording of identical CD spectra for ten-fold diluted samples of the B subunit. In experiments with peptides and lipid vesicles, a 15 μL aliquot of a 2-4 mM stock solution of either peptide dissolved in 50% (v/v) TFE was diluted to a final volume of 2.8 mL in either 5 mM phosphate, pH 7.0 or 5 mM acetate, pH 5.0 and dispensed into a 1 cm pathlength cuvette. The final peptide concentration was typically between 10 and 20 μM. Spectra were collected with an averaging time period of 10 seconds per point. Each point represents the averaged ellipticity value recorded over a 0.5 nm spectral interval. When spectra were recorded in the presence of lipids, the peptide was added directly to a cuvette
containing lipid vesicles suspended in a constantly stirring solution. Estimates of the helical fraction of the peptide were calculated by the method of Greenfield and Fasman (1969) using the following equation:

\[
F_{\text{helix}} = \frac{[\theta]_{222}^{\text{obs}} - [\theta]_{222}^{\text{coil}}}{[\theta]_{222}^{\text{helix}} - [\theta]_{222}^{\text{coil}}}
\]

where the value of \([\theta]_{222}^{\text{helix}}\) for a 27-amino acid long peptide was calculated to be -35,740 deg.cm².dm³ (Chen et al., 1974), and the value for \([\theta]_{222}^{\text{coil}}\) was determined from a peptide solution prepared in 6M guanidine.HCl.

**2.4.3 EPR measurements.** Spectra were collected using a Varian E-104B EPR spectrometer operated in continuous wave mode and equipped with a Varian variable temperature module. Samples were contained in a sealed 50 µL glass micropipette. The peptide concentrations used were typically 20-30 µM and the peptide-to-lipid molar ratio was maintained constant at 1:120. Ascorbic acid causes the reduction of the spin label free radical thus abolishing its EPR spectrum. In the case of ascorbate reduction experiments, 2 µL of 1.0 M sodium ascorbate prepared in either 5 mM phosphate, pH 7.0 or 5 mM acetate, pH 5.0 buffers, was added to 120 µL of the peptide/vesicle mixture immediately before data collection. In some reduction experiments, ascorbate ions were entrapped into SUVs during vesicle formation and the loaded vesicles purified from free ascorbate ions by gel filtration chromatography as described above for calcein. Peptide solutions were then mixed with loaded SUVs just prior to data acquisition. For fast, nearly isotropic motion, the motional parameter \(\tau_0\) was obtained as described elsewhere (Eletr and Keith, 1972). In the case of spectra reflecting
slower motion, relative changes in motion were determined from the maximum hyperfine splitting, $T_{\text{max}}$. However, due to the low amplitude of the spectra and broadness of the outer high field peak, this value was measured as the separation of the outer low field line from the midpoint of the centre peak where it crossed the baseline, rather than as one half the separation of the outer low field line from the outer high field line.
CHAPTER 3

LOCAL CONFORMATIONAL CHANGE IN THE B SUBUNIT OF
SHIGA-LIKE TOXIN AT ENDOSOMAL pH

3.1 Introduction

Understanding the mechanism of entry of protein toxins into cells is of fundamental importance in the development of prophylactic approaches and of strategies to deliver drugs, antigens and bioactive agents to cellular compartments. A particularly successful toxin design is the "two-subunit assembly" where one subunit (A subunit) is an enzyme that specifically modifies a regulatory or essential component of the host cellular machinery while one or more copies of another subunit (B subunit) is (are) involved in the recognition and binding of the toxin to surface receptors located on target cells. Examples of this structural design include diphtheria toxin, cholera toxin, the E. coli heat-labile enterotoxins and the Shiga (ShT) and Shiga-like (SLT) toxins. The B subunits of Shiga and related toxins (Seidah et al., 1986; Calderwood et al., 1987; DeGrandis et al., 1987; Jackson et al., 1987a,b; and Strockbine et al., 1988) represent the smallest subunits (~ 70 amino acids) known to participate in this type of structural motif. These B subunits spontaneously assemble into pentamers in solution (Ramotar et al., 1990; Head et al., 1991; and Saleh & Gariépy, 1993) and bind to unique cell surface glycolipids (Lindberg et al., 1987; Lingwood et al., 1987). The pentamer non-covalently associates with a single A chain and is endocytosed through coated pits (Sandvig et al., 1989). The A chain must traverse vesicular compartments and reach the cytoplasm for the toxin to be effective. In this work, results are presented which suggest that in addition to the three functions already carried out by this small protein (pentamerization, A subunit and glycolipid-binding properties), the B subunit may play a role in the mechanism of translocation of the A chain.

Earlier work on the isolated A subunit of SLT-I had shown that it can interact with DMPC vesicles (change in tryptophan fluorescence; Surewicz et al., 1989). Binding of the A
chain to lipid vesicles may have been due to the hydrophobic nature of its A₂ domain. In fact, the crystal structure of the holotoxin has revealed that the C-terminal residues of the A chain (A₂ domain) are inserted into the hydrophobic central pore of the pentamer (Fraser et al., 1994). The A₂ domain is strongly associated with the B pentamer and it is highly unlikely that this domain would be available to interact with membranes following the internalization of the toxin. One possible mechanism by which the A₂ domain could be released would be through the unfolding of the B subunit as a result of acidification of intracellular vesicles containing the toxin. In addition to releasing the A₂ fragment, unfolding of the B subunit may promote its interaction with membranes and facilitate the release of the enzymatic A₁ fragment to the cytosol.

The objective of this study was to probe for the existence of one or more conformational states for the B subunit of Shiga-like toxin I at acidic pH conditions that would suggest its involvement in the translocation mechanism of the A chain across the endosomal compartment. These states were contrasted with those arising from protein denaturation events based on the sensitivity of its receptor-binding property to protein denaturation.

3.2 Results and Discussion

3.2.1 The fluorescence of tryptophan-34 is perturbed at endosomal pH. The presence of a single tryptophan residue at position 34 (Fig. 1.4) in the sequence of the B subunit allows us to monitor local pH-sensitive perturbations in the structure of the pentamer induced by pH changes using fluorescence spectroscopy. As shown in Figure 3.1, a sharp and reversible transition in fluorescence intensity occurs at pH 4.5. No shift in wavelength maxima (350 nm) was observed under such conditions. From the crystal structure of the B subunit
Figure 3.1 Effect of pH on the tryptophan fluorescence intensity of the B subunit of Shiga-like toxin I. Samples were initially prepared in duplicate in water containing 150 mM NaCl. One sample was titrated to low pH and one to high pH conditions (○) then both were titrated back to neutral pH (●). $\lambda_{\text{exc.}}$=298 nm; $\lambda_{\text{em.}}$=350 nm.
pentamer, the tryptophan side chain in each monomer is located at the N-terminus of the single α-helix (residues 36 to 46; Figure 2A; Stein et al., 1992) forming a "doorway" to the central channel of the pentamer. The loss in fluorescence intensity below pH 5 could be interpreted as the result of an increased exposure of the indole ring to a polar environment.

The crystal structure solved at neutral pH provides a clear indication that tryptophan-34 is exposed in all five subunits of the pentamer (Stein et al., 1992). Aspartic or glutamic acid side chains generally have pKa values between 4.4 and 4.6 in the context of proteins (Cantor & Schimmel, 1980) suggesting that the ionization state of one or more of these side chains may influence the environment of Trp-34. Examination of the crystal structure of the pentamer does not place any such side chains in the vicinity of the indole ring. However, in three of the five subunits of the pentamer, the guanidinium group of arginine-33 is located less than 2.8 Å from side chain oxygens of either Asp 16 or Asp 18 present on a loop region of an adjacent subunit (Figure 3.2). This spatial proximity suggests the existence of a salt bridge that may be disrupted by the protonation of such aspartic acid side chains. The presence of a free guanidinium group at low pH may then influence the polarity of the tryptophan side chain environment or destabilize the N-terminus of the α-helix through an unfavorable interaction with the helix dipole (Hol et al., 1978; 1981; Shoemaker et al., 1987).

3.2.2 The structure of the B subunit is gradually altered by pH conditions ranging from pH 2 to 4.5. The secondary structure of the B subunit of Shiga-like toxin I remains unaffected by pH conditions between 4.5 and pH 10 as identical spectra are recorded by circular dichroism in the far U.V. region (195 to 250 nm). The spectrum of the B subunit is altered as the pH of the sample drops below 5 (Figure 3.3A). These pH-sensitive spectral changes are reversible until pH 2 is reached. The pentamer is significantly denatured at pH conditions below 2 and above 10 (Figures 3.3B and 3.4) as the spectrum of the pentamer at neutral pH cannot be regenerated following the back titration of such samples. The circular
Figure 3.2 Stereoprotjections of the B subunit pentamer of Shiga-like toxin I (adapted from Stein et al., 1992). (A) Ribbon-like representation of the pentamer (Insight II software: Biosym Technologies, San Diego, CA). The single α-helix in each monomer gives rise to a close arrangement of 5 helices in the pentamer. The indole side chain of tryptophan-34 is located at the top of each helix. The B subunits have identical sequences and are composed of 69 amino acids. Secondary structure composition of the B subunit (Stein et al., 1992): β1, residues 3-8; β2, residues 9-14; β3, residues 20-24; β4, residues 27-31; α-helix, residues 36-46; β5, residues 49-53; β6, residues 65-68. The positions of Asp-16, Asp-17, Asp-18 and Arg 33 are illustrated in relation to Trp-34. (B) Identical representation as in A) without the ribbons. Three possible salt bridges are highlighted (Δ) where the distance separating the aspartic acid side chain oxygens of either Asp-16 and Asp-18 and the guanidinium group of Arg-33 is less than 2.8 Å (see text). The subunits of the pentamer are alphabetically labelled from A to E and the putative salt bridges would represent intersubunit contacts.
dichroism spectra of SLT-I B between pH 2 and 10 have negative maxima at 208 and 222 nm supporting the presence of α-helices (Figure 3.3A; Brahms & Brahms, 1980). At neutral pH, residues 36 to 46 adopt an α-helical conformation which represents 16% of the secondary structure elements of the molecule (Stein et al., 1992). A graph of ellipticity values at 222 nm as a function of pH (Figure 3.4) suggests a change in α-helical content starting at pH conditions lower than 4.5, with ellipticity measurements at 222 nm increasing from -8,100 deg.cm².dmol⁻¹ at neutral pH to -9,500 deg.cm².dmol⁻¹ at pH 2 (Fig. 2.4).

The gradual change in conformation observed between pH 2 and 4.5 (Figures 3.3A and 3.4) is not concomitant with the sharp local perturbation observed for Trp-34 (Figure 3.1) and would suggest the existence of at least two conformational stages for the B subunit pentamer at low pH. As discussed below, both states involve stable conformations rather than denatured states of the B subunit since the pentamer retains its ability to bind the glycolipid Gb₃ under these conditions. The conformational transition occurring below pH 4.5 may be shifted to a higher pH (closer to endosomal pH for example) in the context of the B subunit being bound to a Gb₃-containing membrane or in association with the A subunit. Indeed, the pH-induced conformational change in the A chain of diphtheria toxin is sensitive to its environment, with the transition pH between hydrophillic and hydrophobic states being shifted from pH 3.5 to 5 in the presence of small unilamellar vesicles (Zhao & London, 1988). Its isolated B subunit also undergoes a pH-induced exposure of a hydrophobic domain (Boquet et al., 1977; and review by London, E., 1992).

3.2.3 The tertiary and quaternary structure of the B subunit pentamer are stable between pH 2 and 10. The crystal structure of SLT-I B subunit has recently been derived from the X-ray diffraction analysis of crystals grown at neutral pH (pH 6.8-7.0; Stein et al., 1992). The protein exists as a pentamer. The peptide backbone adopts a structure reminiscent of the
Figure 3.3 Effect of pH on the circular dichroism spectrum of the B subunit of Shiga-like toxin I (195-250 nm region). (A) Spectra were recorded under neutral and acidic pH conditions: pH 7.0, pH 4.0, pH 3.0, and pH 2.0 (see legend in figure). (B) Spectra of denatured forms of the B subunit at pH 1.3 and pH 12.0 are contrasted with the reference spectrum of the B subunit recorded at pH 7.0. Each spectrum represents the average of 12 accumulated spectra (refer to experimental methods in chapter 2, page 54).
Figure 3.4 Effect of pH on ellipticity values at 222 nm. Ellipticity measurements (\([\theta]_{222\text{nm}}\)) were derived from spectra recorded as described in Figure 2.3. The graph was constructed using spectra collected from three independent titrations. The pH and protein concentration of each sample were determined prior to and after spectrum acquisition. The ellipticity data was extracted from individual spectra representing the average of 12 accumulated spectra (refer to experimental methods in chapter 2, page 54).
larger B subunit of the *E. coli* heat-labile enterotoxin (Sixma et al., 1991; Stein et al., 1992; Sixma et al., 1992). Each B subunit is composed of 6 β-strands and one α-helix linked together by a series of short turns. Pentamerization occurs as a result of intersubunit contacts between two β-strands (β-2 and β-6) on adjacent B subunits (Figure 3.2A; Stein et al., 1992). The SLT-1 B subunit spontaneously assembles into pentamers in solution, with the presence of monomers or other multimers not being observed by gel filtration chromatography or by sedimentation equilibrium experiments (Ramotar et al., 1990; Saleh & Gariépy, 1993). Monitoring the oligomeric state of the B subunit by high performance gel filtration chromatography at pH 5, thus approximating the pH of the endosomal or lysosomal compartments (Maxfield, 1982; Geisow, 1984; Geisow & Evans, 1984), revealed that the structure of the oligomer remains intact eluting with the expected retention time and showing no sign of disaggregation (results not shown).

A more important criteria proving the integrity of the B subunit structure was to monitor the ability of the pentamer to bind to its natural receptor, the glycolipid globotriaosylceramide (Gb₃) (Lingwood et al., 1987). Indeed, denaturation events such as the reduction of the single disulfide bond in SLT-I B followed by carboxyamidomethylation, lead to the dissociation of the pentamer and result in a loss of receptor binding property (Boyd, 1991). In this assay, Gb₃ was immobilized onto solid phase and radioiodinated SLT-I B was allowed to associate with the bound receptor (Ramotar et al., 1990). The radiolabeled subunit was incubated in a range of pH conditions, prior to and during the binding step. The level of specific binding was assessed by performing the binding step in the presence and absence of an excess of unlabeled B subunit. From the histogram presented in Figure 3.5, one can conclude that the specific binding of the toxin to its receptor is not affected by acidic pH as low as 3.5. A significant level of specific binding still occurs even after exposing SLT-I B to pH levels below 2. A similar result was observed when the
radiolabeled B subunit was allowed to bind initially to Gb₃ at neutral pH, and the complex later exposed to lower pH conditions (pH 3 and pH 5; results not shown). The stability of the tertiary or quaternary structure was more sensitive to alkaline conditions particularly pH levels above 10. Boiling the radiolabeled B subunit for 10 minutes prior to performing the binding assay resulted in a total loss of binding to Gb₃ (Figure 3.5).

In summary, the changes observed in the tryptophan fluorescence and circular dichroism experiments under acidic pH conditions do not parallel a loss in tertiary or quaternary structure. These changes suggest the existence of a discrete conformational transition at endosomal pH's. A similar effect was not observed at alkaline conditions where denaturation as monitored by loss of receptor binding property correlates with large changes in ellipticity values at 222 nm (Figure 3.5).
Figure 3.5 Histogram depicting the effect of pH on the specific binding of radiiodinated Shiga-like toxin I B subunit to its natural receptor Gb3. Open bars, total binding; closed bars, non-specific binding (presence of excess unlabeled B subunit). B, denatured B subunit as a result of boiling the protein sample prior to performing the binding assay. Error bars represent the standard deviation associated with experiments performed in triplicate (refer to experimental methods in chapter 2, page 37).
CHAPTER 4

EFFECT OF pH ON THE CONFORMATION OF THE
HOLOTOXIN

This chapter represents a series of unpublished results and observations relating to the effect of pH on the structure of the holotoxin.
CHAPTER 4

4.1 Introduction

Exposure of internalized bacterial toxins to reduced pH levels may result in conformational changes in one or several parts of the molecule. Work with diphtheria toxin (DT) provided evidence that the A fragment may be responsible for membrane penetration and translocation, a function previously suspected to be encoded by other subunits. Zhao and coworkers (1988) have demonstrated that diphtheria toxin A subunit undergoes a reversible conformational change at pH below 5, which results in the exposure of tryptophanyl residues. These conformational changes also result in the exposure of a hydrophobic domain, as judged by an increase in toxin binding to detergent micelles. Proteolysis protection experiments with Pseudomonas aeruginosa exotoxin A (PE) and diphtheria toxin implicates that both the A and B subunits may function together in a concerted manner during membrane insertion and translocation. Incubation of cells with $^{125}$I-labelled diphtheria toxin followed by treatment of cells with pronase showed that two fragments of 21 kDa and 25 kDa were protected from proteolysis (Moskang et al., 1988). The 21 kDa fragment was identified as the A fragment whereas the 25 kDa represents the B fragment.

Exotoxin A, on the other hand, is a single polypeptide chain of 613 amino acids (Grey et al., 1984) comprising three functional domains (I, II and III). In experiments involving exotoxin A and lipid vesicles, it was found that the toxin induces vesicles permeabilization at acidic pH by formation of ion-conductive channels (Menestrina et al., 1991). The toxin domain interacting with lipids was hypothesized to be the highly helical domain II of the toxin and includes residues 253-300 (Allured et al., 1986). This region falls between domain I (enzymatic domain) and domain III (receptor binding domain). It is
therefore possible that in some protein toxins, domains may function as one unit in membrane penetration. In light of previous results observed with DT and PE, we decided to test whether or not SLT-I may have membrane active properties and to monitor the effect of pH on the structure of the holotoxin. Since most of the hydrophobic regions of SLT-I are buried in the mature structure, the toxin will have to alter its conformation. We hypothesize that the acidification of its environment may trigger a conformational rearrangement. Unlike in the case of diphtheria toxin and exotoxin A, acidic pH does not induce SLT-I to translocate across surface membranes of cells. Proteolysis protection experiments would thus not be revealing. Fluorescence and circular dichroism spectroscopies provide sensitive tools to monitor small changes in the structure of the holotoxin.

The B subunit of SLT-I does undergo a small conformational change at low pH levels but such a change may not be directly involved in membrane translocation of the A subunit (see chapter 3). The A subunit of SLT-I may mediate its own translocation across intracellular membranes, as was observed in diphtheria toxin. Alternatively, the site of membrane translocation by SLT-I has been hypothesized to be in the lumen of the endoplasmic reticulum (ER) of sensitive cells (Garred et al., 1995a), where the pH is near neutrality (Bartido et al., 1995). It can be therefore argued that Shiga and related toxins may not need to change structure at acidic pH since the translocation would occur at neutral pH. Nonetheless, the toxin may require acidic pH to change its conformation in order to alter its interaction with endogenous factors (proteases, lipids or protein translocation factors) en route to the ER. Such interactions would be crucial for subsequent interactions with and translocation across the ER membrane. In this section, we report the effect of acidic pH on the conformation of SLT-I, as determined by fluorescence spectroscopy and CD, and discuss the results in the context of membrane insertion and translocation.
**Figure 4.1** Various representations of the structure of SLT-I highlighting the locations of the tryptophans. (A) The A subunit (yellow) has two Trp residues (stick models in magenta) at positions 203 and 277. Each of the B chains (green colour) has one Trp residue (stick model in magenta) at position 34. (B) The same colour scheme as in (A) in a space-filling model to show the exposure of the Trp side chains to the solvent. It can be seen that the tryptophans in the A subunit are not exposed to the solvent. (C) The same model shown in (B) but the structure is rotated such that the view is at the receptor binding face of the B subunit pentamer. Trp-34 in the B subunit is shown in magenta and unlike those of the A subunit, the B subunit Trp is clearly exposed to the solvent. The C-terminal helix of the A subunit is shown (yellow), as well as a portion of Trp-277 (magenta) through the central pore of the pentamer.
4.2 Results and Discussion.

4.2.1 Acidic pH Alters the Microenvironment of Tryptophan(s). SLT-I contains a total of seven Trp residues; one in each of the five B chains and two in the A subunit (Fig. 4.1). These intrinsic probes allowed us to monitor the conformation of the toxin at different regions in the toxin. The crystal structure of the free SLT-I B subunit and that of the holotoxin show that the five Trp residues (Trp-34) in the B subunit pentamer are clustered and exposed to the solvent (Fig. 4.1). This feature is reflected in the emission fluorescence spectrum of the B subunit (Fig. 4.2), which shows a maximum at about 353 nm. The Trp fluorescence emission spectrum of SLT-I is not symmetrical, indicative of heterogeneity in the environment of Trp residues (Fig. 4.2). The spectrum of SLT-I also shows a distinct plateau at about 335 nm. This blue shift in maxima reflects the shielded nature of Trp in the A subunit (Fig. 4.1).

The pH titration of SLT-I shows a complex titration curve that reflects the heterogenous Trp populations within the toxin. Association of the A subunit with the B pentamer occurs through the central pore and through the A-face of the pentamer (Fig. 4.1) and does not affect Trp-34 in the B subunit. Thus, it would be reasonable to assume that pH will alter the microenvironment of Trp-34 to a similar extent as in the free B subunit. Titration of Trp-34 with pH shows a sharp and reversible quenching of the fluorescence centred at pH 4.5 (see chapter 3). This is not observed in the pH titration of the holotoxin. Instead, the titration curve of SLT-I (Fig. 4.3) shows three distinct regions: a region of small quenching between pH 7 and pH 4, a region of sharp quenching between pH 4 and pH 3 followed by another slow quenching region below pH 3. The changes observed between pH 7 and pH 3 are reversible whereas those observed below pH 3 are not reversible. It will not be possible to assign these regions to specific Trp residues. However, it shows that the
Figure 4.2 Tryptophan fluorescence emission spectra of SLT-I (solid line) and SLT-I B subunit (dashed line) in PBS. The protein concentrations used were 0.18 mg/ml (18 μM Trp) for SLT-I and 0.137 mg/ml (18 μM Trp) for SLT-I B subunit (refer to experimental methods in chapter 2, page 53).
Figure 4.3 Change in tryptophan fluorescence of SLT-I as a function of pH. The pH of the solution (150 mM NaCl in water) containing the toxin was reduced by the addition of 10μl aliquots of 60 mM HCl to the 1.0 cm cuvette, containing a total of 3.0 ml of sample. The solution was continuously mixed. The toxin concentration was 0.18 mg/ml (refer to experimental methods in chapter 2, page 53).
microenvironment of more than one Trp in the holotoxin may be perturbed by acidic pH.

4.2.2 Acidic pH Does Not Alter The Exposure of Tryptophans in SLT-I. Acrylamide quenching was used to monitor the exposure of tryptophans in SLT-I. Quenching of Trp-34 fluorescence in the free B pentamer with acrylamide clearly demonstrates the exposure of the indole ring and correlates with the emission maximum at 353 nm observed in Figure 4.2. The quenching effect can be fitted by the Stern-Volmer equation (Birks, J. B., 1970):

\[ \frac{F_0}{F} = 1 + K_{sv}(\text{eff}) Q \]

which gives a value of 8.1 for \( K_{sv}(\text{eff}) \) at pH 7.4, a value comparable to numbers reported for other proteins with exposed tryptophan (Eftink & Ghiron, 1976; 1981; Macek et al., 1995). The same equation can be used to determine \( K_{sv}(\text{eff}) \) from the initial rate of quenching of SLT-I (Fig. 4.4) and a value of 2.4 was obtained at pH 7.4. This value agrees with other values obtained from proteins containing shielded Trp residues (Eftink & Ghiron, 1976; 1981; Macek et al., 1995) and further supports the results observed in the previous section. The quenching of Trp emission at acidic pH is achieved at a similar rate to that observed at neutral pH (Fig. 4.4), as determined from the initial rate of acrylamide quenching. At higher acrylamide concentrations, the quenching curves deviate from each other, particularly in the case of the holotoxin. The deviation is not large and is primarily due to experimental errors. It can be concluded from this section that the decrease in Trp fluorescence at acidic pH in the holotoxin is not due to exposure of shielded tryptophans but rather due to changes in the polarity of the Trp microenvironment as a result of changes in the ionic state of charged side chains in the vicinity of Trp.
Figure 4.4 Acrylamide quenching of Trp fluorescence in SLT-I and its B subunit at neutral and at acidic pH. The quenching was performed in either 20mM phosphate buffer (pH 7.4) or 20 mM acetic acid (adjusted to pH 3.0 with NaOH). Concentrations of proteins were as specified in Fig. 4.2 (refer to experimental methods in chapter 2, page 53).
4.2.3 pH does not alter the secondary structure of SLT-I. Monitoring the secondary structure of SLT-I provides information about global changes in its conformation that may not be apparent by fluorescence spectroscopy. The CD spectrum of SLT-I in the far UV (Fig. 4.5) shows negative maxima at 222 nm and at 208 nm, which reflect the presence of α-helices. This result was expected since the X-ray structure of the holotoxin shows that the A subunit is made up of a large number of α-helices (Fraser et al., 1994). The CD spectrum of SLT-I at neutral pH gives an ellipticity value of ~ -12,000 deg.cm².dmol⁻¹ at 222 nm (Fig. 4.5). This value translates to an α-helical content of approximately 30%. This value agrees with the calculated α-helical content derived from the X-ray structure of SLT-I (Fraser et al., 1994), where the A subunit is about 30% α-helical and the B subunit is approximately 15% α-helical. The CD spectrum of SLT-I at pH 7.4 and pH 5, representing a range of pH levels expected to be present in intracellular compartments, are identical (Fig. 4.5). This result indicates that the secondary structure of SLT-I is not sensitive to mild acidic conditions.

Both fluorescence and CD observations were confirmed by size exclusion chromatography (Table 4.1). Changes in the folding of a protein results in changes to its volume, which in turn changes its mobility in a high-pressure size exclusion experiment (Corbett and Roche, 1984). As shown in Table 4.1, pH 5.0 does not affect the folding of SLT-I or its B subunit. The differences in retention times seen in Table 4.1 between neutral and acidic pH fall within experimental error. This technique is limited in sensitivity and will not detect small changes in conformation. However, given the scale of conformational changes that would be required to mediate membrane insertion of the holotoxin or its enzymatic fragment, it is clear that endosomal pH alone does not produce such effects on the holotoxin or its B subunit. In contrast to these results, endosomal pH have significant effects on the conformation of DT and PE. Employing similar techniques, Blewitt et al. (1985) have shown that endosomal pH induces a conformational change in DT leading to the exposure of
Figure 4.5 CD spectra of SLT-I in various pH conditions. The spectra of the toxin (0.12mg/ml final concentration) were collected in 20mM phosphate, pH 7.4; 20mM citrate, pH 5.0 and in 20mM acetic acid, pH 3.0. The pathlength was 0.1 cm and the experiments were performed at room temperature (refer to experimental methods in chapter 2, page 54).
Trp residues. Associated with those changes was the exposure of hydrophobic surfaces that lead to increase in the membrane interaction of DT. Similar effects were observed in PE, where low pH was found to induce a change in the structure of the toxin that were suggestive of partial unfolding and increased hydrophobicity (Farahbakhsh et al., 1987; Jiang and London, 1990).

**Table 4.1**

Effect of endosomal pH on the gel permeation properties of SLT-I and its B subunit

<table>
<thead>
<tr>
<th></th>
<th>$V_e/V_o$‡</th>
<th>$V_e/V_o$‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH 7.2</td>
<td>1.36</td>
<td>1.33</td>
</tr>
<tr>
<td>PH 5.0</td>
<td>1.60</td>
<td>1.62</td>
</tr>
</tbody>
</table>

‡See chapter 2, page 49 for details

In conclusion, it was observed that SLT-I does not contain a pH-sensitive molecular "switch", which could potentially lead to the exposure of a previously-shielded hydrophobic domain segments and thus, allow the toxin to insert into the membrane in the appropriate compartment and translocate the A₁ fragment to the cytosol. It is therefore necessary to re-evaluate the search for a pH-triggered switch in the holotoxin and search for another mechanism that members of this family of toxins may utilize to access their target in the
cytosol. As was observed in the case of the isolated SLT-I B subunit (chapter 3), acidic pH induces local conformational changes in the structure of the holotoxin. During intracellular trafficking, such effects may be important for the proper cleavage of the toxin and/or the reduction of the disulfide bond holding the $A_1$ and the $A_2$ fragments. Improper intracellular processing could be detrimental to the delivery of the enzymatic fragment to the cytosol.
CHAPTER 5

INSERTION AND ORIENTATION OF A SYNTHETIC PEPTIDE REPRESENTING THE C-TERMINUS OF THE A1 DOMAIN OF SHIGA TOXIN INTO PHOSPHOLIPID MEMBRANES

CHAPTER 5

5.1 Introduction

Since the target site for the N-glycosidase activity of the A₁ chain is a single adenine base of 28S rRNA (Endo et al., 1988; Saxena et al., 1989), the A₁ subunit must possess a mechanism to translocate to the cytoplasmic side of the ER membrane. In the case of diphtheria toxin, there is evidence that the toxin exploits the acidification of the endosomal compartment to undergo a conformational change and exposes a hydrophobic domain that enables it to translocate across membranes (Donovan et al., 1981; Hu et al., 1984; Blewitt et al., 1985; and Rolf et al., 1993). A similar effect has been observed in the case of Pseudomonas exotoxin A where low pH alters its structure to reveal a membrane binding domain (Farahbakhsh & Wisnieski, 1989). Other toxins such as ShT and ricin appear to lack a response to endosomal pH and may have developed an alternate mechanism for relocating their enzymatic domain to the cytosol.

One possible mechanism is the exposure of a hydrophobic domain as a result of intracellular processing events enroute to the ER. Members of the ShT family of toxins contain a 9-amino acid long segment (residues 246-254) which include the well-defined furin-sensitive motif RXXR (for review, see Gordon & Leppla, 1994). Proteolytic cleavage at multiple sites within this nonapeptide segment takes place during the intravesicular trafficking of the toxin (Takao et al., 1988; Blum et al., 1991; and Garred et al., 1995a,b). An inspection of the A-chain sequence of ShT and related toxins reveals the presence of a hydrophobic signal-sequence-like domain between residues 220 and 246 (Fig. 4.1). This hydrophobic region contains residues usually found in signal peptides and transmembrane protein segments (Perlman & Havorson, 1983; Briggs & Gierash, 1986). The region immediately precedes the start of the nonapeptide cleavage sites (residues 246 to 254) located
in the A-chain protease-sensitive loop (Cys-242 to Cys-261) and would represent the C-terminus of the A₁ fragment. The X-ray structure of Shiga toxin shows that this hydrophobic segment of the A subunit is not initially exposed to solvent in the native toxin but rather hidden at the interface between the A subunit and the B subunit pentamer (Fraser et al., 1994). In the case of ricin, the isolated A subunit shows higher avidity for membranes than the holotoxin, indicating the exposure of a hydrophobic domain upon dissociation from the B subunit (Ishida et al., 1983; Utsumi et al., 1984; Yamasaki et al., 1988; Utsumi et al., 1989; Bilge et al., 1995). We therefore hypothesize that the reduction of the single disulphide bond of the A chain of ShT and its limited proteolysis may facilitate the release of the A₁ fragment from the holotoxin and/or expose its hydrophobic C-terminus thus favouring its interaction with membranes.

5.2 Objectives

In an effort to study the potential of the C-terminus tail of the A₁ domain to interact with membranes, peptides corresponding to residues 220 to 246 of the ShT A subunit were synthesized and their interaction with small unilamellar vesicles (SUV) was characterized. Spectroscopic results demonstrate that this region of the A₁ domain inserts into negatively charged membranes and assumes an α-helical structure with a large fraction of the bound peptide probably spanning the width of a negatively charged lipid bilayer at neutral pH.

5.3 Results and Discussion

The A₁ chain of Shiga and related toxins must transit from the lumenal side of the endoplasmic reticulum to the cytoplasmic side to be able to effectively interact with ribosomal subunits and block protein synthesis. This translocation event may be initiated by a specific
domain of the toxin as in the case of other bacterial toxins (*Pseudomonas* exotoxin A, diphtheria toxin). Such a domain has not been identified in bacterial toxins such as Shiga and Shiga-like toxins. We propose that the C-terminus created as a result of proteolysis and reduction of the A chain in this toxin family may represent a functional domain triggering membrane translocation. Recently, a proline mutation (Pro250Ala) in a 12-residue hydrophobic segment located near the C-terminus of the A-chain of ricin has been shown to affect the ability of ricin to intoxicate Vero cells but did not affect its N-glycosidase activity (Simpson et al., 1995). This finding was interpreted as suggesting that this hydrophobic domain may be involved in membrane translocation.

A representation of the A chain of Shiga toxin in Figure 5.1 (panels A and B), highlights the presence of a proteolytic loop (Cys-242 to Cys-261) flanked on its amino terminus by a 27-amino acid long signal-sequence like domain (residues 220-246). This domain represents the C-terminus of the A1 subunit created as the result of transcytosis modifications. The hydrophobicity profile of the A chain (Fig. 5.1A; derived using the method of Engelman et al., 1986) further suggests that this domain (darkened area) may represent a transmembrane region. Sequence alignments of Shiga and related toxins (Fig. 5.1C) with a segment of the A chain of ricin implicated in membrane translocation (Simpson et al., 1995) illustrates the existence of a common hydrophobic region within these molecules. Ricin and Shiga toxins have A chains with homologous functions. The crystal structure of Shiga toxin (Figure 1.5) has indicated the existence of only a limited number of interactions between its A1 domain and the B subunit pentamer. In particular, residues 226 to 242 are buried under most of the A1 chain, in close proximity to the B pentamer, but making no contact with the pentamer (Fraser et al., 1994). Thus, most of the C-terminus of the A1 chain is not initially accessible to solvent as part of the intact toxin. We hypothesize that this domain becomes exposed after cleaving the protease-sensitive loop at one or more
Figure 5.1  (a) Hydrophobicity profile of the A subunit of Shiga toxin (ShT) using the Goldman, Engelman and Steitz (GES) scale (Engelman et al., 1986). The A chain of Shiga toxin is proteolytically cleaved into two domains, namely a catalytic A₁ domain and a short A₂ domain associated with the binding subunit of the toxin. The shaded area in the hydrophobicity profile identifies the sequence from residues 225 to 242 as representing the most dominant hydrophobic region of the A₁ chain.

(b) Schematic representation of ShT A subunit organization showing the relative location of the hydrophobic region in relation to the known protease sensitive loop (Cys 242 to Cys 261). Established (▲) and putative (cathepsins B and D; †) cleavage sites within the loop are highlighted on the amino acid sequence (residues 220 to 246).

(c) Sequence alignment of the C-terminus of ShT A₁ with regions of other ribosome-inhibiting toxins. Abbreviations and sequences listed in this panel are associated with the following toxins: SLT-IIv, residues 212 to 251 of the A subunit of Shiga-like toxin II variant; RTA, residues 225-268 of the A chain of ricin; AbA, residues 217 to 249 of the A subunit of abrin; DT, residues 341 to 380 of diphtheria toxin. Several protein toxins possess a stretch of hydrophobic amino acids (boxed sequences) flanked by positively charged amino acids at neutral pH (R, Arg) as well as histidines (H) which possess a positive charge at endosomal pH.
(a)

(b)

RVGRISFGSINAILGSVALILNCHHHRASRVARMASDEFPSMC

(c)

ShTA  DYHGQDSVRV GRISFGSINA ILGSVALILN CHHHRASRVAR ....
SLTIvA  EYRGEDGVVR GRISFNNISA ILGTVAVILN CHHQPASRVAR ....
RTA  ...GAFASPIQ LQRRNGSKFS VY DVSILIPILAL MVYRCAPPSSQF
AbA  ..........LTNRNEPVTV DLSHPTVAVLALMLFVQNPPN......
DT  LMAIPLVGE LVD [IGFAAYN FVESIINLFQV] HNSYNPAY
sites and reducing the disulphide bridge between Cys-242 and Cys-261. The newly created signal sequence-like C-terminus may then possess membrane active properties that would facilitate the A1 chain insertion from the lumenal side of the Golgi or ER membranes.

Peptides were thus synthesized to investigate the ability of this region of the A1 domain to interact and insert into phospholipid membranes. Two peptides representing residues 220 to 246 of the A1 domain, were assembled by solid-phase peptide synthesis. Their amino terminus was acetylated with acetic anhydride to insure the absence of an unnatural positive charge. The peptide ShTA(220-246) includes a naturally occurring cysteine residue at position 242 which provided a unique site for the subsequent incorporation of a paramagnetic probe (proxyl group) to monitor the insertion and orientation of the peptide into unilamellar vesicles. The peptide analog, W232A242Sh-TA(220-246), included two mutations, namely Cys242Ala and Ile232Trp to monitor its interaction with membranes by fluorescence spectroscopy. Spectroscopic experiments were performed at two pH values, namely 5.0 and 7.0 to mimic representative pH conditions observed in endosomal and ER compartments respectively.

5.3.1 ShTA(220-246) adopts a partially helical structure in the presence of negatively charged lipid vesicles. The circular dichroism spectrum of ShTA(220-246) dissolved in 5 mM phosphate, pH 7.0, suggests that this peptide lacks structure (random coil) in aqueous solutions (Fig. 5.2). The mean residue ellipticity (MRE) at 222 nm was experimentally determined to be -5,000 ± 600 deg.cm².dmol⁻¹. The propensity of ShTA(220-246) to adopt a partially α-helical conformation in hydrophobic environments was demonstrated in the presence of SDS micelles (MRE of -18,000 ± 900 deg.cm².dmol⁻¹ at 222 nm). This finding was further supported by recording the spectrum of the peptide in 50% (v/v) TFE, where the ellipticity maxima at 222 nm reached a value of -22,000 ± 1,100 deg.cm².dmol⁻¹. The
Figure 5.2  Circular dichroism spectra of the peptide ShTA(220-246) dissolved in either 5 mM phosphate, pH 7.0 (---), in 10% (w/v) SDS (—), or in 50% (v/v) TFE (—). The peptide concentration was 15 µM (refer to experimental methods in chapter 2, page 54).
Figure 5.3  Circular dichroism spectra of the peptide ShTA(220-246) in the presence of small unilamellar vesicles composed of DMPG lipids. The spectrum of the peptide dissolved in 5 mM phosphate buffer, pH 7.0 (---) is compared to that of the peptide exposed to DMPG vesicles at pH 7.0 (- -) and pH 5.0 (—). The peptide concentration was 15 μM (refer to experimental methods in chapter 2, page 54).
peptide also assumed a partially α-helical structure in the presence of vesicles constructed with negatively charged lipids (DMPG; Fig. 5.3). Similar CD spectra were recorded at pH 5.0 and pH 7.0, with a negative maxima at 222 nm of 16,750 ± 1,200 deg.cm².dmole⁻¹ at a peptide-to-lipid ratio of 1:50. This ellipticity value translates to an approximate helical content of 45-50%. Since about half of the added peptide was bound to the lipid (see below), this finding indicates that the membrane-bound peptide is mostly α-helical in structure at pH 7.0 and to a lesser extent at pH 5.0. In the presence of vesicles composed of neutral lipids such as DMPC, the peptide appears to possess significantly less α-helical character with a MRE 222 nm of -11,000 ± 850 deg.cm².dmole⁻¹ at pH 7.0 and half that value at pH 5.0 (Fig. 5.4). A broad negative maxima centred at 218 nm was observed in the spectrum of ShTA(220-246) exposed to DMPC vesicles at pH 7.0 and suggests the existence of one or more peptide conformations that would include β-sheet containing structures. ShTA(220-246) tends to slowly aggregate in aqueous buffers (hours) giving rise to a circular dichroism spectrum showing a minima between 213 and 218 nm, a characteristic feature of β-sheet structure (data not shown). Aggregation may be nucleated by the presence of DMPC vesicles at pH 7.0.

5.3.2 Changes in the fluorescence spectrum of W232A242ShTA(220-246) in the presence of lipid vesicles suggest that this hydrophobic region of the A1 domain partitions readily into a lipid bilayer. Substituting isoleucine with tryptophan at position 232 provided us with an intrinsic probe to monitor the environment of the hydrophobic core of the peptide. The Trp emission spectra of the peptide in solution showed a maximum at 354 nm, indicating that the tryptophan side chain was exposed to the aqueous environment. As DMPG vesicles were added to the peptide solution at pH 7.0, a blue shift (354 nm to 335 nm) in the emission maximum of the peptide was observed (Fig. 5.5A) indicating that the Trp side chain had
Figure 5.4 Circular dichroism spectra of the peptide ShTA(220-246) in the presence of small unilamellar vesicles composed of DMPC lipids. The spectrum of the peptide dissolved in 5 mM phosphate buffer, pH 7.0 (---) is compared to that of the peptide exposed to DMPC vesicles at pH 7.0 (—) and pH 5.0 (—>). The peptide concentration was 15 μM (refer to experimental methods in chapter 2, page 54).
migrated to a hydrophobic environment (Lakowicz, 1983). Changes in the fluorescence intensity at 335 nm (F/Fo) were also monitored as a function of lipid concentration (Fig. 5.5B). The increase in fluorescence intensity correlated with the observed spectral shift to 335 nm (Fig. 5.5A). Both measurements support the concept that the indole ring has relocated to a hydrophobic environment (Wharton et al., 1988). Similar spectral characteristics were observed at pH 5.0 in the presence of DMPG vesicles. Changes in the spectra of W232A242ShTA(220-246) in the presence of DMPC vesicles at pH 7.0, were comparable to those observed in the presence of DMPG vesicles. However, these changes occurred at higher lipid-to-peptide ratios. These results were not observed for the peptide mixed with DMPC vesicles at pH 5.0. Mixtures of acidic and zwitterionic lipids were also used to mimic physiological conditions expected on the inner leaflet of the ER membrane.

The composition of the inner leaflet of rat liver rough endoplasmic reticulum is dominated by zwitterionic (neutral) lipids such as phosphatidylcholine and phosphatidylethanolamine (Bollen & Higgins, 1980; Zachowski, 1993). However, acidic lipids such as phosphatidylserine and phosphatidylinositol account for 7% up to 25% of the rough ER inner leaflet composition (Bollen & Higgins, 1980). This lipid leaflet is thus negatively charged. The association of W232A242ShTA(220-246) with such membranes was assessed using DMPC vesicles containing a level of DMPG (20:80 ratio, DMPG:DMPC) representative of the proportion of negatively charged lipid present in the rat liver ER inner lipid leaflet. The effects on both the fluorescence intensity and shift in wavelength emission maxima of W232A242ShTA(220-246) were similar to those observed for the peptide interacting with vesicles composed of DMPG lipids only (Fig. 5.5A,B), although requiring more lipids. When vesicles were constructed with a 5:95 ratio of DMPG to DMPC, the changes observed were intermediate to those recorded when W232A242ShTA(220-246) was added to either pure DMPG or DMPC vesicles alone (data
not shown). The influence of mixed lipid vesicles on the fluorescence spectral properties of
the peptide suggests that the presence of low levels of negatively charged lipids (such as
DMPG) in membranes can strongly dictate the association of W^{232}A^{242}ShTA(220-246) with
lipid vesicles.

5.3.3 Binding of radiolabeled W^{232}A^{242}ShTA(220-246) to lipid vesicles. A \(^{14}\)C-acetyl group
was initially introduced at the N-terminus of W^{232}A^{242}ShTA(220-246) during synthesis.
The radiolabeled peptide was shown by centrifugation to interact directly with small
unilamellar vesicles. The fraction of bound peptide was determined to be 0.48 ± 0.09 at pH
7.0 and 0.51 ± 0.07 at pH 5.0 in the presence of DMPG vesicles while values of 0.31 ±
0.07 at pH 7.0 and 0.1 ± 0.05 at pH 5.0 respectively, were calculated for the fraction of
peptide bound to DMPC vesicles. These results suggest that W^{232}A^{242}ShTA(220-246)
partitions more favourably into a membrane containing negatively charged lipids such as
DMPG lipids than in neutral lipids exemplified by DMPC. In addition, the reduced fraction
of peptide bound to vesicles containing a zwitterionic lipid (DMPC) at pH 5.0, suggests that
the protonation of the three C-terminal histidines of W^{232}A^{242}ShTA(220-246) may
accentuate its partitioning into the aqueous phase. Since the helical content of the peptide in
the presence of DMPG vesicles at both pH 5.0 and 7.0 was estimated to be approximately
50% and since the CD spectrum of the peptide free in solution (Fig. 5.2-5.4) lacks helical
character, one can conclude that the population of membrane-bound peptide must adopt a
mostly \(\alpha\)-helical structure.

5.3.4 Monitoring the interaction of ShTA(220-246) with membranes by EPR. The naturally
occurring cysteine residue (Cys-242; see Figure 5.1) located near the C-terminus of ShTA(220-246) provided a useful site to insert a paramagnetic proxyl group in the peptide. As monitored by EPR spectroscopy, the covalently attached proxyl probe displayed its expected nearly isotropic mobility when the peptide was dissolved in an aqueous environment. The spectrum of the proxyl-peptide conjugate highlights the anticipated triplet of narrow spectral lines (Figure 5.6A). The value of the proxyl’s motional parameter \( \tau_0 \) was significantly increased (Table 5.1; 0.30 ns) when compared to the free spin label (0.02 ns), reflecting the fact that the probe is less mobile when bound to the peptide. When the labelled peptide was mixed with vesicles of DMPC, there was little effect on the spectrum of the probe except for a small increase in \( \tau_0 \) values (Table 5.1), suggesting that there was little interaction of the peptide with DMPC vesicles at pH 7.0. The circular dichroism spectrum of ShTA(220-246) observed in the presence of DMPC vesicles at pH 7.0 (Figure 5.4), suggests the existence of peptide conformers containing \( \beta \)-sheet structure and indirectly supports the presence of microaggregates of the peptide on the surface of DMPC vesicles. Such an event would explain our binding results where 31% of the peptide population was found associated with DMPC vesicles at a peptide-to-lipid ratio of 1:50.

In the presence of negatively charged DMPG vesicles, the spectrum at 26.5 °C contained two spectral components (Figure 5.6B). One component (mobile component), representing the free peptide, was defined by the set of sharp lines previously observed in the spectrum of the proxyl-peptide in solution (Figure 5.6A). The other spectral component (immobilized component) was broader with greater hyperfine splitting and reflected the more restricted motion of the spin-labelled peptide bound to DMPG vesicles. The amount of immobilized component was greater at pH 5 than at pH 7 (Figure 5.7A and 5.7B). The mobility of the spin-labelled peptide bound to DMPG rose with increasing temperature, as
Figure 5.6 Effect of DMPG vesicles and ascorbic acid on the EPR spectrum of proxyl-labelled ShTA(220-246). The EPR spectrum of the labelled peptide was recorded for the peptide dissolved in 5 mM phosphate buffer, pH 7.0 (spectrum A), in the presence of DMPG vesicles suspended in the same buffer (spectrum B), and after the external addition of 12 mM (final concentration) ascorbic acid to the peptide/vesicles mixture (spectrum C). For details, refer to experimental methods in chapter 2, page 55.
Table 5.1

EPR parameters derived from spectra of spin-labelled ShTA(220-246) in different environments a

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>$T_{\text{max}}$ (G)</th>
<th>$\tau_0$ (nsec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free spin label</td>
<td>7.0</td>
<td>26.5</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>Spin-labelled peptide</td>
<td>7.0</td>
<td>6.5</td>
<td></td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26.5</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37.5</td>
<td>0.23</td>
</tr>
<tr>
<td>Spin-labelled peptide</td>
<td>7.0</td>
<td>6.5</td>
<td></td>
<td>0.57</td>
</tr>
<tr>
<td>+ DMPC vesicles</td>
<td></td>
<td></td>
<td>26.5</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37.5</td>
<td>0.29</td>
</tr>
<tr>
<td>Spin-labelled peptide</td>
<td>7.0</td>
<td>6.5</td>
<td>26.3</td>
<td>26.3</td>
</tr>
<tr>
<td>+ DMPG vesicles</td>
<td></td>
<td></td>
<td></td>
<td>23.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37.5</td>
<td>20.1</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>6.5</td>
<td></td>
<td>25.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26.5</td>
<td>24.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37.5</td>
<td>23.4</td>
</tr>
</tbody>
</table>

a Experimental conditions and the derivation of $T_{\text{max}}$ and $\tau_0$ values are described in the section detailing experimental procedures.
Figure 5.7 Reduction of only the immobilized EPR spectral component by entrapped ascorbic acid. Superimposed EPR spectra of the proxyl-labelled peptide in the presence of DMPG vesicles at pH 7.0 (panel A, solid line spectrum) and of the labelled peptide interacting with DMPG vesicles loaded with ascorbic acid, at pH 7.0 (panel A, dotted line spectrum). Superimposed EPR spectra of the proxyl-labelled peptide in the presence of DMPG vesicles at pH 5.0 (panel B, solid line spectrum) and of the labelled peptide interacting with DMPG vesicles loaded with ascorbic acid, at pH 5.0 (panel B, dotted line spectrum). For details, refer to experimental methods in chapter 2, page 55.
indicated by a decrease in the value of the maximum hyperfine splitting parameter, $T_{\text{max}}$ (Table 5.1). The lipid exists in the gel phase at 6.5 °C, and in the liquid crystalline phase at 37.5 °C. There were no significant changes in line shape when the temperature of the peptide in solution was varied, indicating that the observed changes in the spectra with temperature in the presence of DMPG vesicles were not due to changes in the conformation of the free peptide, but rather due to its binding to the lipid bilayer. The change in mobility as a function of temperature was greater at pH 7 than at pH 5, suggesting that the spin label on the peptide was more sensitive to the DMPG gel to liquid crystalline phase transition at pH 7 than at pH 5 (Table 5.1).

The preceding results demonstrate that the C-terminus of the peptide associates with DMPG membranes. This conclusion is supported by the motional restriction of the proxyl group and its sensitivity to the lipid phase transition. However, motional restriction could occur as a result of either peptide binding to the bilayer surface or peptide insertion into the bilayer. Use of ascorbic acid to reduce the N-O$^*$ group to N-OH, thus abolishing its EPR signal, enabled us to determine the directionality of membrane insertion. Addition of ascorbic acid (10-20 mM) to the outside of DMPG vesicles almost completely reduced the mobile spectral component (sharp lines) as shown in figure 5.6C. Interestingly, only a partial reduction (at most 50%) of the immobilized component was observed at pH 7.0 (Figure 5.6C), indicating that only a fraction of the bound peptide C-terminus is accessible to ascorbic acid from the outside of vesicles. At pH 5, ~ 70% of the immobilized component was reduced by externally added ascorbic acid (data not shown) suggesting a greater access of ascorbic acid to the probe. Ascorbic acid completely reduced the spectrum of the labelled peptide in the presence of DMPC vesicles, indicating that the spin label is completely accessible to the aqueous phase in the presence of this neutral lipid.

To monitor if the C-terminus of the peptide could penetrate the bilayer of DMPG
vesicles, the spin-labelled peptide was added to ascorbic acid-loaded vesicles at pH 7.0. In contrast to the effect of ascorbic acid added to the outside of the vesicles, the ascorbic acid (12 mM) trapped inside the vesicles caused an almost complete reduction of only the immobilized component of the spectrum leaving the mobile component unaffected (Figure 5.7A). The addition of the spin-labelled peptide to vesicles containing ascorbic acid at pH 5.0, did not alter any components of the spin label spectrum (Figure 5.7B). These results suggest that at pH 7, the C-terminus of the bound peptide is accessible to intravesicular ascorbic acid and may thus insert through the lipid bilayer. At pH 5, the proxyl group must reside mostly near the surface of the SUV outer leaflet (Figure 5.7B and preceding paragraph) where it is more accessible to ascorbate added outside the vesicles. The partial or complete protonation of the three C-terminal histidines (positions 243-245) at pH 5.0 may contribute to the lack of penetration of the peptide into the lipid bilayer, thus favouring the orientation of ShTA(220-246) along the surface of the membrane at endosomal/lysosomal pHs (pH 4.5-6.0).

5.3.5 ShTA(220-246) causes leakage of calcein trapped inside DMPG vesicles.

The C-terminus of the A₁ domain may possess membrane-active properties. The addition of ShTA(220-246) to DMPG SUVs at pH 7.0 containing self-quenching concentrations of calcein resulted in a rapid increase in fluorescence intensity at 520 nm, indicating the release of the calcein fluorophore. Similar results were recorded at pH 5.0 (data not shown). The release of calcein was observed at peptide-to-lipid molar ratios of 1:2 and 1:4 (1:4 ratio is shown in Figure 4.8). No significant leakage was observed at lower peptide-to-lipid ratios (1:17 or 1:50; Figure 5.8). Melittin, a pore-forming peptide used as a positive control, required peptide-to-lipid ratios of the order of 1:20 to achieve similar effects on vesicles loaded with calcein as those observed for ShTA(220-246) (Figure 5.8). When
Figure 5.8  Peptide-induced leakage of calcein from DMPG vesicles as a function of time and peptide-to-lipid ratio. The peptide ShTA(220-246) was added to a cuvette containing 5 mM phosphate buffer, pH 7.0 and calcein-loaded DMPG vesicles. The fluorescence excitation wavelength was set at 490 nm and the increase in the emission signal due to the release of calcein was monitored at 520 nm. The molar ratio of ShTA(220-246) to DMPG lipid was either 1:4 (●), 1:17 (□), or 1:50 (○). As a positive control, the peptide melittin was used at a melittin to lipid molar ratio of 1:17 (△). A curve (◇) representing the background leakage of calcein from DMPG vesicles observed in the absence of peptide is also shown. The fluorescence signal corresponding to 100% release of calcein from DMPG vesicles, was determined by adding Triton-X100 (1% (v/v) final concentration) to each peptide:lipid mixture (refer to experimental methods in chapter 2, page 53).
DMPG vesicles were added to clear solutions of ShTA(220-246). The resulting mixtures became turbid at high peptide-to-lipid ratios, suggesting that the vesicles were starting to aggregate and fuse under such conditions. If the lipid vesicles were added to the peptide solution such that the peptide-to-lipid ratio was low (1:50), no turbidity was observed. Consistent with our calcein leakage experiments, this result indicates that leakage of calcein seen at a high peptide-to-lipid ratio may be the result of membrane insertion and disruption (detergent-like property) rather than channel formation.

5.3.6 A model summarizing the interaction of ShTA(220-246) with a negatively charged lipid bilayer. The results in this study indicate that the peptide representing the C-terminus of the A₁ fragment of ShT inserts into DMPG vesicles. Three possible types of interaction of ShTA(220-246) with a DMPG lipid bilayer are presented in Figure 5.9, which explain the restricted motion of the bound peptide and its sensitivity to the lipid phase transition. At pH 7, the N-terminus of ShT(220-246) is positively charged (Arg-220 and Arg-224) while its C-terminus remains mostly electrically neutral. Interactions of the peptide N-terminus with lipid head groups present on the outer leaflet of the vesicles are thus favoured. The remaining segment of the peptide (residues 225-246) may either insert into the bilayer in an orientation parallel to the surface of the membrane (Figure 5.9; conformer A) or may traverse the lipid bilayer (Figure 5.9; conformer B). Results from fluorescence and circular dichroism spectroscopies cannot distinguish between conformers A and B since both membrane conformations would lead to the burying of the tryptophan side chain (fluorescence spectroscopy) and to the observation of a peptide adopting a mostly α-helical structure (circular dichroism). Only the EPR results presented in this study allow us to differentiate between such conformers. Most of the peptide population must traverse the DMPG bilayer with the C-terminus facing the interior of vesicles since the C-terminus proxyl group (1) can
Figure 5.9  A schematic model depicting possible modes of interaction of peptide ShTA(220-246) with the lipid bilayer of DMPG vesicles. At pH 5, the dominant interaction is depicted by conformer A, where the peptide adopts a partially α-helical conformation and partitions within the bilayer with the helix axis being parallel to the normal axis of the membrane. At pH 7, a population of the peptide inserts and traverses the lipid bilayer (conformer B). Populations of both conformers A and B may exist at pH 7. The open star represents the position of the proxyl probe within the peptide while the letter N indicates the location of its amino terminus. Open rectangles depict the length of the putative α-helical region within the peptide. The dotted line as part of the rectangle in conformer B represents an extension of the helical region beyond residue 242 (the site of incorporation of the proxyl label).
be readily reduced by ascorbate entrapped inside vesicles and (2) is reduced to a much greater extent under such conditions than as a result of ascorbate being present on the outside the vesicles. The putative transmembrane segment could include residues 225 to 246 (starting after Arg-224), suggesting that the resulting helix could incorporate up to 22 amino acids, a length sufficient to span most lipid bilayers (Persson and Argos, 1994; Rost et al., 1995). This hypothesis would imply that up to 81% (22 of 27 amino acids) of the peptide residues would have to adopt an α-helical conformation for the proxyl probe (Cys-242) to be located near the surface of the inner leaflet of a bilayer. Circular dichroism results and binding measurements of peptide to lipid vesicles predict that most of the residues in ShTA(220-246) bound to DMPG vesicles assume an α-helical geometry. More precisely, approximately half of the peptide is free in solution in equilibrium with the remaining peptide population existing as one or more α-helical membrane-bound conformers (as demonstrated by binding experiments, circular dichroism and EPR results; Figure 5.3 and 5.6). In summary, the dominant ShTA(220-246) conformer bound to DMPG vesicles at pH 7.0 would be conformer B (Figure 5.9).

At pH 5.0, both the N- and C-termini are positively charged (Arg-220 and -224; His-243, -244 and -245). Reduction experiments with ascorbic acid indicate that the C-terminus of ShTA(220-246) is only accessible from the outer surface of the lipid vesicles, suggesting that the peptide does not traverse the bilayer at pH 5.0. The association of ShTA(220-246) with lipid vesicles at pH 5.0, was confirmed by circular dichroism, fluorescence, and binding experiments with radiolabeled peptide. Thus, the hydrophobic core of the peptide probably inserts into the lipid bilayer in an orientation best depicted by conformer A (parallel to the surface of the bilayer) with its charged termini bound to negatively charged lipid head groups (Figure 5.9). Since the toxin must travel through acidic compartments (pH 5-6) prior to reaching the ER lumen (neutral pH), the insertion of the A1
C-terminus into negatively charged membranes may be restricted to a particular orientation during the transit stage. However, either mode of insertion would be favourable since it means bringing the A1 fragment in contact with the membrane; a required step for membrane translocation.

Finally, oriented CD measurements of magainins, a family of 23-residues frog peptides with antimicrobial properties, demonstrated that they adopt an α-helical conformation with two distinct orientations in relation to a lipid membrane (either inserted parallel or perpendicular to the lipid bilayer) (Ludtke et al., 1994). This report supports our results that membrane-bound ShTA (220-246) can insert into a lipid bilayer in the two proposed orientations.
CHAPTER 6

CONCLUDING REMARKS
Concluding Remarks

6.1 *Does the B subunit play a role in A chain translocation?* The observation of a local conformational change in the B subunit pentamer at acidic pHs predicted for compartments associated with endocytosis, suggested that the B subunit may encode a mechanism for promoting or actuating the processing, release and/or translocation of the A chain to the cytoplasm. The potential role of the B oligomer remains unclear but could involve the five α-helices. This site would be favoured by analogy with the *E. coli* heat-labile enterotoxin where most of the A chain contacts with its B subunit pentamer occur through the central helices (Sixma et al., 1991; Sixma et al., 1992). The change in tryptophan fluorescence intensity (Figure 2.1) between pH 5 and 7 would be related to a local conformational change occurring near the NH$_2$ terminus of these helices. The crystal structure of the B subunit indicates that the tryptophans in the pentamer are already mostly exposed to the solvent at neutral pH. Thus, at pH levels below 5, the indole side chains must either be located in proximity of charged side chains that are revealed within this pH range or that the tryptophans and potentially the α-helices are partially reoriented to a more solvent-exposed environment. Since the B subunit selectively binds the glycolipid Gb$_3$, it suggests that the receptor-binding domain of the B subunit maintains a rigid tertiary or quaternary structure and that the receptor-binding function is not influenced by acidic pH levels (Figure 3.5).

As in the case of the influenza virus hemagglutinin and diphtheria toxin, the ShT B subunit would have to undergo a change in conformation large enough in scale to directly mediate the membrane insertion and translocation of the A$_1$ chain. Given the small size of the
B chain (69 amino acids) and the several functions it is known to perform (binding to the receptor, binding to the A subunit and oligomerization) it does not appear that it can undergo large scale conformational changes without affecting any of those functions. Endosomal pH does not alter the oligomerization of the B subunit or the holotoxin, as determined by gel permeation chromatography (Table 4.1). The B subunit also maintains its receptor binding properties at those pH levels (Figure 3.5). This allowed us to conclude that the B subunit may not be directly involved in the translocation of the A₁ fragment to the cytosol. Instead, it may be required to orient the A subunit for proper proteolytic processing and for the localization of the toxin in the ER.

6.2 Domains analogous to ShTA(220-246) are present on other proteins. In searching for a domain in the holotoxin that may be involved in the A₁ chain translocation, we found a signal sequence-like domain at the C-terminus of this chain (residues 220-246) which may be the sought-after functional domain (chapter 5). Amino acid sequences similar to this peptide sequence can be found in a number of proteins. Of particular interest is the human asialoglycoprotein (ASGP) receptor H1. Protein fusion experiments have shown that this sequence (residues 38-65) was sufficient to result in membrane insertion and translocation of the C-terminal domain of rat α-tubulin (Spiess & Lodish, 1986). The presence of such a hydrophobic segment is also seen in the C-terminus of polyomavirus middle-T antigen (residues 394-421) which is involved in membrane binding (Markland et al. 1986). In diphtheria toxin, the hydrophobic sequence shown in Figure 5.1 is located within the putative membrane translocation domain (Moskaug et al., 1991; Choe et al., 1992). Point mutation studies on this domain have revealed that substituting glutamic acid at position 362 with lysine allowed the toxin to translocate at a higher pH than the wild type (Falnes et al., 1992). More recently, spin-labelled analogs of the isolated transmembrane domain of
diphtheria toxin have been shown to insert into and permeabilize large unilamellar vesicles (Zhan et al., 1995).

6.3 Does ShTA(220-246) and the A1 chain interact with component(s) of a protein transporter located in the ER membrane? The import of nascent protein chains associated with ribosomes into the ER lumen is thought to involve the recognition of signal sequences on newly synthesized proteins by a signal sequence binding protein (itself an ER membrane protein) in association with other known components of the protein translocation machinery to the ER (signal recognition particle (SRP), SRP receptor, ribosome and transmembrane channel protein). One hypothetical scenario may be that the C-terminus of the ShT A1 domain inserts into the ER membrane and allows the catalytic domain to diffuse along the plane of the membrane until it is recognized by a signal sequence binding protein. Subunits of the transmembrane channel protein or recently assembled channel proteins may then be recruited or be already positioned in proximity to the complex of the ShT A1 domain and the signal sequence binding protein, favouring the ultimate translocation of the A1 domain across the ER membrane. As a result of interactions with the ER membrane and associated protein components, the population of bound A1 chain may exist as a series of partly unfolded intermediates, which may then be recognized as appropriate substrates for export (cytosol) through the transmembrane protein channel. The involvement of various ER protein factors linked to the folding of proteins such as the protein disulfide isomerase, p88 or calnexin, HSP47, GRP94 and Bip (Gething and Sambrook, 1992; Gaut and Hendershot, 1993; Simon, S., 1993) may be crucial for the export process to occur.

6.4 The involvement of the ubiquitin-proteasome pathway. Alternatively, the hydrophobic C-terminus of the A1 fragment would be exposed and would contain a free cysteine (Cys-242)
available for reversible disulfide bond formation with resident proteins of the ER compartment. These features have been proposed to mediate protein retention and degradation in the ER (Schmitz et al., 1995; Reddy et al., 1996). One possible scenario is that upon arrival of SLT-I in the pre-Golgi/ER compartments, the exposed hydrophobic region in the C-terminus of the A₁ fragment is recognized by an ER resident protein (such as Bip in eukaryotes or Der1 in yeast) and is then translocated to the cytoplasmic side of the ER to be degraded by the ubiquitin-proteasome pathway. This pathway, which is initiated at the cytosolic side of the ER (Biederer et al., 1996), is the major pathway for degradation of several misfolded and mutated proteins including free MHC class I molecules (Raposo et al., 1995), mutant forms of the cystic fibrosis transmembrane conductance regulator (CFTR) associated with cystic fibrosis (Ward et al., 1995) and Sec61p complex, a key component of the ER protein translocation machinery (Biederer et al., 1996). The interaction of any of those ER proteins mentioned above with SLT-I remains to be investigated.

6.5 A hypothetical model describing membrane insertion and translocation of the SLT-I A₁ fragment. Intoxication of cells by Shiga and Shiga-like toxins occurs through three distinct stages; The binding of the B subunit to its receptor, the intracellular trafficking and proteolytic processing (primarily of the A subunit) of the toxin followed by the membrane translocation of its A₁ domain leading to the catalytic inactivation of the ribosome. Membrane translocation of the enzymatic fragment requires that it comes in close proximity to, and perhaps making contact with, the vesicular membrane. The results of this work and from the work of other laboratories (Surewicz et al., 1989) indicate that the holotoxin possesses little membrane active properties. Processed toxin however, may expose the hydrophobic domain (see chapter 4) which we have shown to have strong membrane active properties. This feature would satisfy the requirement of bringing the enzymatic fragment in close proximity
Figure 5.1 A schematic diagram depicting the proposed mechanism of membrane translocation by SLT-I. **Panel A** This is the initial step in the translocation. The A₁ chain interacts with the ER membrane through its hydrophobic C-terminus either directly (1) or via transient association with an endogenous factor/chaperone of the ER (2). **Panel B** The next stage would involve unfolding of the A₁ chain by ER chaperones followed by the translocation step. The translocated A₁ chain may either remain associated with the ER membrane and gain access to ribosomes docked onto the membrane (3) or be released from the membrane to inactivate free ribosomes (4). **Panel C** An alternate translocation pathway could involve protein-translocating channels. The lateral diffusion of the membrane inserted A₁ may bring this region in close proximity to the translocon (the protein translocation channel) either after unfolding (5) or before unfolding (6) takes place. Because of the homology between the A₁ C-terminus and signal sequences, the translocon may recognize this region of the A₁ and subsequently facilitate its translocation.
A Cytoplasm

B

C

60S 40S

60S 40S

60S 40S
to the vesicular membrane (Figure 5.1; panel A, step 1). The next step could follow one of two routes; the inserted C-terminus could allow the A$_1$ fragment to spontaneously escape the ER compartment, or it could result in the lateral diffusion of the A$_1$ fragment to bring it in close proximity to specialized components of the ER protein translocation complex and allow for the assisted translocation of the catalytic fragment (Figure 6.1). For example, major histocompatibility complex (MHC) molecules targeted to the inner side of the ER membrane require the binding of small peptides for subsequent processing and targeting to the cell surface. In the absence of such peptides, MHC molecules are unstable and are targeted for proteolysis. It is becoming evident that this is accomplished by unfolding and translocation (through specialized protein channels) to the cytosol, where they are recycled by proteolysis in the proteasome (Raposo et al., 1995). The proposed mechanism for the translocation of the SLT-I A$_1$ fragment is attractive in that it implies that the ER protein translocation complex could function in both directions; the normal direction being the translocation of nascent chains from the ribosome on the cytosolic side to the inside of the ER. Although the proposed mechanism is highly speculative, its appeal resides in the facts that A) a machinery provided by the host cell at the level of the ER membrane can recognize a broad array of sequences similar to ShTA(220-246) and B) can transport large proteins across this membrane barrier in association with ribosomes which C) are the target site for the action of the catalytic A$_1$ domain.

6.6 Future Directions. The model in Figure 5.1 highlights the potential existence of two distinct mechanistic events, that may act in concert, in delivering the enzymatic A$_1$ fragment from the ER to the cytosol. This hypothesis raises two questions; one is whether or not the proposed hydrophobic C-terminus of the A$_1$ fragment is actually involved in membrane translocation and the second issue of whether or not any ER resident proteins interact with
the toxin in the ER to affect translocation.

The former question can be effectively addressed by modifying the character of the this region to either reduce or abolish its contribution (if any) to the translocation process. This region of the A₁ has two main features, the uninterrupted hydrophobic core and the positive charges at both termini (Fig. 5.1). Site-directed mutagenesis could be used to alter these features. Certain hydrophobic residues in the core of this region may be converted to bulky charged residues (such as arginines) to interrupt the hydrophobicity of the domain. Similarly, the arginines at the N-terminus or the histidines at the C-terminus may be converted to neutral residues. The effect of such mutations on the toxins’ ability to intoxicate cells may then be determined. For an effective comparison, however, such mutations should not affect the folding, enzymatic activity or intracellular targeting of the toxin.

The latter question concerns the potential interaction of both soluble and membrane-associated ER proteins with the toxin molecule, most probably its A subunit. Such factors could be identified by co-immunoprecipitation experiments, given the availability of antibodies directed against ER resident factors. Other strategies include cross-linking experiments by probing cell extract from cells pretreated with toxin followed by western blot analysis using anti-toxin or anti-ER proteins antibodies.
REFERENCES


Boyd, B., S. Richardson, and J. Gariépy. 1991. Serological responses to the B subunit of Shiga-like toxin 1 and its peptide fragments indicate that the B subunit is a vaccine candidate to counter the action of the toxin. *Infect. Immun.* **59**:750-757.


*Acad. Sci.* USA. **84:**4364-4368.


Lingwood, C. A., H. Law, S. Richardson, M. Petric, J. L. Brunton, S. De Grandis, and


Maloney, M. D., and C. A. Lingwood. 1994. CD19 has a potential CD77 (globotriaosyl ceramide)-binding site with sequence similarity to verotoxin B-subunits: implications of molecular mimicry for B cell adhesion and enterohemorrhagic *Escherichia coli* pathogenesis.


Characterization of Shiga-like toxin 1 B-subunit purified from overproducing clones of the SLT-1 B cistron. *J. Biochem.* **272**:805-811.


Smith, H. W., P. Green, and Z. Parsell. 1983. Vero cell toxins in Escherichia coli and related bacteria: transfer by phage and conjugation and toxic action in laboratory animals,


Wadolkowski, E. A., J. A. Burris, and A. D. O'Brien. 1990b. Mouse model for


Zoja, C., D. Corna, C. Farina, G. Sacchi, C. Lingwood, M. P. Doyle, V. V. Padhye, M.
