Characterization of calmodulin binding properties of a phage display peptide series and the peptide analogues of KirGem sm-MLCK and neuromodulin using fluorescence spectroscopy.

Wendy Joan Stevenson
University of Windsor

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CHARACTERIZATION OF CALMODULIN BINDING PROPERTIES OF
A PHAGE DISPLAY PEPTIDE SERIES AND THE PEPTIDE
ANALOGUES OF KIRGEM, SM-MLCK AND NEUROMODULIN
USING FLUORESCENCE SPECTROSCOPY.

by

Wendy Joan Stevenson

A Thesis
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in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
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Windsor, Ontario, Canada

1997

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ABSTRACT

An M13 Phage Display library experiment by Dr. B. Kay (University of North Carolina) yielded a lone Ca\(^{2+}\) dependent calmodulin-binding peptide (12.1). Point mutations to the C-terminus and the central Gly 13 Gly 14 residues of this peptide were made to study their effect on the peptide binding to Cam. This series of peptides was denoted as the ‘Evolved’ series of peptides (52). Two sources of this peptide series were used during experiments, one provided by Dr. B. Kay, University of North Carolina (K series) and a second from Dr. G. LaJoie, University of Waterloo (UW series). Fluorescence spectral titrations with Cam indicated that the results were source dependent. Secondly, fluorescence titrations indicated that the peptides bound to Cam in 1:1 or 2:1 equivalents of peptide:Cam. Scatchard analysis enabled the \(K_d\)'s one or both complexes to be determined. Comparison of the \(K_d\)'s obtained, (ranging from < 0.098 \(\mu\)M to 2.2 \(\mu\)M for the 1:1 complexes) showed the order of affinity for Cam was E3 > E3-AS > E5 > E4 > E6 >E3-L-TV > 12.1. This showed that peptide amphiphilicity behaviour and long chain hydrophobic residues in the C-terminus were determinants of binding affinity. Hydrophobicity plots and helical wheels confirmed that the peptides were amphipathic. Tb\(^{3+}\) energy transfer experiments and acrylamide quenching experiments of the peptide/Cam complexes were also performed to further characterize the complex. Tb\(^{3+}\) energy transfer data indicated that the K series peptides generally bound such the N-terminal Trp was in close proximity to the 2nd and 3rd Ca\(^{2+}\) binding loops of Cam. Acrylamide quenching experiments determined that the Trp was generally protected from solvent quenching.

A second project studied the binding interactions between the peptide analogues of sm-MLCK, neuromodulin and the Ras-like protein KirGem. Data for sm-MLCK and KirGem indicated 2:1 binding. Scatchard analysis estimated the \(K_d\)'s for sm-MLCK as 0.56 \(\mu\)M for the 1:1 complex and \(\sim 16 \mu\)M for the 2:1 complex and the \(K_d\) of the 2:1 KirGem complex as 7.2 \(\mu\)M. Studies with neuromodulin indicated stoichiometric binding and further that Tyr 138 of Cam participates in binding while Tyr 99 does not.
DEDICATION

I dedicate this thesis to my parents, Murray and Sue, who have always supported me and had unending faith in me and taught me to never give up. Your love and support has helped me to be true to myself and be the best ‘me’ that I can be.
ACKNOWLEDGEMENTS

I would like to thank Dr. A.G. Szabo for funding and support throughout this project. Thank you to Peter Pathrose and Dr. B. Mutus for the generous gift of the calmodulin used in this study. Thank you to Mike Weller for assistance with the computer, Mauro Acchione for critical reading of the manuscript and Bahe Rajendran. Sharon Barker and Shawna Anderson for invaluable support both technical and otherwise throughout this project. Also, thanks to my committee members Dr. L. Lee and Dr. D. Haffner.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xii</td>
</tr>
<tr>
<td>1. Introduction</td>
<td></td>
</tr>
<tr>
<td>1.1 Structure of Cam</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Structure of Cam/Peptide Complex</td>
<td>2</td>
</tr>
<tr>
<td>1.3 Importance of Aromatic and Long Chain Hydrophobic Residues</td>
<td>6</td>
</tr>
<tr>
<td>1.4 Amphiphilic, Basic Requirements for Strong Binding</td>
<td>8</td>
</tr>
<tr>
<td>1.5 Degrado's 2:1 Binding Theory</td>
<td>10</td>
</tr>
<tr>
<td>1.6 Evidence Supporting the Two Globular Domains as Binding Sites</td>
<td>11</td>
</tr>
<tr>
<td>1.7 Phage Display Series</td>
<td>13</td>
</tr>
<tr>
<td>1.8 KirGem, sm-MLCK and Neuromodulin</td>
<td>16</td>
</tr>
<tr>
<td>2. Materials and Methods</td>
<td></td>
</tr>
<tr>
<td>2.1 General Chemicals</td>
<td>18</td>
</tr>
<tr>
<td>2.2 Instrumental Methods</td>
<td></td>
</tr>
<tr>
<td>2.2.1 High Performance Liquid Chromatography</td>
<td>18</td>
</tr>
<tr>
<td>2.2.2 UV-Visible Spectroscopy</td>
<td>19</td>
</tr>
<tr>
<td>2.2.3 Fluorescence Spectroscopy</td>
<td>19</td>
</tr>
<tr>
<td>2.3 Peptides</td>
<td>20</td>
</tr>
<tr>
<td>2.4 Purification of Peptides</td>
<td></td>
</tr>
<tr>
<td>2.4.1 Phage Display Peptides</td>
<td>21</td>
</tr>
<tr>
<td>2.4.2 Berchtold's Peptides</td>
<td>23</td>
</tr>
<tr>
<td>2.5 Peptide Quantification</td>
<td>23</td>
</tr>
<tr>
<td>2.6 Peptide Composition</td>
<td>24</td>
</tr>
<tr>
<td>2.7 Purification of Cam</td>
<td>24</td>
</tr>
<tr>
<td>2.8 Titrations of Peptides by Cam</td>
<td>24</td>
</tr>
<tr>
<td>2.9 Titrations of Cam by Peptides</td>
<td>25</td>
</tr>
<tr>
<td>2.10 Scatchard Analysis</td>
<td>26</td>
</tr>
<tr>
<td>2.11 Acrylamide Quenching Experiments</td>
<td>28</td>
</tr>
<tr>
<td>2.12 Terbium Energy Transfer Experiments</td>
<td>29</td>
</tr>
<tr>
<td>3. Results</td>
<td></td>
</tr>
<tr>
<td>3.1 Cam Activity Assay</td>
<td>30</td>
</tr>
<tr>
<td>3.2 Phage Display Peptide Series</td>
<td>31</td>
</tr>
<tr>
<td>3.2.1 12.1 Peptide</td>
<td>31</td>
</tr>
</tbody>
</table>
3.2.1.1 K 12.1 32
3.2.1.2 UW 12.1 Peptide 37
3.2.2 E3-LTV Peptide 40
  3.2.2.1 K E3-LTV 42
  3.2.2.2 UW E3-LTV 45
  3.2.2.3 K2 E3-LTV 49
3.2.3 E3-AS Peptide 51
  3.2.3.1 K E3-AS 51
  3.2.3.2 UW E3-AS 54
3.2.4 E3 Peptide 58
  3.2.4.1 K E3 58
  3.2.4.2 UW E3-AS 62
  3.2.4.3 K2 E3 65
3.2.5 E4 Peptide 67
  3.2.5.1 K E4 67
  3.2.5.2 UW E4 73
3.2.6 E5 Peptide 76
  3.2.6.1 K E5 77
  3.2.6.2 UW E5 79
3.2.7 E6 Peptide 84
  3.2.7.1 K E6 84
  3.2.7.2 UW E6 87
3.2.8 E2 Peptide 88
3.2.9 E8 Peptide 92
3.2.10 E9 Peptide 93
3.3 The KirGem, sm-MLCK and Neuromodulin Peptides 96
  3.3.1 The KirGem Peptide 96
  3.3.2 The sm-MLCK Peptide 100
  3.3.3 The Neuromodulin Peptide 105

4. Discussion

4.1 Purification and Characterization 109
4.2 Fluorescence Spectra of Peptides and Peptide/Cam Complexes 110
4.3 K Series
  4.3.1 Cam/Peptide Titrations 114
  4.3.2 Binding Analysis and Interpretation 119
  4.3.3 Acrylamide Quenching Data 120
  4.3.4 Tb$^{3+}$ Energy Transfer Data 122
4.4 UW Series
  4.4.1 Cam/Peptide Titrations 124
  4.4.2 Binding Analysis and Interpretation 126
  4.4.3 Hydrophobicity Plots and Helical Wheels 131
4.5 KirGem, sm-MLCK and Neuromodulin
  4.5.1 Cam/Peptide Titrations 135
4.5.2  Binding Analysis and Interpretation  138
4.5.3  Acrylamide Quenching Data  138
4.5.4  Tb$^{3+}$ Energy Transfer Data  140
4.5.5  Hydrophobicity Plots and Helical Wheels  140

5. Conclusions  145

References  147
Vita Auctoris  154
LIST OF TABLES

Table 1.1 — Primary Sequences of Cam-binding Peptides. 7

Table 1.2 — Primary sequences of the peptides to be studied. 15

Table 2.1 — Summary of the Methods of Peptide Purification. 22

Table 3.1 — $F_\infty / \Delta F_\infty$ Values used for Scatchard Analysis. 35

Table 4.1 — Fluorescence Spectra for Peptides and Peptide/Cam Complexes. 111

Table 4.2 — Peptide/Cam Titrations of K Series Peptides. 115

Table 4.3 — Scatchard Analysis Summary of K Series Peptides. 117

Table 4.4 — $K_d$'s of Cam:P and Cam:P$_2$ Complexes of K Series Peptides. 121

Table 4.5 — Stern-Volmer Constants of Various Peptide:Cam Ratios for K Series. 121

Table 4.6 — Tb$^{3+}$ Energy Transfer Data for K Series Peptides. 123

Table 4.7 — Peptide/Cam Titrations of UW Series Peptides. 125

Table 4.8 — Scatchard Analysis Summary of UW Series of Peptides. 127

Table 4.9 — $K_d$'s of Cam:P and Cam:P$_2$ Complexes of UW Series Peptides. 130

Table 4.10 — Peptide/Cam Titrations for KirGem, sm-MLCK and Neuromodulin. 136

Table 4.11 — Scatchard Analysis of KirGem & sm-MLCK Peptides. 139

Table 4.12 — Stern-Volmer Constants of KirGem & sm-MLCK. 139

Table 4.13 — Tb$^{3+}$ Energy Transfer Data for KirGem & sm-MLCK. 142
LIST OF FIGURES

Figure 1.1 -- Ribbon drawing of Ca$^{2+}$-Cam crystal structure. 3

Figure 1.2 -- Ribbon drawing of NMR solution structure of Cam/M13. 5

Figure 1.3 -- Fluorescence titration of Cam with a synthetic Cam-binding peptide. 12

Figure 3.1 -- Terbium energy transfer experiments used to check integrity of Cam. 33

Figure 3.2 -- HPLC trace of K 12.1 peptide. 33

Figure 3.3 -- Typical absorbance spectrum of Trp. 33

Figure 3.4 -- Fluorescence Spectra of K 12.1 peptide. 33

Figure 3.5 -- Sigma Cam titration of K 12.1 peptide. 36

Figure 3.6 -- Scatchard plot of Cam titration of K 12.1 peptide. 36

Figure 3.7 -- K 12.1 titration of 2 μM Sigma Cam. 36

Figure 3.8 -- K 12.1 titration of 1 μM Cam (Dr. Mutus). 36

Figure 3.9 -- Scatchard plot for K 12.1 titration of Cam from Figure 3.8. 39

Figure 3.10 -- HPLC trace of UW 12.1 peptide. 39

Figure 3.11 -- Mass spectrometry trace of UW 12.1 peptide. 39

Figure 3.12 -- Fluorescence spectra of UW 12.1 peptide. 41

Figure 3.13 -- Cam titration of UW 12.1 peptide (3 μM). 41

Figure 3.14 -- Scatchard plot for Cam titration of UW 12.1. 41

Figure 3.15 -- UW 12.1 titration of Cam (1 μM). 41

Figure 3.16 -- Difference curve from UW 12.1 titration of Cam (Figure 3.15). 43

Figure 3.17 -- HPLC trace of K E3-LTV peptide. 43

Figure 3.18 -- Fluorescence spectra of K E3-LTV peptide. 43
Figure 3.19 -- K E3-LTV titration of Cam (1 μM). 43

Figure 3.20 -- Scatchard plot of K E3-LTV titration of Cam. 46

Figure 3.21 -- HPLC trace of UW E3-LTV peptide. 46

Figure 3.22 -- Mass spectrometry trace of UW E3-LTV peptide. 46

Figure 3.23 -- Fluorescence spectra of UW E3-LTV peptide. 48

Figure 3.24 -- Cam titration of UW E3-LTV (3μM). 48

Figure 3.25 -- Scatchard plot for Cam titration of UW E3-LTV. 48

Figure 3.26 -- UW E3-LTV titration of Cam (1 μM). 48

Figure 3.27 -- Difference curve from UW E3-LTV titration of Cam (Figure 3.26). 50

Figure 3.28 -- Scatchard plot of UW E3-LTV titration of Cam. 50

Figure 3.29 -- HPLC trace of K2 E3-LTV peptide. 50

Figure 3.30 -- K2 E3-LTV titration of Cam (1 μM) showing the difference curve only. 50

Figure 3.31 -- Scatchard plot of K2 E3-LTV titration of Cam. 53

Figure 3.32 -- HPLC trace of K E3-AS peptide. 53

Figure 3.33 -- Fluorescence spectra of K E3-AS peptide. 53

Figure 3.34 -- K E3-AS titration of Cam (1 μM). 53

Figure 3.35 -- Scatchard plot of K E3-AS titration of Cam. 55

Figure 3.36 -- Terbium titration curve of a 3:1: Cam:E3-AS sample. 55

Figure 3.37 -- HPLC trace of UW E3-AS peptide. 55

Figure 3.38 -- Fluorescence spectra of UW E3-AS peptide. 55

Figure 3.39 -- Cam titration of 3 μM UW E3-AS peptide. 57
Figure 3.40 -- Cam titration of 1 μM UW E3-AS peptide.
Figure 3.41 -- UW E3-AS titration of Cam (1 μM).
Figure 3.42 -- Difference spectrum of UW E3-AS titration of Cam.
Figure 3.43 -- Scatchard plot of UW E3-AS titration of Cam.
Figure 3.44 -- HPLC trace of K E3 peptide.
Figure 3.45 -- Mass specrometry trace of K E3 peptide.
Figure 3.46 -- Fluorescence spectra of K E3 peptide.
Figure 3.47 -- K E3 titration of 2 μM Sigma Cam.
Figure 3.48 -- K E3 titration of 1 μM Sigma Cam.
Figure 3.49 -- Scatchard plot of K E3 titration of 1 μM Sigma Cam.
Figure 3.50 -- K E3 titration of 1 μM Dr. Mutus' Cam.
Figure 3.51 -- Scatchard plot of K E3 titration of Dr. Mutus' Cam.
Figure 3.52 -- HPLC trace of UW E3 peptide.
Figure 3.53 -- Fluorescence spectra of UW E3 peptide.
Figure 3.54 -- Cam titration of 1 μM UW E3 peptide.
Figure 3.55 -- Difference spectrum of E3 titration of Cam (1 μM).
Figure 3.56 -- HPLC trace of K2 E3 peptide.
Figure 3.57 -- Fluorescence spectra of K2 E3 peptide.
Figure 3.58 -- Difference curve only of K2 E3 titration of Cam (1 μM).
Figure 3.59 -- HPLC trace of K E4 peptide.
Figure 3.60 -- Fluorescence spectra of K E4 peptide.
Figure 3.61 -- K E4 titration of Sigma Cam (1 μM).
Figure 3.62 -- K E4 added to Buffer curve.
Figure 3.63 -- Difference curve representing monomer binding of K E4 to Cam.  

Figure 3.64 -- Scatchard plot of monomer binding to Cam.  

Figure 3.65 -- Dimerization plot of K E4 peptide.  

Figure 3.66 -- Stern-Volmer plot of acrylamide quenching of 1:4: E4:Cam sample.  

Figure 3.67 -- HPLC trace of UW E4 peptide.  

Figure 3.68 -- Fluorescence spectra of UW E4 peptide.  

Figure 3.69 -- Cam titration of UW E4 (1 μM).  

Figure 3.70 -- Scatchard plot of Cam titration of UW E4.  

Figure 3.71 -- UW E4 titration of Cam (1 μM).  

Figure 3.72 -- Scatchard plot of UW E4 titration of Cam.  

Figure 3.73 -- HPLC trace of K E5 peptide.  

Figure 3.74 -- Mass spectrometry trace of K E5 peptide.  

Figure 3.75 -- Fluorescence spectra of K E5 peptide.  

Figure 3.76 -- Cam titration of K E5 peptide (2 μM).  

Figure 3.77 -- K E5 titration of Cam (2 μM).  

Figure 3.78 -- Difference curve of K E5 titration of Cam.  

Figure 3.79 -- Scatchard plot of K E5 titration of Cam.  

Figure 3.80 -- HPLC trace of UW E5 peptide.  

Figure 3.81 -- Fluorescence spectra of UW E5 peptide.  

Figure 3.82 -- Cam titration of UW E5 (1 μM).  

Figure 3.83 -- Scatchard plot of Cam titration of UW E5 peptide.  

Figure 3.84 -- UW E5 titration of Cam (1 μM).
Figure 3.85 -- Scatchard plot of UW E5 titration of Cam. 86
Figure 3.86 -- HPLC trace of K E6 peptide. 86
Figure 3.87 -- Fluorescence spectra of K E6 peptide. 86
Figure 3.88 -- K E6 titration of Dr. Mutus Cam (1 μM). 86
Figure 3.89 -- HPLC trace of UW E6 peptide. 89
Figure 3.90 -- Fluorescence spectra of UW E6 peptide. 89
Figure 3.91 -- Cam titration of UW E6 (1 μM). 89
Figure 3.92 -- Scatchard plot of Cam titration of UW E6. 89
Figure 3.93 -- UW E6 titration of Cam (1 μM). 91
Figure 3.94 -- Scatchard plot of UW E6 titration of Cam. 91
Figure 3.95 -- HPLC trace of E2 peptide. 91
Figure 3.96 -- Fluorescence spectra of E2 peptide. 91
Figure 3.97 -- E2 titration of Sigma Cam (1 μM). 94
Figure 3.98 -- HPLC trace of E8 peptide. 94
Figure 3.99 -- Fluorescence spectra of E8 peptide. 94
Figure 3.100 -- E8 titration of Cam (1 μM). 94
Figure 3.101 -- Fluorescence spectra of E9 peptide. 97
Figure 3.102 -- E9 titration of Sigma Cam (1 μM). 97
Figure 3.103 -- HPLC trace for KirGem peptide. 97
Figure 3.104 -- Fluorescence spectra of KirGem peptide. 97
Figure 3.105 -- Cam titration of KirGem (200 nM). Em. 345 nm. 99
Figure 3.106 -- Cam titration of KirGem (1 μM). Em. 322 nm. 99
Figure 3.107 -- KirGem titration of Cam (1 μM). Em. 345 nm. 99

Figure 3.108 -- Scatchard plot of KirGem titration of Cam @ 345 nm. 99

Figure 3.109 -- KirGem titration of Cam (1 μM). Em. 322 nm. 102

Figure 3.110 -- HPLC trace for sm-MLCK peptide using cation exchange chromatography. 102

Figure 3.111 -- HPLC trace for sm-MLCK peptide using reverse phase chromatography. 102

Figure 3.112 -- Fluorescence spectra of sm-MLCK peptide. 102

Figure 3.113 -- Cam titration of sm-MLCK (830 nM). Em. 325 nm. 104

Figure 3.114 -- sm-MLCK titration of Cam (1 μM). Em. 345 nm. 104

Figure 3.115 -- Difference curve from Figure 3.114 showing early points only. 104

Figure 3.116 -- Scatchard plot of sm-MLCK titration of Cam @ 345 nm. 104

Figure 3.117 -- sm-MLCK titration of Cam (1 μM). Em. 325 nm. 107

Figure 3.118 -- HPLC trace for Neuromodulin peptide. 107

Figure 3.119 -- Neuromodulin titrations of bovine brain Cam and octopus Cam. 107

Figure 4.1 -- Hydrophobicity plots of 12.1 and E4 sequences. 132

Figure 4.2 -- Helical wheels of 12.1, E3, E4 & E5 peptide sequences. 134

Figure 4.3 -- Hydrophobicity plots of sm-MLCK and KirGem. 143

Figure 4.4 -- Helical wheels of sm-MLKC and Neuromodulin. 144
List of Abbreviations

HPLC - High Performance Liquid Chromatography
Trp - Tryptophan
Tyr - Tyrosine
Phe - Phenylalanine
sm-MLCK - smooth muscle myosin light chain kinase
sk-MLCK - skeletal muscle myosin light chain kinase
UV - Ultra Violet
MOPS - (3-[N-morpholino]-propane sulfonic) acid
Cam - calmodulin
Cac - Cacodylic acid buffer
Nata - N-acetyl tryptophanyl amide
[P]_f - concentration of free peptide
[P]_b - concentration of bound peptide
[P]_t - concentration of total peptide
K_d - dissociation constant
K_{SV} - Stern-Volmer constant
NH_4OAc - ammonium acetate
EGTA - Ethylene glycol-bis (β-Aminoethyl ether) N, N', N''-tetraacetic acid
TFA - Trifluoroacetic acid
CD - Circular Dichroism
Ala - Alanine
Leu - Leucine
Val - Valine
Thr - Threonine
Ser - Serine
Arg - Arginine
Glu - Glutamic Acid
Asp - Aspartic Acid
1. Introduction

Calmodulin (Cam) is an acidic 148-residue substrate binding protein which is capable of binding and regulating a variety of proteins within many biochemical systems in a generally calcium-dependent manner. However, exceptions such as neuromodulin (1) have been found to bind to Cam with higher affinity in the absence of calcium. Examples of proteins whose activity is modulated by Cam include NAD kinase, phosphodiesterase (2), smooth muscle myosin light chain kinase (3), calcineurin (4), nitric oxide synthase (5) and phosphorylase kinase (6). This has stimulated a significant interest in elucidating the nature of the interaction of Cam with a variety of different proteins. Smaller segments of these proteins which were identified to be involved in the Cam-protein complex have been prepared and these shorter peptides have been studied as models for elucidating the characteristics of the complexes.

1.1 Structure of Cam

The crystal structure of Cam bound to calcium ion reveals two globular domains separated by a flexible, seven turn alpha helix tether which gives the molecule a dumbbell shape in the unbound form (7, 8, 9). Small angle x-ray scattering studies of Cam in solution reveal a similar tertiary structure; however, the globular domains appear to be closer together on average which presumably would be due to the greater flexibility of the central helix tether in solution than in the crystal structure (10). Further, NMR studies have shown that the middle of this $\alpha$-helical tether between residues 77 and 81 shows a high degree of mobility (11, 12). This suggests that the $\alpha$-helical secondary structure is disrupted in this region in solution, but not in the crystal structure. Similar results have
been reported in other studies of Cam in solution (13, 14). This discrepancy in structure between the crystal and solution structures of Cam is thought to be due to crystal packing forces (12). Figure 1.1 shows the ribbon drawing of the crystal structure of Cam accounting for the random coil structure between residues 77-81 of the interconnecting α-helix.

The globular domains are comprised of two helix-loop-helix conformations called EF hands which bind the four calcium ions required for the binding of Cam to the proteins which it regulates. Adjacent EF hands are connected by short anti-parallel β-sheet structures (7, 8, 9). The N-terminus contains calcium binding domains I and II and the C-terminus calcium binding domains III and IV. Studies focused on the binding affinity of Ca\(^{2+}\) for the calcium binding domains have shown that the Ca\(^{3-}\) has a stronger affinity for the C-terminal globular domains than the N-terminal domains (16, 17). ApoCam (Ca\(^{2+}\) free) structure has been studied using NMR spectroscopy (18). Studies revealed that the N-terminal domain of ApoCam has secondary and tertiary structure homologous to HoloCam (Ca\(^{2+}\) loaded). However, the secondary and tertiary structure of the C-terminal domain of the apo protein was less well defined and appeared to be dynamically disordered.

1.2 Structure of the Cam/Peptide Complex

The crystal structures (19) and NMR (12, 20, 21) studies of Cam bound to the peptide analogs of both sm-MLCK (typically called the RS20 peptide) and sk-MLCK (the M13 peptide) have been resolved. These data have shown that upon binding of the peptide to Cam, the structure of the globular domains remains relatively unchanged but
Figure 1.1 — Ribbon drawing of Ca$^{2+}$-Cam crystal structure with adjustments made in the region of residues 78-81 to account for discrepancies between Cam in solution and its crystalline form (15).
are closer together than in the uncomplexed crystal structure (22). The single peptide binds in an amphipathic manner and lies within a hydrophobic tunnel formed by the globular domains of Cam.

The most dramatic changes to Cam when it binds to the peptide occur in the region of the α-helical tether. In this region, residues 73-77 in the crystal structure become random coil forming a flexible tether (12, 19) while in solution, residues 74-77 become unwound extending the existing loop to residues 74-81 (12, 20). It is generally thought that this unwinding and subsequent bending of the α-helical tether allows the globular domains to come closer together in order for binding to occur. The Cam/peptide complex has an ellipsoidal shape which is markedly different than the dumbbell shape of free Cam. Figure 1.2 shows the NMR structure of the Cam/M13 complex. Other structures of Cam complexes with protein kinase II (23), plasma membrane ATPase, MARKS homolog and glycogen phosphorylase kinase (24) have indicated only slight differences in tertiary structure depending on the target peptide. This indicates a common binding mechanism in which the binding domain of Cam adopts a slightly different tertiary structure to accommodate different target enzymes. Target recognition and regulation is unknown but may involve the globular domain EF hands. Persechini et al. demonstrated that synthetic rearrangement of the EF hands of Cam resulted in significant decreases in the activation of target enzymes (25). Further, studies focused on Ca\(^{2+}\) dissociation from Cam complexes have shown that Ca\(^{2+}\) ion dissociation from the protein/Cam complex is different depending on the enzyme bound (26). This suggests that the EF hands may be important in enzyme recognition and regulation.
Figure 1.2 — (a) NMR solution structure of Cam/M13. Cam is shown as the light ribbon while M13 is shown as the darker ribbon. Note the positions of the Trp-4 and Phe-17 which are thought to act as anchors that bind to the exposed hydrophobic pockets of calmodulin. (b) The structure is rotated to show the hydrophobic channel formed by Cam which encompasses the peptide (15).
The RS20 and M13 peptides (Table 1.1) are observed to bind with 1:1 stoichiometry and form an amphiphilic α-helix where its hydrophobic residues are aligned on one side of the helix and can interact with the hydrophobic patches exposed by Ca\(^{2+}\) binding of the N-and C-terminal globular domains of Cam (12, 19, 20, 27). Hence, binding of the peptide to Cam is thought to be accomplished through mainly hydrophobic interactions (2, 28, 29). However, ionic interaction are also of some importance in stabilizing the binding. For example, in low ionic strength buffers, RS20 was observed to bind to ApoCam but dissociates at physiological ionic strengths (30).

1.3 Importance of Aromatic and Long Chain Hydrophobic Residues

The C-terminus of Cam interacts with the N-terminus of the peptide (21, 28, 31). The Trp, (position five in the RS20 peptide and position four in the M13 peptide), occupies the hydrophobic pocket of the C-domain of Cam and may be a key residue in binding (12, 19, 20, 28). In the C-terminus of the peptides, Phe 17 of the M13 peptide anchors it to the N-terminus of Cam (12, 20, 28). In the RS20 peptide, Ala 14, Ile 15 and Leu 18 have been shown to act as the anchors (19). This suggests that aromatic or long chain hydrophobic residues such as Leu, Ile and Val may be necessary to anchor the C-terminus of the peptide to Cam. Studies in which the Trp 4 and Phe 17 residues of M13 have been interchanged further support the importance of the N-terminal Trp but suggest that its importance may not be absolute. The mutant peptide still binds to Cam with the same orientation as the native peptide but with an eight fold decrease in affinity (31, 32). Similar results were obtained by interchanging Trp 4 and Phe 21 of a peptide from the plasma membrane calcium pump (28). The decrease in affinity could be because the
Table 1.1 – Primary Sequences of Cam-binding Peptides.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Primary Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 (12, 15, 20, 28)</td>
<td>K R R W K K N F I A V S A A N R F K K I S S S K</td>
</tr>
<tr>
<td>RS20 (19)</td>
<td>A R R K W Q K T G H A V R A I G R L S S M A</td>
</tr>
<tr>
<td>Mellitin (37)</td>
<td>G A V L K V L T T G L P A L</td>
</tr>
<tr>
<td>Mastoparan (38, 39)</td>
<td>I N L K A L A A L A K K I L</td>
</tr>
<tr>
<td>Dedman’s (42)</td>
<td>A E W P L S T E I K T L S H F S V P P P P P P</td>
</tr>
<tr>
<td></td>
<td>A E I S L P Q L K W L T H R L K Q P P P P P P</td>
</tr>
<tr>
<td>Degrado’s (40)</td>
<td>W K K L L K K L K K L L K L</td>
</tr>
<tr>
<td>Polistes mastoparan (45, 47)</td>
<td>N V D W K K I G Q H I L S V L</td>
</tr>
<tr>
<td>Mastoparan X (45, 47)</td>
<td>I N W K G I A A M A K K L L</td>
</tr>
</tbody>
</table>

**Note: M13 peptide is the Cam binding domain (residues 577-601) of the sk-MLCK enzyme and RS20 is the Cam binding domain (residues 796-818) of the sm-MLCK enzyme.**
indole ring is larger than the aromatic ring in Phe and hence its surface makes more contacts with the hydrophobic pockets of Cam. Evidence to further support this theory is shown in the binding of the peptide analogs of another Cam binding enzyme, Twitchin, in which Trp is in the C-terminus and was found to bind with lower affinity than other peptides (33).

However, recent evidence has been found which raises questions about the importance of the Trp 4 and Phe 17 amino acid residues. Studies in which Ala substitutions of each residue in turn of the M13 peptide have been shown to have stronger binding affinities than that of the native M13 peptide. This is surprising since Ala is neither aromatic nor a long-chain hydrophobic residue both of which have been proposed to be required as anchors for binding (34). This suggests that the aromatic residues may not be as important as the primary sequence of the peptide.

1.4 Amphiphilic, Basic Requirements for Strong Binding

Naturally occurring peptides such as β-endorphin (29, 35, 36), melittin (37) and mastoparan (38, 39), ideal amphiphilic (40, 41) and phage display (42) synthetic peptides (Table 1.1) of no physiological function have been found to bind to Cam. As with the RS20 and M13 analogs, these peptides are also basic and from circular dicroism (CD) difference studies and other methods have been estimated to form amphiphilic α-helices when bound to Cam.

This apparent basic, amphiphilic requirement of peptides for binding has been further investigated through the synthesis of idealized peptides fulfilling these requirements (41, 43, 44). Studies indicated that these amphiphilic, basic, positively
charged peptides bound with high affinity while amphiphilic, acidic, negatively charged peptides did not (41, 44). This suggests that the amphiphilic nature of the peptide is not the only requirement for peptide binding. Instead, these results suggest that charge and basicity are also requirements for binding.

Further, although peptides have been observed to be α-helical when bound to Cam, circular dichroism studies have shown these peptides to be random coil (39, 44) and even partially β-sheet (42) in the unbound state and become mostly α-helical upon binding to Cam. This suggests that although an amphipathic secondary structure may be required for binding, the peptides do not necessarily have this secondary structure before binding to Cam, but rather it is formed upon binding of the peptide to Cam.

It is of interest to note that the primary sequences of these naturally occurring and synthetic peptides (Table 1.1) for the most part do not contain Trp residues unless fluorescence methods of study are being utilized to study their binding properties (40, 42, 45). However in place of this essential Trp, β-endorphin does contain Phe and Tyr residues and mastoparan contains long chain hydrophobic residues further supporting the notion that either aromatic or long chain hydrophobic residues are necessary anchors in peptide binding to Cam.

Fluorescent studies of various peptides containing a Trp have shown that the local environment of the bound fluorescent Trp varies between peptides. This suggests subtle differences in binding of the Trp such that the Trp residue may occupy slightly different subsites within the peptide binding site (42, 45). Further, acrylamide quenching
experiments suggest that the Trp fluorophore is buried within the hydrophobic interior of the Cam binding domain (44).

1.5 Degrado’s 2:1 Binding Proposal

It has been suggested by O’Neill and Degrado that there is more than one peptide binding site in Cam (2). This theory contradicts the NMR and crystallographic studies of the RS20 and M13/Cam complexes. Evidence supporting this proposal has been found with both naturally occurring peptides and synthetic peptides. Mastoparan which is a fourteen residue peptide has shown evidence of 2:1 binding. Further results indicated that in a 1:1 complex all of the loops participate in binding but upon addition of a second equivalent of mastoparan, there is evidence of a 2:1 complex (38).

Cross-linking studies with β-endorphin, a thirty-one residue neuropeptide (29), and a deletion peptide comprised of residues 14-25 of β-endorphin (46) have shown formation of similar 2:1, peptide:Cam complexes. However, Malencik and Anderson in spectroscopic studies with β-endorphin and Cam have reported a 1:1 complex (35). This contradiction could be due in large to the relative concentrations of Cam and peptide employed in experiments such that the concentrations utilized by Malencik and Anderson may be below the dissociation constant (K_d) of the 2:1 complex and hence only the 1:1 complex was observed. With the higher concentrations utilized by Giedroc in cross-linking studies, the concentrations of Cam and peptide presumably exceeded the K_d of the second binding site (40).

Maulet and Cox have shown using fluorescence spectroscopy that mellitin can bind as a 2:1, mellitin:Cam complex in the absence of calcium with a K_d of 10 μM at
high concentrations of mellitin and further can form complexes having a 5:1 ratio. In the presence of Ca$^{2+}$, it is found to bind stoichiometrically (37).

Erickson-Viitanen and Degrado have demonstrated the formation of a 2:1 complex with synthetic peptides (40). Using fluorescence spectroscopy, a sample of Cam was titrated with aliquots of a Trp containing peptide to a final ratio of 5:1, excess peptide. A plot of the change in fluorescence minus the fluorescence of the peptide versus equivalent of peptide (Figure 1.3) showed that at a 1:1 ratio the slope of the curve started to become more shallow and at a 2:1 ratio it plateaued. This suggests the presence of two distinct quantum yields ($\phi$) which presumably relate to the two distinct complexes i.e. a 1:1 complex (Cam:P) and a 2:1; peptide:Cam complex (Cam:P$_2$). Similar results were obtained with CD spectroscopy and clearly show the formation of a 2:1 complex (40). Hence, much evidence supporting the existence of a second peptide binding site on Cam has been substantiated. However, this evidence seems to also point to the presence of both a high and low affinity binding site as shown by the fact that peptides appear to bind sequentially (29, 38, 40). However, how Cam binds two equivalents of peptide and where the second binding site resides is unknown. Degrado has suggested that each of the globular domains acts as a separate binding site (2).

1.6 Evidence Supporting the Two Globular Domains as Binding Sites

Studies of trypsin digested fragments of Cam, such that the N and C globular domains are separated, indicated from binding studies with Polistes mastoparan and mastoparan X (Table 1.1) that the C-terminal fragment bound stoichiometrically with $K_d$'s of $\sim$0.2 $\mu$M while the N-terminal fragment bound 100-fold more weakly with
Figure 1.3 — Fluorescence titration of Cam with a synthetic Cam-binding peptide. Note the change in slope at 1:1 and 2:1 equivalents. This change in slope indicates three distinct quantum yields. The first indicates binding of the first equivalent of peptide to Cam, the second indicates binding of the second equivalent of peptide to Cam and the third indicates no further changes in fluorescence and hence binding was complete (40).
dissociation constants of \( \sim 20 \, \mu M \) \((45, 47)\). Intact Cam was found to bind with a
dissociation constant of \( 0.9 \, nM \) \((45)\). This enforces Degrado’s 2:1 binding proposal in
that both domains can bind to peptides independently but also suggests that \textit{in vivo} intact
enzymes are probably regulated by Cam as a whole. Further, enzyme activation studies
conducted with these N-and C-terminal fragments have shown that neither of the
fragments are able to activate protein phosphatase, calcineurin or cAMP
phosphodiesterase; however, the C-terminal fragment has been shown to activate
phosphorylase kinase but with much lower affinity than intact Cam \((48)\). Similar studies
with sk-MLCK, sm-MLCK and neural nitric oxide synthase (nNOS) have shown that
there is a specificity for the N-terminal fragment to site B on the enzyme and the C-
terminal fragment to site A suggesting that not only is binding of the enzyme to both
domains necessary for activation but also that each lobe makes a distinct contribution to
enzyme activation. However, other results also suggested that binding of these fragments
were not specific to one site. Instead, each fragment was found to bind to either site and
were capable of displacing one another if the concentration of one was in great excess
over the other \((49)\). Further, although both domains appear to be necessary for
regulation, studies have suggested that the C-terminus is of greater significance \((49, 50,
51)\).

1.7 Phage Display Series

Within the Human Genome Bank, the primary sequences of all of the proteins
within the human body have been recorded. However, the functions and biochemical
pathways of these proteins are unknown. Phage display libraries have been used to
generate peptides of random primary sequence (42, 52). These peptides were than tested for Cam-binding thus yielding novel Cam-binding sequences. Comparison of these sequences with those primary sequences within the Human Genome Bank may help to elucidate which of these sequenced proteins are regulated by Cam and hence aid in the discovery of the functions and biochemical pathways of these proteins.

A single 25 amino acid peptide identified by Dr. B. Kay from a random phase display library was designated as the 12.1 peptide and found to bind to Cam (52). A series of evolved peptides based on the primary sequence of 12.1 were synthesized where different amino acids were substituted in a limited number of selected locations. Each of the peptides contained at least one Trp residue to facilitate steady state fluorescence characterization of the local structure of the bound peptide and provide a convenient method to determine the binding affinities. The primary structures of these peptides are shown in Table 1.2.

The notes above suggest that there is sufficient contradictory evidence for how Cam recognizes and regulates its targets and what properties and amino acids are essential and/or preferred for binding to occur. It was the intention of this study to elaborate how subtle changes in primary sequence affected the binding affinities and properties of Cam binding. Thus determinants of 2:1 stoichiometry, preferred amino acids, cationic, basic and amphiphilic requirements and subtleties surrounding the local environment of the Trp may be understood. The understanding of the characteristics of Cam binding peptides may permit the recognition of which other proteins from the Human Genome Bank are regulated by Cam.
Table 1.2: Primary sequences of the peptides to be studied.

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>PRIMARY SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.1</td>
<td>STVPRWIEDSLRGGAAARATQRRLASA</td>
</tr>
<tr>
<td>E3</td>
<td>STVPRWIEDSLRASAAARATQLTVAS (A)</td>
</tr>
<tr>
<td>E3-AS</td>
<td>STVPRWIEDSLRASAAARATQRRLASA</td>
</tr>
<tr>
<td>E3-LTV</td>
<td>STVPRWIEDSLRGGAAARATQLTVASA</td>
</tr>
<tr>
<td>E4</td>
<td>VPRWIEDSLRELATATQSSVASA</td>
</tr>
<tr>
<td>E5</td>
<td>VPRWIEDSLRAATAAQAVLASA</td>
</tr>
<tr>
<td>E6</td>
<td>VPRWIEDSLRCAAATAQTSLASA</td>
</tr>
<tr>
<td>E2</td>
<td>STVPRWIEDSLRDWAARAQSSIPAS</td>
</tr>
<tr>
<td>E8</td>
<td>STVPRWIEDSLRDWAARAQSETASA</td>
</tr>
<tr>
<td>E9</td>
<td>STVPRWIEDSLRWAAARAQAQSASA</td>
</tr>
<tr>
<td>KirGem (53)</td>
<td>KARRFWGKIVAKNNKMNMAFKLKS KS</td>
</tr>
<tr>
<td>sm-MLCK (19)</td>
<td>ARRKNQKTGHA VRAI GR LSSMA</td>
</tr>
<tr>
<td>Neuromodulin</td>
<td>AHKAAATKIQASFRGHI TRKKLKG</td>
</tr>
<tr>
<td>(1, 56, 57, 59)</td>
<td></td>
</tr>
</tbody>
</table>

**Note: the peptide analogue of sm-MLCK is the Cam binding domain of the sm-MLCK enzyme (residues 796-818), the peptide analogue of neuromodulin is the Cam binding domain of the neuromodulin enzyme (residues 39-56) and the KirGem peptide is the Cam binding domain of the KirGem enzyme (residues 264-288).**
1.8 KirGem, sm-MLCK & Neuromodulin

A second set of peptides was also studied in collaboration with Dr. M. Berchtold (Zurich, Switzerland). This focused project studied three peptide analogs of proteins that are known suspected to bind to Cam. The first peptide is an analog of the Ras-like GTPase KirGem protein (53). The particular Ras-like protein on which this peptide analog is based, differs from other Ras proteins in that it has a 30 residue extension of the C-terminus that upon inspection appears to exhibit many of the same features that other Cam binding peptides are found to possess. The binding affinity of this 30 residue peptide to Cam was studied and was found to bind to dansyl Cam with 1 nM affinity in a calcium dependent manner. Further studies, in which the Trp was replaced by a Gly residue significantly reduced the affinity of the peptide for Cam suggesting the importance of this residue (53). The single Trp residue in the KirGem peptide also allowed the use of Trp residue fluorescence as an intrinsic probe of its binding. The binding of the KirGem peptide was compared to that of a peptide analog of sm-MLCK (RS20 according to the literature) and neuromodulin. Neuromodulin protein (or P57 as it has been called) has been isolated from the cerebral cortex membranes (54) and has been found to bind to Cam in a 1:1 ratio with higher affinity in the absence of Ca$^{2+}$ (230 nM) than in its presence (1 μM) in low ionic strength buffers. However, this was reversed in higher ionic strength buffers (e.g. 150 mM KCl) for which the affinity of neuromodulin for Cam decreased for both the calcium dependent (3 μM) and the calcium-independent (3.4 μM) forms of Cam, suggesting the importance of ionic interactions for binding of this peptide (55, 56, 57, 58). This is significantly different than the chemistry of binding
interactions seen with most other peptides and proteins in which hydrophobic interactions seem to be key while ionic interactions are less significant (2, 19, 20). A 9-residue Cam binding domain (residues 43-51) of the neuromodulin protein has been isolated and found to bind in the presence and absence of calcium (59).

This peptide is of interest for two reasons. Firstly, it has unique binding interactions with Cam and secondly, it does not contain the fluorophore Trp. This enabled the study of the interactions upon binding using the Tyr residues found in Cam which ordinarily cannot be monitored due to the overlapping and much stronger spectral properties of the Trp. By focusing study on the Tyr residues, the local environment of these residues could be monitored as binding of the neuromodulin peptide occurred. For this study two types of Cam were utilized, the bovine brain Cam used in all of the studies presented and octopus brain Cam. Minor differences in primary structure exist between the two Cams, the one of importance to this study being the substitution of the Tyr 99 in bovine brain Cam for a spectroscopically weak Phe (60). This allowed for differentiation in signal between Tyr 99 and Tyr 138 found in bovine brain Cam as Tyr 138 remained in the octopus Cam. Primary sequences of these peptides are shown in Table 1.2.
2. Materials and Methods

2.1 General Chemicals

All samples were prepared in cacodylic Acid buffer or Mops buffer. Both buffers were prepared by dissolving 50 mM cacodylic acid (Sigma, St. Louis, MO) or MOPS (Sigma, St. Louis, MO) and 100 mM KCl (BDH Inc., Toronto, Ont.) in 18 MΩ resistance Milli-Q water and adjusting the pH to 7. Stocks of CaCl₂ (BDH Inc., Toronto, Ont.), Acrylamide (BDH Inc., Toronto, Ont.), EGTA (Sigma, St. Louis, MO) and terbium were prepared in cacodylic acid buffer. G-25 Fine Sephadex used for de-salting peptides was obtained from Pharmacia Biotech (Uppsala, Sweden).

2.2 Instrumental Methods

2.2.1 High Performance Liquid Chromatography (HPLC)

HPLC was conducted using a Gilson HPLC system (Gilson, Middleton, WI) with Gilson 306 pumps and a Gilson 811b dynamic mixer. Detection of eluant was achieved using a Gilson 117 detector and monitoring the absorbance at 280 nm unless otherwise stated. An IBM compatible 386 computer with Gilson 715 HPLC system controller software controlled the instrument and data collection. BioRad (Mississauga, Ont.) C18 and C8 reverse phase columns were used with eluant solvents of Milli-Q water and Spectrograde acetonitrile (OmniSolv, BDH Inc., Toronto, Ont.) to which TFA (0.1 %) (Pierce, Rockford, IL.) was added. Cation exchange chromatography was conducted using a Progel-TSK CM-5PW cation exchange column (Supelco Canada Ltd., Mississauga, Ont.) and salt solutions of NaCl (BDH Inc., Toronto, Ont.) and ammonium acetate (BDH Inc., Toronto, Ont.).
2.2.2 UV-Visible Spectroscopy

UV spectra were collected using an SLM-Aminco DW-2000 spectrophotometer (SLM Instruments Inc., Urbana, IL.). An IBM 286 compatible computer with SLM version 1.1A software. interfaced to the spectrophotometer, controlled the monochromator and recorded the spectral data. Light was provided by a deuterium lamp in the ultraviolet range and a quartz iodine lamp beyond 360 nm. Spectra were collected by monitoring absorbance between 240 nm and 350 nm. The absorbance of the solvent was subtracted in each case.

2.2.3 Fluorescence Spectroscopy

Fluorescence measurements were done on a Spex Fluorolog DM 3000F Fluorometer (Spex, Edison, NJ) with a scanning double monochromator on the excitation side of the instrument and a single or double scanning monochromator on the emission side. The detector was a photomultiplier tube cooled by a Peltier cooler. An IBM compatible 486 computer with DM3000 software, interfaced to the flurometer, controlled the monochromators and recorded spectral data. Excitation of the sample was achieved using a xenon lamp. Fluorescence measurements were corrected for variations in the excitation source intensity by the use of a Rhodamine-B quantum counter and a beam splitter.

For acrylamide and Cam/peptide titrations, excitation was at 295 nm to avoid excitation of the tyrosine residues on Cam and emission was monitored at 345 nm which shows the maximum intensity for solvent exposed Trp unless otherwise stated. For neuromodulin, excitation was 280 nm to excite the Tyr residues and emission was
monitored at 302 nm, the maximum intensity for Tyr. The excitation slit was set to a 4 nm bandpass and the emission bandpass was 4 nm or 8 nm. Samples were continuously stirred. The fluorescence intensity was integrated for 60 seconds for each measurement. For terbium titrations of Cam/peptide samples excitation was at 295 nm, while for Cam samples alone and neuromodulin/Cam samples excitation was at 280 nm. Tb$^{3+}$ emission was monitored at 545 nm (a luminescence line of Tb$^{3+}$).

Fluorescence spectra of each of the peptides alone and complexed to Cam were collected with excitation at 295 nm (280 nm for neuromodulin) and the fluorescence was measured between 300 nm and 450 nm (290 - 350 nm for Cam/neuromodulin) at 0.5 nm intervals and with 2 second integration. A buffer only blank, collected under similar conditions, was subtracted from each spectrum. Spectra were uncorrected and smoothed.

2.3 Peptides

The peptide samples to be studied are listed with their primary sequences in Table 1.2. A first set of E2, E3, E4, E5 and E6 peptides were obtained from Chiron Mimotopes Peptide Systems (San Diego, CA). The E3-AS, E3-LTV, 12.1, E8 and E9, were obtained directly from Dr. Brian Kay at the University of North Carolina. These samples were obtained in an unpurified form and required purification. A second set of peptides (12.1, E3-AS, E3-LTV, E3, E4, E5 and E6) designated as the UW peptides were obtained from Dr. Giles Lajoie at the University of Waterloo (Waterloo, Ont.). These peptides were subject to preliminary HPLC purification and characterized by mass spectrometry by Dr. G. Lajoie. A second sample of the E3 and E3-LTV peptides were obtained from Dr. B.
Kay. These peptides are referred to as K2 E3 and K2 E3-LTV. These samples required purification.

The peptides designated as KirGem, sm-MLCK and Neuromodulin, were obtained from Dr. Martin Berchtold (University of Zurich, Zurich, Switzerland). These peptide samples required purification.

2.4 Purification of Peptides

2.4.1 Phage Display Peptides

The purity of the K 12.1 peptide was confirmed using HPLC and a BioRad C8 reverse phase column (Table 2.1). The K 12.1 sample eluted using a gradient of water and acetonitrile. All other K peptides were purified using a CM-5PW cation exchange column and a gradient of water and 0.2 M ammonium acetate (Table 2.1). In the case of the E2, E3, E3-AS, E3-LTV, E4, E5, E6 and E8 peptides, the largest of the peaks was collected. The collected fractions were checked for Trp absorbance, pooled, lyophilized and re-dissolved in MOPS buffer or water to give a concentrated sample of purified peptide.

The purity of the UW peptides were verified using HPLC with a C18 column and a gradient of water and acetonitrile (Table 2.1). The peptides, K2 E3 and K2 E3-LTV, were purified by reverse phase HPLC in the same manner as the UW peptides. These samples were collected, monitored for Trp absorbance, lyophilized and re-dissolved in water.

The flowrate used for these samples was 0.75 mL/min and the gradients are shown in the sample HPLC traces for each peptide (Chapter 3).
<table>
<thead>
<tr>
<th><strong>Method</strong></th>
<th><strong>Peptides</strong></th>
</tr>
</thead>
</table>
| C8 column  
A: water/0.1% TFA  
B: acetonitrile/0.1% TFA  
Flowrate 0.75 mL/min | K 12.1 |
| CM-5PW column  
A: water  
B: 0.2 M NH₄OAc  
Flowrate 0.75 mL/min | E2, K E3,K E3-AS.  
KE3-LTV, K E4, K E5, K E6. E8 |
| C18 column  
A: water/0.1 % TFA  
B: acetonitrile/ 0.1% TFA  
Flowrate 0.75 mL/min | UW 12.1, UW E3, UW E3-AS.  
UW E3-LTV, UW E4, UW E5, UW E6.  
K2 E3, K2 E3-LTV, sm-MLCK |
| CM-5PW column  
A: water/Acetonitrile  
B: 0.2 M NH₄OAc /Acetonitrile  
Flowrate 0.5 mL/min | sm-MLCK, KirGem & Neuromodulin |
2.4.1 Berchtold's Peptides

The Berchtold peptides were purified by HPLC using a CM-5PW cation exchange column and eluted with solvent containing varying percentages of acetonitrile to counteract the added hydrophobic interactions of the peptides to the column (61) (Table 2.1). The gradient used for elution had solvent A as aqueous 5 mM NaCl and acetonitrile, pH 6. solvent B was aqueous 0.5 M NaCl and acetonitrile with the same pH. The percentages of acetonitrile used in solvents A and B were 40% for the KirGem peptide, 30% for neuromodulin and 25% for sm-MLCK. Neuromodulin was followed at both 260 nm, the absorbance maximum of Phe, and 220 nm (peptide absorbance). Samples were lyophilized and re-injected to confirm their purity. Samples were then desalted using a G-25 column. Samples of each individual peptide were pooled, lyophilized and re-dissolved in Milli-Q water to give a salt-free, concentrated sample of purified material. The flowrate was 0.5 mL/min and the gradients for each peptide are shown in the sample HPLC traces (Chapter 3).

Sm-MLCK could also be purified by reverse phase chromatography using a C18 column and the same method as the UW peptides. This was the preferred purification method.

2.5 Peptide Quantification

Concentrations of the peptide stock solutions were determined from the peptide absorbance spectrum at 280 nm. An extinction coefficient at 280 nm of 5559 M⁻¹cm⁻¹ for Trp was used (62).
The sample of neuromodulin, which lacks a strongly absorbant residue, could not be quantified in the same manner. Therefore, an aliquot of this peptide was quantified by amino acid analysis (kindly determined by Dr. M. Yaguchi, Biological Sciences, NRC, Ottawa).

2.6 Peptide Composition

The composition of the peptides purchased from UW and obtained from other sources were characterized by mass spectrometry. The amino acid analysis of the neuromodulin sample confirmed its composition.

2.7 Purification of Cam

Both the bovine brain Cam and the octopus Cam were generously purified (63) and provided by Dr. B. Mutus and Mr. Peter Pathrose (University of Windsor, Windsor, Ont.). Their concentrations were determined by absorption spectroscopy at 280 nm, with an extinction coefficient of 1400 M\(^{-1}\)cm\(^{-1}\) per tyrosine residue (Bovine Cam 2 Tyr; Octopus Cam, 1 Tyr) (62). Other bovine brain Cam was obtained from Sigma Chemicals (St. Louis, MO).

2.8 Titrations of Peptides by Cam

Solutions of 2 mL of either 1 μM, 2 μM, or 3 μM peptide (see Figure caption), and 100 μM CaCl\(_2\) were prepared in Cac buffer. Aliquots of bovine brain Cam were added to a final ratio of greater than 1:1. The change in peptide fluorescence measured at selected wavelengths was monitored. The fluorescence wavelength used for each peptide was chosen according to the fluorescence maximum of the complex determined separately and corresponded to 322 nm, 325 nm, 347 nm, 345 nm and 350 nm for
KirGem; sm-MLCK; UW E3; K 12.1, UW E4, UW E5 and UW E6; and UW E3-AS, UW E3-LTV and UW 12.1, respectively. Fluorescence measurements were corrected for dilution.

2.9 Titrations of Cam by Peptides

Samples of 1 μM Cam, 50 μM CaCl₂ in Cac buffer were titrated using similar methods as the Cam titrations of the peptides. Fluorescence wavelengths were at 322 nm, 325 nm, 347 nm, 345 nm and 350 nm for KirGem; sm-MLCK; UW E3; UW E4, UW E5, UW E6, the K series and the K2 peptides; and UW E3-AS, UW E3-LTV and UW 12.1. Cam samples were titrated with 0.1 μM aliquots of peptide to a ratio of 1:1, peptide:Cam, followed by larger aliquots of peptide beyond a ratio of 1:1. At the same time the fluorescence of the peptide alone (blank titration) was determined for each addition of peptide.

Fluorescence measurements for both the Cam/peptide and peptide blank titrations were dilution corrected. The ‘net’ fluorescence change was obtained by subtracting the peptide blank titration from the Cam/peptide titration values. Titration curves show the peptide blank, the titration of Cam/peptide and the net fluorescence versus the calculated ratios of peptide/Cam at each point.

Samples of 10 μM Cam in Cac buffer and in the presence of calcium were titrated with 10 μL aliquots of neuromodulin. In this titration, the fluorescence of the Tyr residues were monitored. Due to the presence of a fluorescent impurity in the neuromodulin, a titration of neuromodulin in buffer was also measured and the fluorescence contribution by the fluorescent impurity was subtracted from the
In order to estimate the binding affinity of the peptide to Cam, Scatchard analysis or a modified version of it was used (64). Scatchard analysis assumes that the binding is both weak and stoichiometric. Therefore, for the formation of a 1:1 complex:

\[
P + \text{Cam} \longrightarrow \text{Cam:P}
\]

where: \( P \) is defined as the peptide and

\( \text{Cam:P} \) is defined as the Cam/peptide complex.

From this equation it follows that \( K_d \) is:

\[
K_d = [P]_f [\text{Cam}]_f / [\text{Cam:P}]
\]

For the titrations in which \( \text{Cam} \) is added to the peptide, \( \text{Cam} \) is defined to be the ligand while the peptide is the acceptor. Therefore, \( r \) is defined to be:

\[
r = [\text{Cam}]_b / [P]_f
\]

\[
r = [\text{Cam:P}] / [P]_f + [\text{Cam:P}]
\]

where:

\([\text{Cam}]_b\) represents the concentration of bound \( \text{Cam} \)

\([P]_f\) represents the concentration of free peptide

\([P]_f\) represents the total concentration of peptide

From equation 2.2:

\[
[P]_f = K_d [\text{Cam:P}] / [\text{Cam}]_f
\]

Therefore,

\[
r = [\text{Cam:P}] / \{ K_d [\text{Cam:P}] / [\text{Cam}]_f + [\text{Cam:P}] \}
\]

\[
r = 1 / \{ K_d / [\text{Cam}]_f + 1 \}
\]

\[
r = [\text{Cam}]_f / K_d + [\text{Cam}]_f
\]

Re-arrangement of equation 2.4 yields the Scatchard equation.
\[ r = \frac{1}{\{ \frac{K_d}{[Cam]_t} + 1 \}} \]

\[ r = \frac{[Cam]_t}{K_d} + [Cam]_t \]

Re-arrangement of equation 2.4 yields the Scatchard equation.

\[ r \ (K_d + [Cam]_t) = [Cam]_t \]
\[ r \ K_d = [Cam]_t - r[Cam]_t \]
\[ \frac{r}{[Cam]_t} = \frac{1}{K_d} - \frac{r}{K_d} \]

Therefore, a plot of \([Cam]_b/[P]_t\) / \([Cam]_t\) vs \([Cam]_b/[P]_t\) will yield a linear plot with a slope of \(-1/K_d\). In the titrations in which Cam is being added to the fluorescent peptide, the change in fluorescence signal is a result of the change in fluorescence quantum yield of the peptide, \(\phi_F\), as the peptide binds to Cam. The fraction of peptide bound, \(P_b\), at any addition of Cam is determined from 2.6;

\[ \text{Fraction } P_b = \frac{F - F_0/F_\infty - F_0}{\Delta F / \Delta F_{\text{MAX}}} \]

where: \(F_t\) is the observed fluorescence of the Cam:P mixture at any \([Cam]\)

\(F_0\) is the fluorescence intensity in the absence of Cam

\(F_\infty\) is the maximum plateau fluorescence signal

Scatchard analysis assumes stoichiometric binding,

\[ [Cam]_b = [P]_b = \text{fraction peptide bound} \times [P]_t \]

and

\[ [Cam]_t = [Cam]_t - [Cam]_b \]

A similar method of analysis can be used to determine the dissociation constant of the peptide titrations of Cam. However, in this case, Cam is defined as the acceptor while the peptide becomes the ligand. Hence the function, \(r\), becomes

\[ r = \frac{[P]_b}{[Cam]_t} \]
It follows that the Scatchard equation is therefore,
\[
\frac{r}{[P]_f} = \frac{1}{K_d} - \frac{r}{K_d}
\]  
2.10

Therefore, from a plot of \([P]_b/[\text{Cam}]_t\) vs \([P]_f\) vs \([P]_b/[\text{Cam}]_t\), the slope is \(-1/K_d\).

Since, in this case, the fluorescent peptide is being added to the sample, the change in fluorescence is a result of both the increased concentration of fluorophore and the changing \(\phi_F\) of the peptide as binding occurs. The difference in fluorescence signal is the total fluorescence, \(F\), due to that of the complex and of free peptide less that of an equivalent concentration of total peptide, \(F_0\).
\[
\Delta F = F - F_0
\]  
2.11

Assuming stoichiometric binding,
\[
\text{Fraction } P_b = \frac{\Delta F}{\Delta F_{\text{MAX}}} \times [\text{Cam}]_t
\]  
2.12

such that \(\Delta F_{\text{MAX}}\) is the maximum fluorescence observed from equation 2.11 when all Cam sites are saturated with peptide. Finally,
\[
[P]_f = [P]_t - [P]_b
\]

2.11 Acrylamide Quenching Experiments

Acrylamide quenching experiments were carried out in the following manner. For the Kay series of peptides, samples of 1 \(\mu M\) peptide, 4 \(\mu M\) Cam and 100 \(\mu M\) Ca\(^{2+}\) in Cac buffer were prepared. Samples were titrated with 0.02 M aliquots of acrylamide in Cac buffer to a final concentration of 400 mM. Samples with free peptide were titrated in the same manner with aliquots of 0.04 M acrylamide to a final concentration of 800 mM. A 2 \(\mu M\) sample of NATA was titrated using the same procedure above. This served as a reference point for the Stern-Volmer constant (\(K_{SV}\)) of a fully solvent exposed Trp.
Fluorescence measurements were corrected for dilution. The Stern-Volmer equation (13) was employed to generate Stern-Volmer plots (65) and to find the $K_{SV}$'s for each sample.

$$\frac{F_0}{F} = 1 + k_q \tau [Q]$$  \hspace{1cm} (13)

where:
- $F$ is the fluorescence
- $F_0$ is the initial fluorescence of the sample
- $[Q]$ is the concentration of quencher added at any point
- $k_q$ is the rate constant for quenching
- $\tau$ is the excited state decay time of the fluorophore
- $K_{SV}$ is the product $\tau k_q$

From the equation, it can be seen that a plot of $F_0/F$ vs $[Q]$ should be linear with slope equal to $K_{SV}$.

2.12 Terbium Energy Transfer Experiments

Samples of varying ratios of peptide:Cam and Cam alone were titrated with TbCl$_3$ in Cac buffer. The aliquots are added so that there were five points per Ca$^{2+}$ binding loop to a final ratio of 6:1 Tb$^{3+}$. The luminescence measurements at 545 nm were corrected for dilution and plotted versus Tb$^{3+}$/Cam. Stocks of TbCl$_3$ were stored at pH 1 at -20 °C and diluted to 100 μM into buffer as required for titrations.
3. Results

3.1 — Cam Activity Assay

In order to test the integrity of the Cam, Tb$^{3+}$ energy transfer experiments were carried out. The binding of MLCK based peptides to Cam requires that the four EF hand calcium binding loops of Cam be filled to expose the hydrophobic patches to which the peptides bind (12, 19, 20, 27). The lanthanide, Tb$^{3+}$, is isomorphous to Ca$^{2+}$ and will replace Ca$^{2+}$ in the binding loops (66, 67, 68). However, Tb$^{3+}$ is luminescent and can be excited by energy transfer from a chromophore provided it is close ($\sim 10 \text{ A}^0$) to the bound Tb$^{3+}$ (69). Bovine brain Cam has two Tyr residues, one in proximity to the loop III at position 99 and the other in position 138 in proximity to loop IV. If Cam is viable then when the Tyr residues are excited at 280 nm, Tb$^{3+}$ luminescence is observed as a result of energy transfer from Tyr 99 to Tb$^{3+}$ as it fills binding loop III. As Tb$^{3+}$ binds into loop IV a further small increase in luminescence is observed due to energy transfer from Tyr 138 to the Tb$^{3+}$ in loop IV. The larger enhancement seen with Tyr 99 is due to the closer proximity of Tyr 99 to the binding loops than Tyr 138. The binding of Tb$^{3+}$ into the loops of Cam mimics the binding of Ca$^{2+}$ into these loops and the Tb$^{3+}$ titration curve becomes an assay for ensuring the integrity of the Cam and that valid peptide binding might occur. Figure 3.1 compares the fluorescence energy transfer from two different samples of Cam, one that demonstrates the behaviour of native intact Cam while the other is that of ‘inactive’ Cam. Samples of Cam were checked regularly for integrity of structure. Activity assays of this nature became necessary as it was observed that Cam (from both
sources) became inactive when stored in MOPS buffer. No effort was made to determine the origin of this denaturation.

3.2 - Phage Display Peptide Series

As indicated in the introduction (Section 1.7), from phage display methods, a single peptide was found to bind with high affinity to Cam. It was isolated and its sequence determined by Dr. B. Kay. This peptide was denoted the 12.1 peptide. Select residues within the primary sequence of this peptide were changed by Dr. Kay in an attempt to elucidate how changes within the primary sequence affected the binding of the peptide to Cam. These mutant peptides are known as the ‘evolved’ series of peptides and were denoted as E3, E3-AS, E3-LTV, E4, E5, E6, E2, E8 and E9 (Table 2.1). The properties and interaction of these peptides with Cam will be discussed in turn. The data shows that the results were dependent upon the source of the peptides. One source was purchased by Dr. Kay and provided to us. As they were consumed another set was purchased from Dr. G. Lajoie (UW). Two sources of Cam were also used, one was purchased from Sigma and the other was purified from bovine brain and was generously supplied to us by Dr. B. Mutus and Mr. Peter Pathrose. Most experiments utilized the later source of Cam and unless otherwise stated, this was the source of Cam used.

3.2.1 12.1 Peptide

The primary sequence of the 12.1 peptide was:

\[ \text{CH}_3\text{CO-S T V P R W I E D S L R G A A R A Q T R L A S A-NH}_2 \]

During the course of experiments, two sources of this peptide were used. The first source was received from Dr. Kay in North Carolina. The second source was obtained from Dr.
G. Lajoie at the University of Waterloo. The experimental results from these peptide sources are summarized separately. The peptides themselves will be referred to as K 12.1 and the UW 12.1 peptide, respectively.

3.2.1.1 K 12.1 Peptide

Purification and Characterization

The K 12.1 peptide was checked for purity by HPLC using a C8 reverse phase column. An elution gradient from 100% water to 100% acetonitrile over 30 min was used. The peptide eluted at approximately 40% acetonitrile as a single band (Figure 3.2). The band was collected and checked by UV spectroscopy for the characteristic Trp absorption spectrum. Figure 3.3 shows an example of a typical absorption spectrum of this peptide. Based on the HPLC trace and the absorption data, the 12.1 peptide was deemed to be pure and no further purification was required. No further characterization of this peptide was done.

Fluorescence Spectra of K 12.1 Peptide

Fluorescence spectra for the K 12.1 peptide alone and complexed to Cam with an excess of K 12.1 in a 3.5:1 ratio are shown in Figure 3.4. The fluorescence maximum of the spectra did not appear to shift; however, there was an enhancement in fluorescence intensity as the peptide bound to Cam.

Cam Titration of K 12.1

A 2 μM sample of K 12.1 peptide was titrated by adding Sigma Cam to the sample. The resulting fluorescence titration curve (em. 345 nm) is shown in Figure 3.5. As binding occurred the fluorescence of the Trp was quenched. The fluorescence intensity
Figure 3.1 — Terbium energy transfer experiments used to confirm the integrity of Cam. Comparison of titrations with native Cam (closed circles) and denatured Cam (open circles) is shown. Ex. 280 nm, Em. 545 nm.

Figure 3.2 — HPLC trace of the K 12.1 peptide. The single band (continuous line) indicates that the peptide is pure. Dashed line represents the gradient.

Figure 3.3 — Typical absorbance spectrum of Trp.

Figure 3.4 — Fluorescence spectra of K 12.1 alone (continuous line) and complexed to Cam in a 3.5:1 ratio (dotted line). Double monochromator and 4 nm bandpass on excitation and emission sides. Ex. 295 nm, Em. 300-450 nm.
did not level until a ratio of 2:1, Cam:peptide was reached. This suggested that Scatchard analysis of the binding may be applicable. $F_e$ was estimated from the plateau fluorescence (Table 3.1) and an attempt to fit the data to a Scatchard plot showed that binding of the K 12.1 peptide to Cam was quite strong. The data showed that there was no free Cam during the first fifteen additions or a 1.5:1 ratio of peptide:Cam. The remaining data, including the points near the end of the titration that appear to have formed a plateau, were fit to a Scatchard plot (Figure 3.6). From this plot, the $K_d$ was estimated to be 203 nM with an $x$-axis intercept of 1 and correlation coefficient 0.649.

**K 12.1 Titrations of Cam**

Two different samples of Cam were titrated with K 12.1 peptide. Peptide aliquots were added to a sample of 2 μM Sigma Cam to a ratio of 4:1, peptide:Cam and this titration is shown in Figure 3.7. Scatchard analysis was not applicable due to the scatter of points within the binding curve. A second sample of Dr. Mutus' Cam of concentration 1 μM was also titrated to a ratio of 3.5:1, peptide:Cam. (Figure 3.8). Scatchard analysis, with $ΔF_e$ estimated from the plateau (Table 3.1), indicated that the $K_d$ was 204 nM with correlation coefficient of 0.977 if the first 10 points and plateau points were omitted. Figure 3.9 shows the Scatchard plot for this titration with all points included. Comparison between these two titrations showed that the trend was the same for both sources of Cam. However, there was an enhancement in peptide fluorescence signal (em. 345 nm) as the peptide:Cam complex was formed until an approximate ratio of 2:1, peptide:Cam at which point the fluorescence intensity levelled off.
### Table 3.1 – $F_\infty / \Delta F_\infty$ Values used for Scatchard Analysis

<table>
<thead>
<tr>
<th>Peptide Titration</th>
<th>$F_\infty / \Delta F_\infty$ Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cam $\rightarrow$ K 12.1</td>
<td>$5.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>K 12.1 $\rightarrow$ Cam</td>
<td>$1.55 \times 10^{-5}$</td>
</tr>
<tr>
<td>Cam $\rightarrow$ UW 12.1</td>
<td>$8.9 \times 10^{-4}$</td>
</tr>
<tr>
<td>K E3-LTV $\rightarrow$ Cam</td>
<td>$1.02 \times 10^{-6}$</td>
</tr>
<tr>
<td>Cam $\rightarrow$ UW E3-LTV</td>
<td>$4.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>UW E3-LTV $\rightarrow$ Cam</td>
<td>$-8.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>K2 E3-LTV $\rightarrow$ Cam</td>
<td>$-3.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>K E3-AS $\rightarrow$ Cam</td>
<td>$7.4 \times 10^{+5}$</td>
</tr>
<tr>
<td>UW E3-AS $\rightarrow$ Cam</td>
<td>$5.3 \times 10^{+3}$</td>
</tr>
<tr>
<td>K E3 $\rightarrow$ Sigma Cam</td>
<td>$1.73 \times 10^{+5}$</td>
</tr>
<tr>
<td>K E3 $\rightarrow$ Cam</td>
<td>$8.8 \times 10^{+5}$</td>
</tr>
<tr>
<td>K E4 $\rightarrow$ Cam</td>
<td>$3.6 \times 10^{+5}$</td>
</tr>
<tr>
<td>Cam $\rightarrow$ UW E4</td>
<td>$1.85 \times 10^{-4}$</td>
</tr>
<tr>
<td>UW E4 $\rightarrow$ Cam</td>
<td>$6.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>K E5 $\rightarrow$ Cam</td>
<td>$-4.8 \times 10^{-4}$</td>
</tr>
<tr>
<td>Cam $\rightarrow$ UW E5</td>
<td>$3.85 \times 10^{-4}$</td>
</tr>
<tr>
<td>UW E5 $\rightarrow$ Cam</td>
<td>$-2.03 \times 10^{+3}$</td>
</tr>
<tr>
<td>Cam $\rightarrow$ UW E6</td>
<td>$2.0 \times 10^{+4}$</td>
</tr>
<tr>
<td>UW E6 $\rightarrow$ Cam</td>
<td>$1.2 \times 10^{+5}$</td>
</tr>
<tr>
<td>KirGem $\rightarrow$ Cam #1</td>
<td>$1.7 \times 10^{+5}$</td>
</tr>
<tr>
<td>KirGem $\rightarrow$ Cam #2</td>
<td>$1.1 \times 10^{+5}$</td>
</tr>
<tr>
<td>sm-MLCK $\rightarrow$ Cam #1</td>
<td>$2.6 \times 10^{+5}$</td>
</tr>
<tr>
<td>sm-MLCK $\rightarrow$ Cam #2</td>
<td>$6.6 \times 10^{+5}$</td>
</tr>
</tbody>
</table>
Figure 3.5 – Sigma Cam titration of K 12.1 peptide. Double monochromator and 4 nm bandpass excitation and emission sides of instrument. Ex. 295 nm; Em. 345 nm.

Figure 3.6 – Scatchard plot of Cam titration of K 12.1 peptide.

Figure 3.7 – K 12.1 titration of 2 μM Sigma Cam. Double monochromator and 4 nm bandpass excitation and emission sides of instrument. Ex. 295 nm, Em. 345 nm.

Note: For titrations of this nature, squares represent the peptide added to buffer, triangles represent peptide titration of Cam and circles represent the difference curve of the titration of buffer subtracted from titration of Cam.

Figure 3.8 – K 12.1 titration of 1 μM Cam (Dr. Mutus). Double monochromators and 4 nm bandpass on excitation and 8 nm bandpass emission sides of instrument. Ex. 295 nm, Em. 345 nm.
Acrylamide Quenching

Acrylamide quenching experiments were completed to measure the solvent accessibility of the Trp in the complex. However, previous work on this peptide by Brennan et al. (52) included acrylamide titration experiments and hence experiments with a representative 1:1 sample were not completed. Cam/Peptide titrations of K12.1 indicated the formation of a 2:1 complex (see discussion) and hence in order to examine the solvent accessibility of the Trp residues within the Cam:P2 complex acrylamide titrations at ratios representative of this complex were conducted. Hence a sample of 2 μM K12.1, 1 μM Cam was prepared and titrated with acrylamide to a final concentration of 8 M acrylamide. The resulting linear Stern-Volmer plot yielded a $K_{SV}$ value of 8.16 M$^{-1}$ with a correlation coefficient of 0.999.

3.2.1.2 UW 12.1 Peptide

Purification and Characterization

The UW 12.1 peptide was synthesized and purified at the University of Waterloo by Dr. G. Lajoie's group. The purity of the peptide was confirmed using HPLC with a C18 reverse phase column. The elution gradient ran between 5% aqueous acetonitrile and 100% acetonitrile over 30 min. The peptide was found to elute as mainly a single band with a smaller band associated with the main band at approximately 20 min or 48% acetonitrile as shown in Figure 3.10. The smaller peaks at approximately 35 and 44 min elution were present in the blank elution.

The peptide was characterized by MALDI mass spectrometry by Dr. G. Lajoie. The mass spectrometry analysis (in duplicate) found the molecular weight of the peptide
to be 2711.00 g/mole and 2710.88 g/mole which was in excellent agreement with the true molecular weight of 2711.31 g/mole. A mass spectrometry trace for the later trial is shown in Figure 3.11.

Fluorescence Spectra of the UW 12.1 Peptide

Fluorescence spectra were collected for a 3 µM sample of UW 12.1 peptide and a 1:1, Cam:peptide complex with Cam and a 2:1 ratio (Figure 3.12) Upon binding of the peptide to Cam, the Trp fluorescence blue shifted from 350 nm to 344 nm as more Cam was added to the system. The fluorescence intensity decreased as Cam was added to the peptide.

Cam Titration of UW 12.1

A 3 µM sample of UW 12.1 peptide was titrated with Cam to a ratio of 2:1, Cam:peptide. The titration curve for this experiment is shown in Figure 3.13. From the titration curve it can be clearly seen that the fluorescence intensity was reduced as the peptide bound to Cam. Further, the fluorescence intensity did not fully level off even at a ratio of 2:1, Cam:peptide indicating that Scatchard analysis may be applicable. A double reciprocal plot of 1/Fluorescence vs 1/Cam/peptide allowed for the estimation of $F_{∞}$. (Table 3.1) Based on this, a Scatchard plot was generated and is shown in Figure 3.14. From the Scatchard plot, two distinct regressions are shown. Regression of the initial 8 points (approximately 1:1 ratio) indicated that the $K_d$ was 8.9 µM with a correlation coefficient of 0.533. The latter seven points indicated a $K_d$ of 2.2 µM with a correlation coefficient of 0.942 and x-axis intercept of approximately 1. This contrasts with a value of nearly 100 nM for K 12.1.
Figure 3.9 — Scatchard analysis of K 12.1 titration of Cam (Dr. Mutus). All points have been included although all points were not included in the final regression.

Figure 3.10 — HPLC trace of UW 12.1 peptide. Absorbance intensity is represented by the continuous line while the dashed line represents the gradient.

Figure 3.11 — Mass spectrometry trace of UW 12.1 Peptide.
UW 12.1 Titration of Cam

A sample of 1 μM Cam was titrated with the UW 12.1 peptide to a final ratio of 6:1, UW 12.1:Cam. The titration curve is shown in Figure 3.15 and upon initial inspection, there did not appear to be a change in fluorescence as binding of the peptide to Cam occurred. Upon expansion of the difference curve (Figure 3.16), the quenching trend seen in both the fluorescence spectra and the Cam titration of UW 12.1 is apparent in the early stages of the titration to a ratio of about 2:1 and then the fluorescence increases to a ratio of nearly 6:1; peptide:Cam where it had not yet plateaued. This suggests the formation of a 2:1 complex (see discussion).

3.2 E3-LTV Peptide

The E3-LTV peptide has the following primary sequence:

\[\text{CH}_3\text{CO-} \text{SVPRWIEDSLGRGARALKTVASANH}_2\]

This sequence is completely homologous to the 12.1 peptide except that the 'TRL' in the C-terminus of the 12.1 peptide has been changed to 'LTV' (highlighted in sequence) in the E3-LTV peptide. Titration experiments with this peptide were conducted with 3 different sources of peptide. The first source was the original sample of peptide sent from Dr. Kay. The second source was synthesized at UW and the final source was a second sample of E3-LTV peptide sent by Dr. Kay. These were designated as K E3-LTV, the UW E3-LTV peptide and K2 E3-LTV peptide respectively.
Figure 3.12 — Fluorescence spectra of UW 12.1 peptide alone (continuous line), 1:1 ratio (dotted line) and 2:1; Cam: UW 12.1 ratio (dot-dashed line). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 300–450 nm, single monochromator, 4 nm bandpass.

Figure 3.13 — Cam titration of UW 12.1 (3 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 350 nm, single monochromator, 4 nm bandpass.

Figure 3.14 — Scatchard plot for Cam titration of UW 12.1 peptide.

Figure 3.15 — UW 12.1 titration of Cam (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 350 nm, single monochromator, 4 nm bandpass.
3.2.2.1 K E3-LTV Peptide

Purification and Characterization

Attempts were made to purify this peptide by HPLC using a C8 reverse phase column with a water/acetonitrile gradient. However, the peptide did not appear to elute from the column. A CM-5PW cation exchange column was used and eluted with an aqueous/0.2 M NH$_4$OAc gradient over 20 min. A peptide eluted at approximately 15 min or 25 % NH$_4$OAc (Figure 3.17). This band was checked for Trp absorption and found to contain Trp. Other bands eluted in the water phase and at 100 % NH$_4$OAc. These samples were also collected and checked for the Trp absorption. No strong Trp absorption was observed. Owing to degradation on storage, this peptide was not characterized by mass spectrometry.

Fluorescence Spectra of K E3-LTV Peptide

Fluorescence spectra for the E3-LTV peptide alone and complexed to Cam with an excess of E3-LTV in a 3:1 ratio is shown in Figure 3.18. As with the fluorescence spectra for the K 12.1 peptide, there was no shift in the spectral maximum of this latter sample compared to that of the peptide alone. There was a large enhancement in fluorescence intensity.

K E3-LTV Titration of Cam

A 1 μM sample of Cam was titrated with K E3-LTV peptide to a 3:1 excess of E3-LTV. Figure 3.19 shows there is a very large enhancement of fluorescence intensity as binding occurs. The total fluorescence curve and that of the difference fluorescence intensity follow one another until a 0.5:1; peptide:Cam ratio was reached at which point
Figure 3.16 — Difference curve from UW 12.1 titration of Cam (Figure 3.15).

Figure 3.17 — HPLC trace of K E3-LTV peptide. Continuous line represents the absorbance while the gradient is shown by the dashed line.

Figure 3.18 — Fluorescence spectra of K E3-LTV peptide alone (continuous line) and 3:1 K E3-LTV-Cam complex (dotted line). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 300-450 nm, 8 nm bandpass.

Figure 3.19 — K E3-LTV titration of Cam (1 μM). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 345 nm, 8 nm bandpass.
the titration of Cam began to curve such that the difference curve plateaued at a ratio of 2:1; peptide:Cam. This indicates very strong binding of the peptide to Cam up to a 1:1 ratio and negligible free peptide was present to this ratio. Points beyond this ratio were fit to a Scatchard plot (Figure 3.20) using the plateau value as ΔF∞ (Table 3.1). Analysis indicated the $K_d$ to be 46 nM with a correlation coefficient of 0.964 and x-axis intercept of 1.

**Acrylamide Quenching**

Acrylamide quenching titrations were carried out for K E3-LTV/Cam samples in ratios of 1:4 and 2:1 peptide:Cam to final concentrations of acrylamide of 400 mM and 800 mM respectively. From the Stern-Volmer plots (data not shown), the 1:4 ratio sample was found to have a $K_{SV}$ of 2.6 M⁻¹ with a correlation coefficient of 0.998 while the $K_{SV}$ for the 2:1 sample was determined to be 1.4 M⁻¹ with a correlation coefficient of 0.993.

**Tb³⁺ Energy Transfer**

The location of the Trp on the peptide within the Cam:peptide complex may be found using Tb³⁺ energy transfer. This was accomplished by exciting the Trp (295 nm) and monitoring Tb³⁺ luminescence (545 nm) as Tb³⁺ fills the Ca²⁺ binding loops of Cam. Since binding of the Tb³⁺ to Cam’s loops is sequential and provided that the order of binding does not change for the binding of Tb³⁺ to the complex, hence it is possible to locate the loop or loops that are near the Trp fluorophore.
Tb$^{3+}$ energy transfer titration experiments were performed for 3:1 and 1:3 peptide:Cam samples. Excitation of the samples at 295 nm did not show any Tb$^{3+}$ luminescence.

### 3.2.2.2 The UW E3-LTV Peptide

**Purification and Characterization**

The UW E3-LTV peptide was checked for purity using the method outlined for the UW 12.1 peptide (C18 reverse phase chromatography). The HPLC trace of UW E3-LTV showed two bands one at 20 min (50% acetonitrile) and the other at 22 min (57% acetonitrile), the latter being the larger band. The UW E3-LTV peptide was poorly soluble in water and a saturated solution was 25 μM in peptide. Other peaks present in the peptide HPLC trace were also present in the blank HPLC trace and in some cases larger than those of the E3-LTV peptide (Figure 3.21). No further purification was completed.

Duplicate samples of the peptide were further characterized by mass spectrometry showing molecular weights of 2653.38 g/mole and 2654.00 g/mole, in excellent agreement with the calculated molecular weight of 2654.26 g/mole (Figure 3.22).

**Fluorescence Spectra of UW E3-LTV Peptide**

Fluorescence spectra of the E3-LTV peptide alone and complexed to Cam in a 1:1 ratio 2:1 ratio are shown in Figure 3.23. The spectra show that as Cam was added there was a slight blue shift in the wavelength maximum from approximately 350 nm to 344 nm and the fluorescence intensity was reduced in contrast to the observation of K E3-LTV.
Figure 3.20 — Scatchard plot of K E3-LTV titration of Cam. The continuous line represents absorbance while the gradient is shown by the dashed line.

Figure 3.21 — HPLC trace of UW E3-LTV peptide. Continuous line represents absorbance while the gradient is shown by the dashed line.

Figure 3.22 — Mass spectrometry trace of UW E3-LTV Peptide.
Cam Titration of the UW E3-LTV Peptide

A 3 μM sample of the UW E3-LTV peptide was titrated with Cam to a final ratio of 2:1, Cam:UW E3-LTV. From Figure 3.24, it can be seen that the fluorescence intensity is quenched as binding occurred and further that the fluorescence intensity did not reach a plateau. In order to estimate the fluorescence at a high ratio of Cam:UW E3-LTV, a double reciprocal plot of 1/F vs 1/ Ratio was used and the resulting $F_\infty$ is shown in Table 3.1. A Scatchard plot was then generated and is shown in Figure 3.25. The $K_d$ was found to be 1.4 μM with a correlation coefficient of 0.953 and x-axis intercept of approximately 1.

UW E3-LTV Titration of Cam

A 1 μM sample of Cam was titrated with UW E3-LTV to an excess UW E3-LTV of 3:1. The results of this titration are shown in Figure 3.26. Similar to the UW 12.1 peptide, it may appear that this peptide did not to bind to Cam. However, when the difference curve was expanded, there was clearly a reduction in fluorescence intensity to a peptide/Cam ratio of 1:1 at which point the difference fluorescence began to increase. (Figure 3.27) Also shown in Figure 3.27 is the difference spectrum of an earlier titration showing that these results are reproducible. From the second plot, Scatchard analysis was performed using $\Delta F_\infty$ as the point where the fluorescence intensity plateaued before it increased (Table 3.1). The resulting Scatchard analysis (Figure 3.28), omitting the first eleven points (approximately 1:1 ratio) indicated the $K_d$ to be 540 nM with correlation coefficient of 0.562 and x-axis intercept of approximately 1.4.
Figure 3.23 – Fluorescence spectra of UW alone (continuous line), 1:1 ratio (dotted line) and 1:0.75 UW E3-LTV ratio (dot-dashed line). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 300–480 nm, single monochromator, 4 nm bandpass.

Figure 3.24 – Cam titration of UW E3-LTV (3 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 350 nm, single monochromator, 4 nm bandpass.

Figure 3.25 – Scatchard plot for Cam titration of UW E3-LTV.

Figure 3.26 – UW E3-LTV titration of Cam (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 350 nm, single monochromator, 4 nm bandpass.
3.2.2.3 K2 E3-LTV Peptide

Purification and Characterization

Due to the dissimilar behaviour in the titration curves of K E3-LTV peptide and UW E3-LTV peptide, a second sample of the E3-LTV peptide from Dr. B. Kay was obtained (K2 E3-LTV). This sample appeared to be very soluble in water unlike the UW E3-LTV peptide. The purity of this second sample of peptide was checked using the HPLC conditions developed for the UW E3-LTV peptide. This sample of peptide eluted from a C18 column (Figure 3.29). The largest band eluted at 23 min (60% acetonitrile) similar to the elution of UW E3-LTV and was found to have characteristic Trp absorption. This band was collected, pooled, lyophilized and redissolved in water for quantification. This K2 E3-LTV peptide was analysed by MALDI mass spectrometry. It had a molecular weight of 2653.50 g/mole in excellent agreement with both the molecular weights obtained for the UW E3-LTV peptide and the expected molecular weight.

K2 E3-LTV Titration of Cam

When a 1 μM sample of Cam was titrated with K2 E3-LTV peptide, the resulting titration curve was almost identical to the titration curve found for the UW E3-LTV peptide. In the resulting difference curve (Figure 3.30), the fluorescence intensity diminished to a ratio of pepide:Cam of 4:1 where it began to level off. Scatchard analysis was done estimating ΔF∞ as the plateau region (Table 3.1). Omitting the first 16 points (1:1 ratio), the resulting plot (Figure 3.31) yielded a Kd of 4 μM with a correlation coefficient of 0.848 and x-axis intercept of 2.
Figure 3.27 — Difference curve of UW E3-LTV titration of Cam (Figure 3.26) (closed circles) and difference curve of an earlier titration (open circles). Em. 345 nm.

Figure 3.28 — Scatchard plot of UW E3-LTV titration of Cam.

Figure 3.29 — HPLC trace of K2 E3-LTV peptide. Continuous line represents the absorbance while the gradient is shown by the dashed line.

Figure 3.30 — K2 E3-LTV titration of Cam (1 μM) showing difference curve only. Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 345 nm, single monochromator, 4 nm bandpass.
3.2.3 -- E3-AS Peptide

The E3-AS peptide has the following primary sequence:

\[ \text{CH}_3\text{CO-}STVP\text{RW1EDSLRASAAURALARTHASNH}_2 \]

It is completely homologous to the 12.1 peptide except that G13 and G14 have been changed to an A13 and S14. As with the 12.1 and E3-LTV peptide, more than one source of peptide was used for titration experiments and the results were quite different.

3.2.3.1 K E3-AS Peptide

Purification and Characterization

Due to difficulties with purification of the K E3-LTV peptide using reverse phase HPLC methods, the K E3-AS peptide was also purified on a CM-5PW cation exchange column. The gradient ran between water and 100% 0.2 M NH\textsubscript{4}OAc over 15 min with an initial 10 minute aqueous phase to start and a final 10 minute NH\textsubscript{4}OAc phase. Two bands eluted from the column, one at 21 min (73% NH\textsubscript{4}OAc) within the gradient and the other at 34 min (100% NH\textsubscript{4}OAc) (Figure 3.32). Both bands were checked using absorption spectroscopy for the characteristic Trp spectrum. The band at 21 min showed a very weak Trp signal while the band at 34 min exhibited a strong Trp signal. The band at 34 min was chosen to be the correct band based on its intensity and positive Trp absorption and was collected. Owing to degradation on storage, this peptide was not characterized by mass spectrometry.

Fluorescence Spectra for K E3-AS Peptide

Fluorescence spectra of a 3.5 \( \mu \)M sample of the K E3-AS peptide alone and a 3.5 \( \mu \)M K E3-AS/1 \( \mu \)M Cam sample were collected. There appeared to be a blue shift in
spectral maximum. An estimate of the Cam:P₂ complex spectrum was generated by subtracting 1.5/3.5 of the K E3-AS alone spectrum from that of the complex having an excess of K E3-AS. The Cam:P complex spectrum was estimated in the same manner except that a 2.5/3.5 ratio representative of a 1:1 ratio of peptide:Cam was used. The resulting spectra are shown in Figure 3.33. It can be seen that the complex blue shifted from 350 nm to 346 nm for both the 1:1 and 2:1 complexes. Since the concentration of fluorophore was not constant in these estimated spectra, comparison of fluorescence intensity was not possible.

**K E3-AS Titration of Cam**

A 1 μM sample of Cam was titrated with K E3-AS peptide to a final ratio of 3.5:1. The resulting titration curve is shown in Figure 3.34. The fluorescence intensity did not change for the first five additions of peptide and then increased until it began to plateau near the end of the titration. This can be seen in both the difference curve and the peptide titration of Cam curve where distinct changes in slope can be observed. These curves suggested that a 2:1 complex was being formed (see discussion). Scatchard analysis was performed estimating ΔF∞ from a double reciprocal plot (Table 3.1) and omitting the first 18 points (approximately 2:1 ratio). Analysis indicated a Kₐ of 1.4 μM with correlation coefficient of 0.943 and x-axis intercept of 1. (Figure 3.35)

**Acrylamide Quenching**

Due to the apparent 2:1 binding of K E3-AS to Cam, acrylamide studies were performed at two different ratios of peptide:Cam corresponding to the Cam:P and Cam:P₂ complexes. Hence, samples of 1:4 and 3:1 K E3-AS:Cam were titrated with acrylamide to final concentrations of acrylamide of 400 mM and 800 mM respectively. The results
Figure 3.31 — Scatchard plot of K2 E3-LTV titration of Cam.

Figure 3.32 — HPLC trace of K E3-AS peptide. Continuous line represents the absorbance while the dashed line represents the gradient.

Figure 3.33 — Fluorescence spectra of K E3-AS alone: 3.5 μM (continuous line), 3.5:1 ratio; 3.5 μM (dotted line), estimated 2:1 complex; 2 μM (dot dashed line) and estimated 1:1 complex, 1 μM (grey dotted line).

Double monochromators both sides. Ex. 295 nm, 4 nm bandpass.
Em. 300-450 nm, 8 nm bandpass.

Figure 3.34 — K E3-AS titration of Cam (1 μM). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass.
Em. 345 nm, 8 nm bandpass.
from the Stern-Volmer plots showed the $K_{SV}$ of the 1:4 complex to be 2.6 M$^{-1}$ with a correlation coefficient of 0.999 and the 3:1 sample to have a $K_{SV}$ of 1.4 M$^{-1}$ with a correlation coefficient of 0.999.

**Tb$^{3+}$ Energy Transfer**

Tb$^{3+}$ energy transfer data with excitation at 295 nm was collected for a 3:1 sample of Cam:K E3-AS. This ratio represented the Cam:P complex. The titration curve in Figure 3.36 shows that Tb$^{3+}$ luminescence enhancement occurred as the Tb$^{3+}$ cation bound into the 2nd and 3rd binding loops of Cam.

**3.2.3.2 UW E3-AS Peptide**

**Purification and Characterization**

The UW E3-AS peptide was checked for purity by C18 reverse phase HPLC. The HPLC trace shown in Figure 3.37 indicated that the peptide eluted as a single band with a small shoulder at 20 min (50% acetonitrile). Peaks at 32 and 44 min are also present in the blank.

The peptide was characterized by MALDI mass spectrometry in duplicate and showed molecular weights to be 2756.00 g/mole and 2754.75 g/mole in agreement with the true molecular weight of 2755.35 g/mole.

**Fluorescence Spectra for UW E3-AS Peptide**

Fluorescence spectra of UW E3-AS alone and complexed to Cam in 1:1 and 2:1 ratios of Cam:UW E3-AS are shown in Figure 3.38. The spectra show that on peptide binding, the wavelength maximum blue shifted from 350 nm to 340 nm for both the 1:1 and 2:1 complexes and the fluorescence intensity decreased.
Figure 3.35 — Scatchard plot of $K_{E3-AS}$ titration of Cam.

Figure 3.36 — Terbium titration curve of a 3:1: Cam:K E3-AS sample. Double monochromators both sides. Ex. 295 nm. 4 nm bandpass. Em. 545 nm. 8 nm bandpass.

Figure 3.37 — HPLC trace of UW E3-AS peptide. Continuous line represents the absorbance intensity while the dashed line represents the gradient.

Figure 3.38 — Fluorescence spectra of UW E3-AS alone (continuous line), 1:1 ratio (dotted line) and 2:1: Cam:UW E3-AS ratio (dot dashed line). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 300-450 nm, single monochromator, 8 nm bandpass.
Cam Titration of UW E3-AS Peptide

Cam titrations were carried out for 3 µM and 1 µM samples of UW E3-AS and are shown in Figures 3.39 and 3.40 respectively. The fluorescence intensity was quenched as binding occurred and it levelled off at a ratio of 1:1. This is indicative of the formation of a 1:1 complex and that the binding was very strong. This precluded Scatchard analysis for this system. It is noteworthy that the first addition of Cam to the peptide did not lead to a decrease in fluorescence intensity. Rather the intensity remained the same as that of the initial fluorescence (Figure 3.39) or showed only slight quenching (Figure 3.40).

UW E3-AS Titration of Cam

A 1 µM sample of Cam was titrated with UW E3-AS to a final excess of E3-AS of 3:1. The resulting titration curve is shown in Figure 3.41. The fluorescence difference spectrum is expanded in Figure 3.42. It can be seen that the fluorescence intensity is diminished upon binding of the peptide to Cam and plateaued near the 2:1 ratio before rising again throughout the third equivalent of added peptide. Scatchard analysis, estimating $\Delta F_{\infty}$ to be the plateau value near 2:1 and including only the first eleven points (1:1 ratio), indicated the $K_d$ to be 98 nM with a correlation coefficient of 0.890 and x-axis intercept of 0.6 (Figure 3.43).
Figure 3.39 — Cam titration of UW E3-AS (3 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 350 nm, single monochromator, 4 nm bandpass.

Figure 3.40 — Cam titration of UW E3-AS (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 350 nm, single monochromator, 4 nm bandpass.

Figure 3.41 — UW E3-AS titration of Cam (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 350 nm, single monochromator, 4 nm bandpass.

Figure 3.42 — Difference curve of UW E3-AS titration of Cam.
3.2.4 — E3 Peptide

The E3 peptide has the following primary sequence:

\[ \text{CH}_3\text{CO} - \text{S T V P R W I E D S L R A S A A R A Q L T V A S A } - \text{NH}_2 \]

This peptide incorporates the changes made to the primary sequence of both the E3-AS and E3-LTV peptides (highlighted in sequence). Three sources of peptide were used for experiments. The first sample was obtained from Chiron Mimotopes Peptide Systems (San Diego, CA), the second was synthesized at UW and the final source was sent by Dr. B. Kay and is of unknown commercial source. These were designated K E3, UW E3 and K2 E3 respectively. The UW E3 peptide differed slightly in that an extra alanine was present in the C-terminus (underlined).

3.2.4.1 K E3 Peptide

Purification and Characterization

Attempts to purify the K E3 peptide by HPLC using a reverse phase C8 column were not successful. It could be purified using a CM-5PW cation exchange column with a water/0.5 M NH₄OAc (pH 7) gradient. The peptide eluted from the column in the long water phase of the gradient. (Figure 3.44) The largest of the peaks eluted at 19 min (0% NH₄OAc) and was collected and found to have the characteristic Trp absorption spectrum. This peak was therefore collected, pooled, lyophilized, re-dissolved in MOPS buffer and the solution concentration determined.

This peptide was characterized by MALDI mass spectrometry at the University of Waterloo. Results indicated a major peak corresponding to a molecular weight of 2626.00 g/mole. (Figure 3.45) This is in good agreement with the theoretical molecular
Figure 3.43 — Scatchard plot of UW E3-AS titration of Cam.

Figure 3.44 — HPLC trace of K E3 peptide. Continuous line represents absorbance while dashed line shows the gradient.

Figure 3.45 — Mass spectrometry trace of K E3 Peptide.
weight of 2627.21 g/mole. Many polymers were also found suggesting degradation of
the sample after long storage.

**Fluorescence Spectra of the K E3 Peptide**

Fluorescence spectra were collected for K E3 alone and complexed to Dr. B.
Mutus' Cam in a 1:3 ratio of K E3:Cam. (Figure 3.46) The spectra indicated that the
maximum wavelength blue shifted from 349 nm to 337 nm as binding occurred. Further,
the fluorescence intensity was quenched.

**K E3 Titrations of Cam**

Samples of 2 µM and 1 µM Sigma Cam were titrated with K E3 peptide to ratios
of excess peptide of 3:1 and are shown in Figures 3.47 and 3.48 respectively. These
titration curves show similar patterns. The fluorescence intensity did not change during
the first few additions of K E3 and then was enhanced until a ratio of approximately 2:1
where it plateaued. Scatchard analysis of the latter sample (Figure 3.49) indicated a $K_d$ of
980 nM with a correlation coefficient of 0.891 and x-axis intercept of 0.85 omitting the
first 12 points (1:1 ratio) and estimating $\Delta F_\infty$ from a double reciprocal plot (Table 3.1). A
1 µM sample of Dr. Mutus' Cam was also titrated with K E3 peptide to an excess of K E3
of 7:1. (Figure 3.50) The initial points of the difference curve to a ratio of about 0.5:1
showed that there was very little enhancement as binding occurred. This was similar to
results found with the Sigma Cam. Beyond 0.5, the enhancement was much greater as
K E3 bound to Dr. Mutus' Cam. Scatchard analysis, estimating $\Delta F_\infty$ from a double
reciprocal plot (Table 3.1) and omitting the first 18 points (2:1 ratio), indicated a $K_d$ of
Figure 3.46 — Fluorescence spectra of K E3 alone (continuous line) and in a 1:3 K E3:Cam ratio (dotted line). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 300-450 nm, 8 nm bandpass.

Figure 3.47 — K E3 titration of Cam (2 µM). Double monochromators and 4 nm bandpass both sides. Ex. 295 nm, Em. 345 nm.

Figure 3.48 — K E3 titration of Sigma Cam (1 µM). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 345 nm, 8 nm bandpass.

Figure 3.49 — Scatchard plot of K E3 titration of Sigma Cam.
460 nM with a correlation coefficient of 0.992 and x-axis intercept of 1 (Figure 3.51). These results suggested the formation of a 2:1 complex (see Discussion).

**Acrylamide Quenching**

Samples of 1 µM K E3, 1 µM K E3/4 µM Cam and 2.5 µM K E3/1 µM Cam were titrated with acrylamide to final concentrations of 400 mM acrylamide for the first two samples and 800 mM acrylamide for the latter sample. Stern-Volmer plots were generated for each experiment and the $K_{SV}$'s determined to be 10.3 M$^{-1}$ with a correlation coefficient of 0.999, 2.8 M$^{-1}$ with a correlation coefficient of 0.997 and 1.7 M$^{-1}$ with a correlation coefficient of 0.999 respectively.

**Tb$^{3+}$ Energy Transfer**

Tb$^{3+}$ energy transfer experiments were completed for a 1 µM K E3/3 µM Cam sample and a 10 µM K E3/1 µM Cam sample. Results showed an enhancement in luminescence in the 2nd and 3rd loops of the former and the 3rd and 4th loops of the latter sample. This discrepancy in energy transfer loops may or may not be real since the concentration of the Cam in the second sample was a third of that in the first sample. Therefore, due to the low concentration of the second sample, it was difficult to estimate which loop was showing enhancement due to the low signal to noise ratio.

**3.2.4.2 UW E3 Peptide**

**Purification and Characterization**

The UW E3 peptide was checked for purity by reverse phase C18 HPLC. The HPLC trace shown in Figure 3.52 indicated that the peptide eluted as a single band at 21 min (55% acetonitrile). Peaks at 32 and 44 min are also present in the blank.
The peptide was characterized by MALDI mass spectrometry in duplicate and showed molecular weights to be 2698.00 g/mole and 2697.00 g/mole which is in excellent agreement with the true molecular weight of 2698.3 g/mole.

**Fluorescence Spectra of the UW E3 Peptide**

Fluorescence spectra were collected for UW E3 alone (1 μM) and complexed in 1:1 and 2:1 ratios of Cam:UW E3 (Figure 3.53). Spectra indicated that upon binding the wavelength maximum blue shifted from 347 nm to 334 nm for both the 1:1 and 2:1 ratios. Further, the fluorescence intensity was quenched as binding occurred.

**Cam Titration of UW E3 Peptide**

A 1 μM sample of the UW E3 peptide was titrated with Cam to a final excess of Cam of 1.5:1. Figure 3.54 indicated that the fluorescence intensity was quenched upon the addition of Cam until the ratio of Cam:UW E3 was stoichiometric at which point the fluorescence intensity plateaued indicating that binding was complete. This suggested that the binding of UW E3 to Cam was very strong and precluded Scatchard analysis.

**UW E3 Titration of Cam**

A 1 μM sample of Cam was titrated with the UW E3 peptide to an excess of peptide of 3:1. The resulting titration curve looked very similar to those shown for UW E3-AS, UW E3-LTV and UW 12.1 such that at first glance the peptide did not appear to be binding. Expansion of the difference curve indicated that the fluorescence intensity was quenched to a ratio of approximately 2.5:1 where it began to plateau. (Figure 3.55) Due to the lack of points between a 1:1 and 2:1 ratio, Scatchard analysis was not applicable.
Figure 3.50 — K E3 titration of Dr. Mutus' Cam (1 μM). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 345 nm, 8 nm bandpass.

Figure 3.51 — Scatchard plot of K E3 titration of Dr. Mutus' Cam.

Figure 3.52 — HPLC trace of UW E3 peptide. Continuous line represents the absorbance and the dashed line shows the gradient.

Figure 3.53 — Fluorescence spectra of UW E3 peptide alone (continuous line), 1:1 ratio (dotted line) and a 2:1: Cam:UW E3 ratio (dot dashed line). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 300-450 nm, single monochromator, 4 nm bandpass.
### 3.2.4.3 K2 E3 Peptide

**Purification and Characterization**

The K2 E3 peptide was purified by C18 reverse phase HPLC. The major band eluted at 23 min (60% acetonitrile) and was found to have the characteristic Trp absorption spectrum. (Figure 3.56) This peak was collected, pooled, lyophilized, redissolved in water and quantified. The retention time was similar to the UW E3 peptide. The molecular weight of the purified K2 E3 peptide was found to be 2626.38 g/mole which is in agreement with the expected molecular weight of the 2627.21 g/mole.

**Fluorescence Spectra of the K2 E3 Peptide**

Fluorescence spectra were collected for K2 E3 alone (6 μM) and complexed to Cam in a 6:1 ratio of K2 E3:Cam. Spectra showed that the maximum wavelength blue shifted from 350 nm to 348 nm and the fluorescence intensity was quenched (Figure 3.57).

**K2 E3 Titration of Cam**

A 1 μM sample of Cam was titrated with K2 E3 to an excess of 6:1. The resulting titration curves resembled the UW E3 peptide. Expansion of the difference curve (Figure 3.58) indicated that the fluorescence intensity was quenched to a ratio of 2:1 where the fluorescence intensity plateaued indicating that the binding was complete. Scatchard analysis was attempted; however, the resulting curve indicated a positive slope which is not reasonable for Scatchard analysis.
Figure 3.54 – Cam titration of UW E3 (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 347 nm, single monochromator, 4 nm bandpass.

Figure 3.55 – Difference curve of UW E3 titration of Cam (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 347 nm, single monochromator, 4 nm bandpass.

Figure 3.56 – HPLC trace of K2 E3 peptide. Continuous line represents absorbance while dashed line shows the gradient.

Figure 3.57 – Fluorescence spectra of K2 E3 peptide alone (continuous line) and in a 6:1: K2 E3:Cam ratio (dotted line). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 300-450 nm, single monochromator, 4 nm bandpass.
3.2.5 – E4 Peptide

The E4 peptide has the following primary sequence:

\[ \text{CH}_3\text{CO} - \text{VPRWIEDSLRELAA\text{T}\text{A}Q\text{S}SSV\text{A}A-N\text{H}_2} \]

This sequence involved several changes from the 12.1 peptide (highlighted). The Gly 13 and Gly 14 were changed to Glu 11 and Leu 12 and the N-terminal Ser and Thr have been removed. Also the C-terminus was changed from Thr 20, Arg 21 and Leu 22 to Ser 18, Ser 19 and Val 20 and Arg 17 was changed to Thr 15. Two sources of this peptide were used, the first was obtained from Chiron Mimotopes Peptide Systems (K E4) and the second was obtained from the University of Waterloo (UW E4).

3.2.5.1 K E4

Purification and Characterization

Mass spectrometry quality assurance data accompanying the K E4 peptide stated that the peptide was approximately 60% pure and that the molecular weight of the peptide sample was 2498.7 g/mole. The expected molecular weight of this peptide is 2503.07 g/mole.

Attempts to purify the K E4 peptide by HPLC using a C8 reverse phase column were unsuccessful. The peptide did not appear to elute from the column under a variety of conditions. The K E4 peptide was purified using a CM-5PW cation exchange column and eluting with a water/0.2 M NH\text{4}OAc gradient (Figure 3.59). Peaks eluted within the long water phase of this gradient and some separation of peaks within the water phase and the gradient were observed. Reproducibility was evident within the peaks that eluted within the gradient; however, the peaks that eluted within the water phase were
sometimes separated to a greater degree than shown in Figure 3.59. The peaks that eluted
during the water phase were collected and checked for the characteristic Trp absorption
spectrum. The first peak did not contain Trp while the second and largest peak at 15 min
(\textasciitilde0\% NH₄OAc) did. This peak was collected, pooled, lyophilized, re-dissolved in MOPS
buffer, and the solution concentration determined. Owing to degradation of the sample,
the K E4 peptide was not characterized by mass spectrometry.

**Fluorescence Spectra of K E4 Peptide**

Fluorescence spectra were collected for K E4 alone and complexed in a 3:1.
Cam:K E4 ratio. Spectra indicated that the fluorescence maximum did not blue shift upon
binding; however, there was a slight quenching (Figure 3.60). This was in contrast to the
titration curves which showed an enhancement in fluorescence intensity as peptide bound
to Cam.

**K E4 Titration of Cam**

A 1 \(\mu\)M sample of Sigma Cam was titrated with K E4 to a final excess of peptide
of 3.5:1. The difference curve indicated that the fluorescence intensity was enhanced as
binding occurred and plateaued at a ratio of 1.5:1 (Figure 3.61). The interesting part
resided in the curve in which K E4 was added to buffer (Figure 3.62). This curve
indicated that the fluorescence signal was not linear with peptide concentration. This
suggested that the peptide was forming a dimer (see Discussion). Scatchard analysis of
the difference curve was not applicable because it represented two processes. To
eliminate the change in fluorescence contributed by the dimer, the initial points of the
buffer curve were extrapolated and a corrected difference curve generated that was
Figure 3.58 — Difference curve of K2 E3 titration of Cam (1 μM). Ex. 295 nm, double monochromators, 4 nm bandpass. Em. 345 nm, single monochromator, 4 nm bandpass.

Figure 3.59 — HPLC trace of K E4 peptide. Continuous line represents absorbance while dashed line represents the gradient.

Figure 3.60 — Fluorescence spectra of K E4 alone (continuous line) and in a 3:1 Cam:K E4 ratio (dotted line). Double monochromators on both sides. Ex. 295 nm, 4 nm bandpass. Em. 300-450 nm, 8 nm bandpass.

Figure 3.61 — K E4 titration of Cam (1 μM). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 345 nm, 8 nm bandpass.
representative of monomer binding to Cam (Figure 3.63). This curve indicated fluorescence enhancement to a 1.5:1 ratio, excess K E4 in contrast to the fluorescence spectra. Scatchard analysis of this curve, estimating ΔF∞ as the plateau value and omitting the first 9 points (1:1 ratio), indicated a K_d of 43 nM with a correlation coefficient of 0.915 and x-axis intercept of 1 (Figure 3.64).

**Determination of the K_d of the Monomer-Dimer Complex of K E4**

The formation of the dimer complex complicated the study of the binding of the peptide to Cam because it was a competitive process. Therefore, it was necessary to know how strong the self association of the K E4 peptide was in order to gauge the degree of competition this side reaction offered that of the Cam binding.

The dimerization process is:

\[ P_1 + P_1 \rightarrow P_2 \]

where P_1 is the monomer and P_2 is the dimer.

It follows that:

\[ K_A = \frac{[P_2]}{[P_1][P_1]} \]  \hspace{1cm} 3.1

The fluorescence intensity can be defined as follows providing that all of the instrumental parameters remain unchanged.

\[ F = \phi_M [P_1] + \phi_D [P_2] \]  \hspace{1cm} 3.2

where: \( \phi_M \) represents the quantum yield of the monomer

\( \phi_D \) represents the quantum yield of the dimer

\( F \) represents the fluorescence intensity for any \([P]\)

At any concentration of peptide:

\[ [P_T] = [P_1] + [P_2] \]  \hspace{1cm} 3.3
\[ = [P_1] + 2 [P_1] \]
\[ = 3 [P_1] \]

If a point is chosen where \([P_1] = [P_2]\), equation 3.1 becomes:
\[ K_A = 1 / [P_1] \]
\[ 3.4 \]

and equation 3.2 becomes:
\[ F = \phi_M [P_1] + \phi_D [P_1] \]
\[ F = (\phi_M + \phi_D) [P_1] \]
\[ F/[P_T] = (\phi_M + \phi_D) [P_1] / 3 [P_1] \]
\[ F/[P_T] = (\phi_M + \phi_D) / 3 \]
\[ 3.5 \]

From a plot of \( F/[P_T] \) vs \([P_T]\), \( \phi_M \) is taken as the plateau value at low concentrations of peptide since negligible amounts of dimer are present at these concentrations and hence \( P_T = P_1 \). At high concentrations of peptide, negligible amounts of monomer are present and hence \( P_T = 2 P_2 \) (since two equivalents of \( P \) form the dimer). Hence, \( \phi_D \) is taken as twice the plateau value at high concentrations of peptide. A sample of this plot is shown in Figure 3.65 for K E4. For this sample, \( \phi_M \) was estimated from the initial points as no initial plateau was evident.

The estimated values of \( \phi_M \) and \( \phi_D \) were applied to equation 3.5 to obtain a value for \( F/[P_T] \) and using the plot of \( F/[P_T] \) vs \([P_T]\), the concentration of \( P_T \) can be read directly from the plot at the point chosen. Knowing \([P_T]\), equation 3.3 can be used to find \([P_1]\) and equation 3.4 can be used to calculate the association constant (\( K_A \)) for the self association of the K E4 peptide. The resulting \( K_d \)'s for the dimerization of K E4, done in triplicate, were found to be between 400-700 nM.
Figure 3.62 – K E4 added to Buffer curve.

Figure 3.63 – Difference curve representing monomer binding of K E4 to Cam.

Figure 3.64 – Scatchard plot of monomer binding of K E4 to Cam.

Figure 3.65 – Dimerization plot for K E4 peptide.
Acrylamide Quenching

Acrylamide quenching titrations were completed for a sample of 1 μM E4/4 μM Sigma Cam (Figure 3.66) and 2 μM E4/1 μM Cam to final concentrations of acrylamide of 400 mM and 800 mM respectively. Two separate slopes were observed for each experiment. These slopes were regressed separately and for the former sample indicated $K_{SV}$ values of 4.9 M$^{-1}$ with a correlation coefficient of 0.984 for the initial points and 2.2 M$^{-1}$ with correlation coefficient of 0.989 for the latter points. Regressions of the latter sample indicated $K_{SV}$ values of 6.7 M$^{-1}$ with correlation coefficient of 0.997 and 4.7 M$^{-1}$ with correlation coefficient of 0.999 for the initial and latter points respectively.

Tb$^{3+}$ Energy Transfer

A 1:3 ratio of K E4:Cam was titrated with Tb$^{3+}$. Results indicated that there was an enhancement in luminescence intensity as Tb$^{3+}$ cation bound into the second and third loops of Cam.

3.2.5.2 UW E4

Purification and Characterization

The UW E4 peptide was checked for purity by reverse phase C18 HPLC. The HPLC trace shown in Figure 3.67 indicated that the peptide did not elute from the column as the peaks at 32 and 44 min are also present in the blank. This was unusual; however, problems with purification using reverse phase columns had plagued the project all along and hence the peptide was assumed to be pure and experiments continued.
The UW E4 peptide was characterized by MALDI mass spectrometry and the molecular weight was found to be 2498.00 g/mole which is in agreement with the theoretical molecular weight of 2503.07 g/mole.

Fluorescence Spectra of the UW E4 peptide

Fluorescence spectra were collected for a 1 μM sample of UW E4 alone and complexed to Cam in 1:1 and 2:1 ratios. Figure 3.68 indicated that the 1:1 and 2:1 ratio spectra were virtually identical suggesting that the UW E4 peptide formed a strong 1:1 complex with Cam. Comparison with the spectrum of UW E4 alone showed that the wavelength maximum blue shifted from 350 nm to 344 nm and the fluorescence intensity was quenched upon binding. Fluorescence spectra were also collected for a 2.5 μM sample of UW E4 alone and complexed to Cam in a 2.5:1 ratio of excess peptide. Results indicated that the fluorescence maximum did not blue shift and there was a 1.3 fold enhancement in fluorescence intensity.

Cam Titration of the UW E4 Peptide

A 1 μM sample of UW E4 was titrated with Cam to an excess of 2:1. (Figure 3.69) Fluorescence intensity was greatly quenched during the first few additions of Cam and then was quenched to a lesser degree as more Cam is added. This fast initial quenching may have suggested the formation of a 2:1 complex that dissociated to form the final 1:1 complex that was observed from the fluorescence spectra. Attempts were made to determine the dissociation constant of this final 1:1 complex using Scatchard analysis. The initial fluorescence was taken as the fourth point of the titration curve where the fluorescence begins to quench at a slower rate and F∞ was taken to be the point
Figure 3.66 — Stern-Volmer plot of acrylamide quenching of K E4:Cam sample.

Figure 3.67 — HPLC trace of UW E4 peptide. Continuous line represents absorbance while dashed line represents the gradient.

Figure 3.68 — Fluorescence spectra of UW E4 peptide alone (continuous line) and in 1:1 (dotted line) and 2:1 Cam:UW E4 ratios (dot-dashed line). Ex. 295 nm, double monochromator. 4 nm bandpass. Em. 300-450 nm, single monochromator, 4 nm bandpass.

Figure 3.69 — Cam titration of UW E4 (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 345 nm, single monochromator, 4 nm bandpass.
where the fluorescence intensity plateaued. Scatchard analysis (omitting the initial 8 points) indicated a $K_d$ of 0.21 μM with a correlation coefficient of 0.888 (Figure 3.70).

**UW E4 Titration of Cam**

A 1 μM sample of Cam was titrated with UW E4 to an excess of peptide of 2.5:1 (Figure 3.71). Fluorescence intensity was enhanced as binding occurred but did not plateau indicating that the binding was very weak. Scatchard analysis, estimating $ΔF_x$ from a double reciprocal plot (Table 3.1) and omitting the initial 13 points (1.5:1 ratio), indicated a $K_d$ of 1.3 μM with a correlation coefficient of 0.932 and x-axis intercept of 1 (Figure 3.72). Secondly, it should be noted that the curve depicting the addition of UW E4 to buffer was linear indicating that the UW E4 peptide did not dimerize in contrast to the K E4 peptide.

**3.2.6 — E5 Peptide**

The E5 peptide has the following primary sequence:

CH$_3$CO -V P R W I E D S L R D A A A T A Q A V L A S A-NH$_2$

The changes in this peptide from the 12.1 peptide are in bold. Again, Gly 13 and Gly 14 have been changed, this time to Asp 11 and Ala 12, the C-terminus has been changed from Thr 20, Arg 21 and Leu 22 to Ala 18, Val 19 and Leu 20, Arg 17 becomes Thr 15 and the N-terminal Ser Thr have been dropped. Two sources of this peptide were used, one was obtained from Chiron Mimotopes Peptide Systems Inc. (K E5) and the second was obtained from the UW (UW E5).
3.2.6.1 K E5

Purification and Characterization

Mass spectrometry data that accompanied the K E5 peptide stated that it was 64% pure with stated molecular weight of 2452.7 g/mole which is in agreement with the theoretical molecular weight of 2452.99 g/mole. Similar to the K E4 peptide, the K E5 peptide would not elute from a reverse phase column. It was HPLC purified using a CM-5PW cation exchange column with an aqueous/0.2 M NH₄OAc gradient. Three peaks were found to elute within the long water phase of the gradient (Figure 3.73). These fractions were collected and checked for the characteristic Trp absorption spectrum. The second and third peaks were found to contain Trp while the first did not. The larger of the Trp peaks was collected, pooled and lyophilized. A portion of the lyophilized powder was re-dissolved in MOPS buffer and its solution concentration determined. The remaining sample was stored dry at -20°C.

The purified sample was characterized by mass spectrometry. The results showed the molecular weight of the lyophilized sample to be 2451.75 g/mole which is in agreement with the theoretical molecular weight of 2452.99 g/mole (Figure 3.74).

Fluorescence Spectra of the K E5 Peptide

Fluorescence spectra were collected for a 7 μM sample of K E5 alone and complexed to Sigma Cam in a 3.5:1 ratio, excess K E5 (Figure 3.75). Comparison between the two spectra indicated that wavelength maximum blue shifted from 360 nm to 352 nm upon binding to Cam and there was a quenching of fluorescence intensity.
Figure 3.70 — Scatchard plot of Cam titration of UW E4.

Figure 3.71 — UW E4 titration of Cam (1 μM). Ex. 295 nm. double monochromator, 4 nm bandpass. Em. 345 nm, single monochromator, 4 nm bandpass.

Figure 3.72 — Scatchard plot of UW E4 titration of Cam.

Figure 3.73 — HPLC trace of K E5 peptide. Continuous line represents absorbance while the dashed line shows the gradient.
Cam Titration of K E5

A 2 μM sample of K E5 was titrated with Sigma Cam (Figure 3.76). The fluorescence intensity was quenched as binding occurred and plateaued at a ratio of 1:1. This indicated very strong binding and precluded Scatchard analysis.

K E5 Titration of Cam

A 2 μM sample of Sigma Cam was titrated with K E5 peptide to an excess of 3.5:1 (Figure 3.77). Expansion of the difference curve indicated that there was a weak fluorescence quenching as binding occurred that plateaued at a 1.5:1 ratio (Figure 3.78). Scatchard analysis, estimating ΔF∞ from a double reciprocal plot (Table 3.1) and omitting the initial points (1:1 ratio), indicated a Kd of 0.58 μM with a correlation coefficient of 0.800 and x-axis intercept of 1.5 (Figure 3.79).

3.2.6.2 UW E5

Characterization and Purification

The purity of the UW E5 peptide was confirmed by HPLC using C18 reverse phase chromatography. The peptide eluted in a single band at 29 min or 80% acetonitrile (Figure 3.80). The peaks at 33 and 42 min are also present in the blank.

Mass spectrometry data indicated that the molecular weight of the UW E5 peptide was 2453.00 g/mole which is in agreement with the theoretical molecular weight of 2452.99 g/mole.
Figure 3.74 — Mass spectrometry trace of K E5 Peptide.

Figure 3.75 — Fluorescence spectra of K E5 peptide alone (continuous line) and in a 3.5:1: K E5:Cam ratio (dotted line). Double monochromators and 4 nm bandpass both sides. Ex. 295 nm, Em. 300-450 nm.

Figure 3.76 — Cam titration of K E5 (2 μM). Double monochromators and 4 nm bandpass both sides. Ex. 295 nm, Em. 345 nm.
Figure 3.77 — K E5 titration of Cam (2 μM). Double monochromators and 4 nm bandpass both sides. Ex. 295 nm, Em. 345 nm.

Figure 3.78 — Difference curve of K E5 titration of Cam.

Figure 3.79 — Scatchard plot of K E5 titration of Cam.

Figure 3.80 — HPLC trace of UW E5 peptide. Continuous line represents absorbance while dashed line represents the gradient.
Fluorescence Spectra of the UW E5 Peptide

Fluorescence spectra were collected for a 1 μM sample of UW E5 alone and complexed to Cam in 1:1 and 2:1 ratios, excess Cam (Figure 3.81). Spectra indicated that the 1:1 and 2:1 ratio spectra were identical and that the wavelength maximum blue shifted from 341 nm to 334 nm and the fluorescence intensity was quenched. It is interesting to note that the spectral maximum of the UW E5 peptide alone was at 341 nm which is blue shifted compared to the other peptides which are usually around 350 nm.

Cam Titration of UW E5

A 1 μM sample of UW E5 was titrated with Cam to an excess of 2:1 (Figure 3.82). The fluorescence intensity was quenched as binding to Cam occurred but did not plateau by a 2:1 ratio suggesting that the binding was weak. Scatchard analysis, estimating $F_\infty$ from a double reciprocal plot (Table 3.1) and omitting the scatter points shown around the regression line, estimated the $K_d$ to be 120 nM with a correlation coefficient of 0.839 and x-axis intercept of 1 (Figure 3.83).

UW E5 Titration of Cam

A 1 μM sample of Cam was titrated with UW E5 to an excess of 2.6:1 (Figure 3.84). From the difference curve, the fluorescence intensity was quenched to a ratio of approximately 2:1 where it plateaued. Scatchard analysis, estimating $\Delta F_\infty$ from the plateau and omitting the initial seven points (1:1 ratio), indicated a $K_d$ of 50 nm with a correlation coefficient of 0.688 and an x-axis intercept of 1.2 (Figure 3.85). This was in agreement with K E5 peptide which behaved in the same manner.
Figure 3.81 — Fluorescence spectra of UW E5 peptide alone (continuous line), 1:1 (dotted line) and 2:1: Cam:UW E5 ratio (dot-dashed line). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 300-450 nm, single monochromator, 4 nm bandpass.

Figure 3.82 — Cam titration of UW E5 (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 345 nm, single monochromator, 4 nm bandpass.

Figure 3.83 — Scatchard plot of Cam titration of UW E5.

Figure 3.84 — UW E5 titration of Cam (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 345 nm, single monochromator, 4 nm bandpass.
3.2.7 -- E6 Peptide

The E6 peptide has the following primary sequence:

\[ \text{CH}_3\text{CO}-\text{VPRWIEDSLREAATAQTSLASA-NH}_2 \]

The changes in this peptide from the 12.1 peptide are in bold. The Gly Gly helix breaker has been altered to Glu Ala, the C-terminus has been changed to Thr 18, Ser 19 and Leu 20. Arg 17 of the 12.1 peptide has been changed to a Thr 15 and the initial Ser Thr on the N-terminus have been removed. Two sources of this peptide were used, one was obtained from Chiron Mimotopes Peptide Systems Inc.(K E6) and the second from the UW (UW E6).

3.2.7.1 K E6

Purification and Characterization

The K E6 peptide was obtained 60% pure by mass spectrometry. The molecular weight of 2484.7 g/mole which was in agreement with the theoretical molecular weight of 2484.99 g/mole. The K E6 peptide would not elute from a reverse phase column. It was purified on a CM-5PW cation exchange column using a water/0.5 M NH$_4$OAc gradient with a long initial water phase. Peaks eluted in the water phase and at 100% NH$_4$OAc (Figure 3.86). The three peaks in the water phase were collected and checked for the characteristic Trp absorption spectrum. Only the third peak was found to contain Trp. This peak was collected, pooled, lyophilized, re-dissolved in MOPS buffer and the solution concentration determined.

Mass spectrometry characterization of this peptide could not be done owing to degradation of the sample upon storage.
Fluorescence Spectra of the K E6 Peptide

Fluorescence spectra were collected for a 3.5 μM sample of K E6 and complexed to Cam in 3.5:1, K E6:Cam ratio (Figure 3.87). Spectra showed that as binding occurred, the wavelength maximum did not shift but the fluorescence intensity was quenched.

K E6 Titration of Cam

A 1 μM sample of Sigma Cam was titrated with K E6 to an excess of 3.5:1 (Figure 3.88). The fluorescence intensity was quenched as binding occurred. The titration curve depicting the addition of K E6 to buffer was interesting as it showed a very definite curvature in what should have been a linear relationship. This same experiment was repeated with Dr. Mutus’ Cam and the results were reproducible. This suggests that, similar to the K E4 peptide, the K E6 peptide also dimerized. The $K_d$ of this self association was determined using the same method as outlined for the K E4 peptide for both titrations discussed above. The resulting $K_d$ was found to be on the order of 800 nM. Due to the extent of the fluorescence change from the self-association, Scatchard analysis was not possible.

Acrylamide Quenching

Acrylamide quenching titrations were carried out with a 4 μM Sigma Cam/1 μM K E6 sample to a final concentration of acrylamide of 400 mM. Results showed two distinct slopes. Regression of these slopes indicated $K_{SV}$'s of 5.8 M$^{-1}$ with a correlation coefficient of 0.998 and 4.8 M$^{-1}$ with a correlation coefficient of 0.996.
Figure 3.85 – Scatchard plot of UW E5 titration of Cam.

Figure 3.86 – HPLC trace of K E6 peptide. Continuous line represents the absorbance while the dashed line shows the gradient.

Figure 3.87 – Fluorescence spectra of K E6 alone (continuous line) and 3.5:1 K E6:Cam ratio (dotted line). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 300-450 nm, 8 nm bandpass.

Figure 3.88 – K E6 titration of Cam (1 µM). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 345 nm, 8 nm bandpass.
**Tb$^{3+}$ Energy Transfer**

Tb$^{3+}$ titrations were carried out with a 1 μM K E6/3 μM Cam sample. The resulting titration curve indicated fluorescence luminescence in the 2nd, 3rd and 4th equivalents.

### 3.2.7.2 UW E6

**Purification and Characterization**

The purity of the UW E6 peptide was confirmed using HPLC and C18 reverse phase chromatography (Figure 3.89). The peptide eluted in a single band at approximately 25 min (67% acetonitrile). The peaks at 33 and 44 min are also present in the blank.

Mass Spectrometry data indicated that the molecular weight of the UW E6 peptide was 2484.00 g/mole which is in agreement with the theoretical molecular weight of 2484.99 g/mole.

**Fluorescence Spectra of the UW E6 Peptide**

Fluorescence spectra were collected for a 1 μM sample of UW E6 alone and complexed to Cam in 1:1 and 2:1 ratios, excess Cam (Figure 3.90). Spectra indicated that the wavelength maximum did not shift but remained fixed at 347 nm while the fluorescence intensity was quenched. At a 2.4:1; UW E6:Cam ratio, fluorescence spectra indicated that the maximum wavelength did not shift but there was an enhancement in fluorescence intensity.
Cam Titration of UW E6

A 1 μM sample of UW E6 was titrated with Cam to an excess of 2:1 (Figure 3.91). The fluorescence intensity was quenched very quickly in the first two points followed by a gradual quenching to a 2:1 ratio where fluorescence began to plateau. Scatchard analysis was done using the average of the second and third points of the titration curve as \( F_0 \) and estimating \( F_\infty \) from a double reciprocal plot. Omitting the initial eight points (1:1 ratio), the \( K_d \) was estimated to be 266 nM with correlation coefficient of 0.969 and x-axis intercept of 1 (Figure 3.92).

UW E6 Titration of Cam

A 1 μM sample of Cam was titrated with UW E6 to a final excess of 2.7:1 (Figure 3.93). The difference curve indicated a decrease in fluorescence to a ratio of 0.5:1 followed by an enhancement to a ratio of 2.7:1 where the fluorescence had not yet plateaued. Scatchard analysis, estimating \( \Delta F_\infty \) from a double reciprocal plot and omitting the initial 12 points (2:1 ratio), indicated a \( K_d \) of 5.94 μM with a correlation coefficient of 0.765 and x-axis intercept of 1.3 (Figure 3.94).

3.2.8 E2 Peptide

The E2 peptide has the following primary sequence:

\[
\text{CH}_3\text{CO-STVPRWIESDLRDWAAARQSPIAS-NH}_2
\]

In this peptide D13 and W14 replace G13 and G14. In addition there is another substitution in the C-terminus where the T20, R21 and L22 is changed to S20, I21 and P22. Only one source of peptide was used in studies with this peptide.
Figure 3.89 – HPLC trace of UW E6 peptide. Continuous line represents absorbance while dashed line shows the gradient.

Figure 3.90 – Fluorescence spectra of UW E6 peptide alone (continuous line), 1:1 (dotted line) and 2:1; Cam:UW E6 ratios (dot dashed line). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 300-450 nm, single monochromator, 4 nm bandpass.

Figure 3.91 – Cam titration of UW E6 (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 345 nm, single monochromator, 4 nm bandpass.

Figure 3.92 – Scatchard plot of Cam titration of UW E6.
Purification and Characterization

The E2 peptide was checked for purity using HPLC and a CM-5PW cation exchange column and eluted with a water/0.2 M NH₄OAc gradient over 35 min. The peptide eluted as a single band at approximately 23 min and 51 % 0.2 M NH₄OAc. (Figure 3.95) The collected aliquot was checked for Trp absorption and was found to contain Trp. The elution of a single band suggests that the peptide was pure and no further purification was required. No additional characterization of this peptide was performed.

Fluorescence Spectra of the E2 Peptide

Fluorescence spectra of a 4 μM sample of E2 and a 4 μM E2/1 μM Sigma Cam sample are shown in Figure 3.96. There was a large intensity enhancement and there was negligible wavelength maximum shift.

E2 Titration of Cam

A 1 μM sample of Sigma Cam was titrated with E2 peptide to an excess of 3.7:1. The resulting titration curve is shown in Figure 3.97. The fluorescence intensity is increased as binding of the peptide to Cam occurred. The initial difference fluorescence followed the fluorescence intensity of the total fluorescence of the sample indicating very strong binding in the early region of the curve. At a ratio of approximately 1:1, the net fluorescence began to deviate from the total fluorescence suggesting that perhaps a second equivalent of peptide was binding to Cam with a lower affinity.

Acrylamide Quenching

Acrylamide quenching experiments were conducted with a 1:4; E2:Cam sample and a 2.5:1; E2:Cam sample. The 1:4 sample was titrated to a final concentration of acrylamide of 400 mM while the 2.5:1 sample was titrated to 800 mM acrylamide. The
Figure 3.93 — UW E6 titration of Cam (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 345 nm, single monochromator, 4 nm bandpass.

Figure 3.94 — Scatchard plot of UW E6 titration of Cam.

Figure 3.95 — HPLC titration of E2 peptide. Continuous line represents absorbance and dashed line shows the gradient.

Figure 3.96 — Fluorescence spectra of E2 alone (continuous line) and in a 4:1; E2:Cam ratio (dotted line). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 300-450 nm, 8 nm bandpass.
resulting Stern-Volmer plots had $K_{SV}$'s of 0.63 $M^{-1}$ with a correlation coefficient of 0.999 and 0.62 $M^{-1}$ with a correlation coefficient of 0.998.

**Tb$^{3+}$ Energy Transfer**

Tb$^{3+}$ titrations were conducted for a 2.3:1; E2:Sigma Cam sample and 1:3; E2:Cam sample. The former sample showed Tb$^{3+}$ luminescence enhancement during the addition of the 3rd and 4th equivalents of Tb$^{3+}$ while the latter reported enhancement in the 2nd, 3rd and 4th equivalents.

**3.2.9 — E8 Peptide**

The primary sequence of the E8 peptide is as follows:

CH$_3$CO-STMVRWIDESLDRDWARAQSETASSA-NH$_2$

The changes made from the 12.1 peptide are shown in bold. As with the E2 peptide, G13 and G14 have been changed to D13 and W14. Only one source of this peptide was used for the titrations.

**Purification and Characterization**

Attempts made to purify the E8 peptide by HPLC using a C8 column were not successful. The peptide did not appear to elute from the column under a variety of gradient conditions. It could be purified using a CM-5PW column and eluting with a water/0.2 M NH$_4$OAc gradient. Figure 3.98 shows the HPLC trace for this purification. The major peak at ~ 30 min and 100 % mobile phase B was collected and found to have the characteristic tryptophanyl absorption spectrum. This band was collected, pooled, lyophilized, re-dissolved in water and the solution concentration determined. No further characterization of the peptide was done.
Fluorescence Spectra for the E8 Peptide

Fluorescence spectra were collected for a 3 μM sample of E8 alone and complexed to Cam in a 3:1; E8:Cam and 1:1 complex (Figure 3.99). The spectra show that the wavelength maximum blue shifts from 349 nm to 338 nm in the 3:1 complex and further to 328 nm in the 1:1 complex with an enhancement in fluorescence intensity.

E8 Titration of Cam

A 1 μM sample of Cam was titrated to a 3:1 excess of E8. The resulting titration curves are shown in Figure 3.100. The net fluorescence intensity is enhanced and plateaus at approximately 1:1 indicating very strong binding for which Scatchard analysis is not applicable.

Tb$^{3+}$ Energy Transfer

A sample of a 3:1; E8:Cam complex was titrated with Tb$^{3+}$ cation. The resulting titration curve showed Tb$^{3+}$ luminescence in the 2nd and 3rd molar equivalents.

3.2.10 — The E9 Peptide

The E9 peptide has the following primary sequence:

\[
\]

The changes from the 12.1 peptide are shown in bold. The ‘G G’ of the 12.1 peptide has been replaced with an ‘I W’ and the C-terminus of the peptide has been altered. The E9 peptide came directly from Dr. B. Kay and was the only source of peptide used for experiments.
Figure 3.97 — E2 titration of Sigma Cam (1 μM). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 345 nm, 8 nm bandpass.

Figure 3.98 — HPLC trace of E8 peptide. Continuous line represents absorbance while dashed line shows the gradient.

Figure 3.99 — Fluorescence spectra of E8 alone (continuous line), 3:1 E8:Cam ratio (dotted line) and an estimated 1:1 ratio (dot-dashed line). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 300-450 nm, 8 nm bandpass.

Figure 3.100 — E8 titration of Cam (1 μM). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 345 nm, 8 nm bandpass.
Purification and Characterization

Owing to the small amount of the E9 sample, the peptide could not be purified. No further characterization was done.

Fluorescence Spectra of the E9 Peptide

Fluorescence spectra were collected for a 3.5 \( \mu \text{M} \) sample of E9 alone and a complex of 3.5:1; E9:Cam (Figure 3.101). The figure indicated that the wavelength maximum blue shifted from 349 nm to 333 nm and that fluorescence intensity was enhanced.

E9 Titration of Cam

A 1 \( \mu \text{M} \) sample of Cam was titrated with E9 to an excess of 3.5:1. The resulting titration curves are shown in Figure 3.102. The net fluorescence intensity did not plateau even at a ratio of 3.5:1 suggesting that the binding affinity of this peptide for Cam was very weak.

Acrylamide Quenching

A 1:4; E9:Cam sample was titrated with acrylamide to a final acrylamide concentration of 400 mM. The resulting Stern-Volmer plot indicated that the \( K_{SV} \) was 1.9 M\(^{-1}\) with a correlation coefficient of 0.999.

Tb\(^{3+}\) Energy Transfer

A 3.5:1; E9:Cam sample was titrated with Tb\(^{3+}\) cation. Fluorescence enhancement was observed to be within the 3rd and 4th molar equivalents of Tb\(^{3+}\).
3.3 -- The KirGem, sm-MLCK and Neuromodulin Peptides

A second set of peptides representative of two peptide analogs of proteins known to bind Cam, sm-MLCK and Neuromodulin, were obtained from Dr. M. Berchtold (Zurich, Switzerland). A peptide analog of a specific Ras-like GTPase Kir/Gem protein referred to as the KirGem peptide was also provided by that group.

3.3.1 The KirGem Peptide

The primary sequence of the KirGem peptide is

\[
\text{NH}_2-\text{K A R R F W G K I V A K N N K N M A F K L K S K S-COCH}_3
\]

Purification and Characterization

The KirGem peptide was purified by HPLC using a CM-5PW cation exchange column. The largest band eluted at approximately 31 min (70% \text{NH}_4\text{OAc}) and was found to have the characteristic Trp absorbance. This band was collected and pooled. The HPLC trace of the unpurified sample is shown in Figure 3.103. A sample of the purified peptide was re-injected and was found to elute in a single band confirming the purity of the sample.

The KirGem sample was further characterized by mass spectrometry. The results indicated that the molecular weight of the KirGem sample was 2952.00 g/mole which is in good agreement with the theoretical molecular weight of 2951.82 g/mole.

Fluorescence Spectra of the KirGem Peptide

Fluorescence spectra were collected for a 10 \text{\mu M} sample of KirGem alone and in a 3:1; Cam:KirGem complex and a 1:10; Cam:KirGem ratio (Figure 3.104). It can be seen
Figure 3.101 — Fluorescence spectra of E9 alone (continuous line) and in a 3.5:1; E9:Cam ratio (dotted line). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 300-450 nm, 8 nm bandpass.

Figure 3.102 — E9 titration of Cam (1 μM). Double monochromators both sides. Ex. 295 nm, 4 nm bandpass. Em. 345 nm, 8 nm bandpass.

Figure 3.103 — HPLC trace of KirGem peptide. Continuous line represents the unpure sample, dotted line shows the purified sample and dashed line represents the gradient.

Figure 3.104 — Fluorescence spectra of KirGem alone (continuous line) and in 3:1 (dotted line) and 1:8.5; Cam:KirGem ratios (dot-dashed line). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 300-450 nm, single monochromator, 4 nm bandpass.
that upon binding of the peptide analog to Cam, the fluorescence maximum shifted from 350 nm to 322 nm for the 3:1 complex and the fluorescence intensity was enhanced.

**Cam Titrations of KirGem**

A 200 nM sample of KirGem was titrated with Cam to an excess of 1.4:1 while monitoring the fluorescence at 345 nm. The resulting titration curve is shown in Figure 3.105 and indicates that there was an enhancement in fluorescence intensity which plateaued at a ratio of approximately 1:1. A second titration of 1 µM KirGem to an excess of Cam of 2:1 while monitoring at 322 nm (Figure 3.106) indicated identical results. Both samples showed the binding to be too strong for Scatchard analysis to be applicable.

**KirGem Titrations of Cam**

A 1 µM sample of Cam was titrated with KirGem to an excess of 10:1. The resulting titration curves are shown in Figure 3.107. From the net fluorescence curve a slight enhancement of fluorescence on binding occurred but it did not appear to plateau. A second sample of 0.5 µM Cam was titrated and indicated reproducible results. This suggested a very weak binding of the peptide to Cam. Scatchard analysis of both samples indicated Kₐ's of 7.3 µM with correlation coefficient of 0.973 and x-axis intercept of 0.7 and 7.2 µM with correlation coefficient of 0.981 and x-axis intercept of 1.5 respectively. Figure 3.108 shows the Scatchard plot for the former titration, omitting the initial 20 points (6:1 ratio) and estimating ΔF₀ from a double reciprocal plot (Table 3.1). For the latter sample, ΔF₀ was also estimated from a double reciprocal plot and the first five points were omitted (4:1 ratio). A second sample of 1 µM Cam was titrated with KirGem to an excess of 1.6:1 using 322 nm for monitoring the titration (the maximum wavelength
Figure 3.105 — Cam titration of KirGem (200 nM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 345 nm, single monochromator, 4 nm bandpass.

Figure 3.106 — Cam titration of KirGem (1 μM). Ex. 295 nm double monochromator, 4 nm bandpass. Em. 322 nm, single monochromator, 4 nm bandpass.

Figure 3.107 — KirGem titration of Cam (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 345 nm, single monochromator, 4 nm bandpass.

Figure 3.108 — Scatchard plot of KirGem titration of Cam @ 345 nm.
of the 3:1; Cam:KirGem fluorescence spectrum). The resulting titration curve is shown in Figure 3.109. This titration curve shows very different results indicating that a plateau was achieved at a ratio of 0.5:1, KirGem:Cam suggesting strong binding which precludes Scatchard analysis.

**Acrylamide Quenching**

A 3 μM Cam/1 μM KirGem sample was titrated with acrylamide to a final concentration of acrylamide of 400 mM. The Stern-Volmer plot showed that two distinct slopes were apparent. Regression analysis of this data indicated that the $K_{SV}$ of the initial points was 2.6 M$^{-1}$ with a correlation coefficient of 0.989 while that of the latter points was 1.8 M$^{-1}$ with a correlation coefficient of 0.997.

**Tb$^{3+}$ Energy Transfer**

A 3:1; Cam:KirGem sample was titrated with Tb$^{3+}$ cation. Luminescence enhancement was found to occur within the first equivalent of Tb$^{3+}$ added.

### 3.3.2 The sm-MLCK peptide

Smooth muscle myosin light chain kinase is an enzyme that is known to be regulated by Cam. The Cam binding domain of this peptide is known and the resulting peptide analog, denoted as RS20 in the literature, has been used in many studies of this nature. The primary structure of this peptide analog is:

$$\text{NH}_2-\text{A R R K W Q K T G H A V R A I G R L S S M A- COCH}_3$$

**Purification and Characterization**

The first sample of sm-MLCK peptide was purified by HPLC using a CM-5PW cation exchange column. The largest band eluted at approximately 39 min (96%
NH₄OAc) and was found to have the characteristic Trp absorbance. This band was collected and pooled. The HPLC trace of the unpurified sample is shown in Figure 3.110. A sample of the purified peptide was re-injected and was found to elute in a single band indicating the sample purity. A sample of this peptide was also injected onto a C18 column and eluted using the method outlined for the UW peptides. The pure peptide eluted as a single band at approximately 18 min or 45% acetonitrile. Peaks at 34 min and 42 min are also present in the blank. A second sample of this peptide obtained from Dr. M. Berchtold was purified using a C18 column. The HPLC trace for this sample is shown in Figure 3.111.

The sm-MLCK sample was further characterized by mass spectrometry. The molecular weight of the sm-MLCK sample was found to be 2479.2 g/mole, in good agreement with the expected molecular weight of 2481.23 g/mole.

**Fluorescence Spectra of the sm-MLCK Peptide**

Fluorescence spectra were collected for an 10 μM sample of sm-MLCK alone and a 3:1; Cam:sm-MLCK ratio and a 1:10; Cam:sm-MLCK ratio (Figure 3.112). From these spectra, it can be seen that upon binding of sm-MLCK to Cam, the fluorescence maximum shifted from 350 nm to 325 nm for the 3:1 complex and 336 nm for the excess of sm-MLCK complex with a fluorescence intensity enhancement.

**Cam Titrations of sm-MLCK**

An 830 nM sample of sm-MLCK was titrated with Cam to a final excess of 2:1 using 325 nm for monitoring the changes. The resulting titration curve is shown in Figure 3.113 and indicated that the fluorescence intensity rose up to a ratio of 1:1 at which point a plateau value was seen. This is consistent with the results obtained for the
Figure 3.109 — KirGem titration of Cam (1 μM). Ex. 295 nm double monochromator, 4 nm bandpass. Em. 322 nm, single monochromator, 4 nm bandpass.

Figure 3.110 — HPLC trace of sm-MLCK peptide using cation exchange chromatography. Continuous line represents the unpure peptide, dotted line the purified sample and dashed line the gradient.

Figure 3.111 — HPLC trace of sm-MLCK peptide using reverse phase chromatography. Continuous line represents the absorbance while the dashed line shows the gradient.

Figure 3.112 — Fluorescence spectra of sm-MLCK alone (continuous line) and in 3:1 (dotted line) and 1:8.5 Cam:sm-MLCK ratios (dot-dashed line). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 300-450 nm, single monochromator, 4 nm bandpass.
sm-MLCK titration of Cam at the same emission wavelength and indicated that at this wavelength, the data suggested that a 1:1 complex is formed that binds strongly to Cam. The binding is too strong to allow for Scatchard analysis.

**Sm-MLCK Titrations of Cam**

A 1 μM sample of Cam was titrated with sm-MLCK to an excess of 10:1 at 345 nm (Figure 3.114). These results were highly reproducible. From the net fluorescence curve, there was a large enhancement within the first few additions of Cam to a ratio of about 1:1 (Figure 3.115) followed by a less steep enhancement that did not plateau even at a ratio of 10:1. This suggested that a 2:1 complex was being formed. Scatchard analysis, estimating ΔF∞ from a double reciprocal plot (Table 3.1), indicated two distinct slopes. When regressed separately, the K_d’s were found to be 548 nM for the initial points with correlation coefficient of 0.741 and x-axis intercept of 0.2 and 14.2 μM with correlation coefficient of 0.919 and x-axis intercept of 1 for the latter points (Figure 3.116). Scatchard analysis of a second sample titration indicated very similar results (Table 4.3). Emission at 345 nm does not give an optimal fluorescence change (Figure 3.112). A second sample of Cam (1 μM) was titrated with sm-MLCK while monitoring the fluorescence at 325 nm. The resulting titration curves are shown in Figure 3.117 and indicate that the net fluorescence increased to a ratio just past 1:1 and than plateaued. The slight decrease in intensity within the second equivalent added may be due to an overcorrection of the blank.

**Acrylamide Quenching**

Samples of 2:1 and 1:3; sm-MLCK:Cam were titrated with acrylamide to an acrylamide concentration of 400 nM. From Stern-Volmer plots, the KSV’s for these
Figure 3.113 — Cam titration of sm-MLCK (830 nm). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 325 nm, single monochromator, 4 nm bandpass.

Figure 3.114 — Sm-MLCK titration of Cam (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 345 nm, single monochromator, 4 nm bandpass.

Figure 3.115 — Difference curve of sm-MLCK titration of Cam showing early points only.

Figure 3.116 — Scatchard plot of sm-MLCK titration of Cam.

@ 345 nm.
samples were found to be 5.7 M\(^{-1}\) and 0.52 M\(^{-1}\) respectively. A sample of 10 μM sm-MLCK alone and a separate sample of Nata (2 μM) were also titrated with acrylamide to acrylamide concentrations of 800 mM and 400 mM respectively. The initial points of the sm-MLCK titration reported a \(K_{SV}\) of 7.8 M\(^{-1}\) with a correlation coefficient of 0.999 while the latter points indicated that the \(K_{SV}\) was 12.3 M\(^{-1}\) with a correlation coefficient of 0.993. The \(K_{SV}\) for the NATA sample was found to be 15.3 M\(^{-1}\) with a correlation coefficient of 0.999. The NATA sample can be used as a reference (assuming that the lifetimes of all tryptophans are similar) for the \(K_{SV}\) of a fully solvent exposed Trp.

**Tb\(^{3+}\) Energy Transfer**

Samples of 1:3 and 3:1; Cam:sm-MLCK were titrated with Tb\(^{3+}\). The resulting titration curves indicated fluorescence enhancement within the 2nd and 3rd equivalents.

### 3.3.3 The Neuromodulin Peptide

The primary sequence of the peptide analog of neuromodulin used in this study is as follows. The nine residue Cam binding domain is in bold.

\[
\text{NH}_2-\text{A H K A A T K I S F R G H I T R K L K G -COCH}_3
\]

**Purification and Characterization**

The neuromodulin peptide was HPLC purified using a CM-5PW cation exchange column. The resulting HPLC trace is shown in Figure 3.118. The major band at approximately 35 min (83%) was collected and pooled. This purified sample was re-injected and the resulting HPLC trace reported a single band indicating that the neuromodulin sample was pure. The peptide composition was confirmed by amino acid analysis.
Fluorescence Spectra of the Neuromodulin Peptide

Fluorescence spectra were collected for an approximately 12 μM sample of neuromodulin alone and complexed to 10 μM samples of octopus Cam and bovine brain Cam in the presence of calcium and 100 mM KCl. Spectra indicated, in both the octopus Cam and the bovine brain Cam, that the fluorescence maximum did not shift upon complex formation but the fluorescence intensity was enhanced.

Neuromodulin Titration of Cams

Neuromodulin was added to Cam in 10 μL aliquots in the presence of calcium until the fluorescence intensity was observed to plateau. This was done for both octopus and bovine brain Cams and an addition to buffer (due to the presence of a weak fluorescence impurity within the neuromodulin sample). The intensity of the fluorescence impurity was directly subtracted from the fluorescence values obtained with the titrations of the Cam. This net fluorescence intensity was plotted vs the ratio of neuromodulin:Cam and is shown in Figure 3.119 for both samples. Results indicated that the fluorescence enhancement was identical for both the bovine brain and octopus samples and that the binding was strong which precluded Scatchard analysis.

A similar experiment was performed with the bovine brain Cam in the presence of EGTA. These results showed that there was no change in fluorescence intensity as the neuromodulin bound to the apo form of the Cam.

Tb$^{3+}$ Energy Transfer

In studies that were completed for the Phage display peptides, KirGerm and sm-MLCK, the order of fill of Tb$^{3+}$ to Cam was assumed to be the same in the complexed
Figure 3.117 — Sm-MLCK titration of Cam (1 μM). Ex. 295 nm, double monochromator, 4 nm bandpass. Em. 325 nm, single monochromator, 4 nm bandpass.

Figure 3.118 — HPLC trace of Neuromodulin peptide. Continuous line shows the unpure peptide, dotted line the purified sample and dot-dashed line the gradient.

Figure 3.119 — Neuromodulin titrations of Bovine Brain Cam (open circles) and Octopus Cam (closed circles). Ex. 280 nm, double monochromator, 4 nm bandpass. Em. 302 nm, single monochromator, 4 nm bandpass.
and uncomplexed forms of Cam. In order to test this assumption, Tb\(^{3+}\) titrations were completed for both octopus and bovine brain Cam free and bound to neuromodulin. Results of both titrations indicated that the order of fill does not change when peptide is bound to Cam validating the assumption made in studies with the Phage display peptides and sm-MLCK and KirGem.
4. Discussion

4.1 Purification and Characterization

HPLC traces and mass spectrometry data after purification for the UW peptide series, K2 E3, K2 E3-LTV, sm-MLCK, KirGem and neuromodulin confirmed the purity and composition of these peptide samples. The need to assess the purity and composition of the first K series peptide samples was not evident until the set of UW peptides were obtained. Mass spectrometry data accompanying the K E4, K E5 and K E6 samples provided by Chiron Mimotopes Peptide Systems indicated the correct molecular weights but the samples as purchased were only 60-70% pure. It would be highly speculative as to how a correct molecular weight might be obtained on such crude samples. A lyophilized sample of purified K E5 indicated the molecular weight of the sample to be correct. Samples of K 12.1, K E3-LTV, K E3-AS, E8 and E9 obtained from Dr. B. Kay may have had a biotin tag but now the nature of these peptides has been lost. None of these samples had been checked for composition and were purified assuming them to be the correct sequence. Purification of this latter group was completed using ion exchange chromatography as the peptides did not appear to elute from C8 or C18 reverse phase columns despite repeated attempts. Yet the UW series of peptides could all be eluted and purified using a C18 column. Lastly, K E3 and E2 were obtained from Chiron Mimotope Peptide Systems Inc. but were not accompanied with mass spectrometry data. Mass spectrometry analysis of the purified K E3 sample indicated that it was of the correct molecular weight.
As the earlier supply of peptides was consumed, additional peptides were obtained from the peptide synthesis facility at the University of Waterloo. These UW peptides, all greater than 90% pure, were checked for purity by HPLC on a reverse phase column (in contrast to some of the peptides received from Dr. Kay), and had the correct molecular weights. From HPLC elution profiles (eg. Figure 3.10) of some of these UW peptides, it was clear that there was a small (<10%) fraction of another peptide which was hardly separable from the target peptide. This small component could not be separated from the main component in any preparative chromatography. One possibility is that the impurity peptide may have had one residue less since the retention times were so similar.

It should be noted that while the mass spectrometry data indicated the UW E3-LTV and K2 E3-LTV peptides to have the same molecular weights, they had vastly different solubilities. The UW sample was found to have limited solubility in water and could only be dissolved to approximately 25 µM while the K2 sample was highly soluble in water and easily reached concentrations in excess of 200 µM. This discrepancy cannot be rationalized.

4.2 Fluorescence Spectra of Peptides and Peptide/Cam Complexes

Uncorrected fluorescence spectral data for all of the peptides both alone and complexed to Cam are summarized in Table 4.1. The maximum wavelengths of the fluorescence spectra of all of the peptides except K E5 and UW E5 were approximately 350 nm. This is typical of a solvent exposed Trp (69). As CD spectra were not measured, the secondary structure of this peptide could not be estimated but they likely existed as random coils. In the case of the E5 peptides, the fluorescence of K E5 had a wavelength maximum at 360 nm, while the fluorescence maximum for the UW E5 was
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>( \lambda_{\text{MAX}} )</th>
<th>FLUORESCENCE INTENSITY CHANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>K 12.1</td>
<td>349 nm</td>
<td>50% Enhanced</td>
</tr>
<tr>
<td>R = 3.5:1</td>
<td>349 nm</td>
<td></td>
</tr>
<tr>
<td>UW 12.1</td>
<td>350 nm</td>
<td>25% Quenched</td>
</tr>
<tr>
<td>R = 1:2</td>
<td>343.5 nm</td>
<td></td>
</tr>
<tr>
<td>K E3-LTV</td>
<td>348 nm</td>
<td>240% Enhanced</td>
</tr>
<tr>
<td>R = 3:1</td>
<td>348 nm</td>
<td></td>
</tr>
<tr>
<td>UW E3-LTV</td>
<td>350 nm</td>
<td>25% Quenched</td>
</tr>
<tr>
<td>R = 1:1; 2:1</td>
<td>344 nm</td>
<td></td>
</tr>
<tr>
<td>K2 E3-LTV</td>
<td>350 nm</td>
<td>16% Quenched</td>
</tr>
<tr>
<td>R = 8:1</td>
<td>350 nm</td>
<td></td>
</tr>
<tr>
<td>K E3-AS</td>
<td>347.5 nm</td>
<td>60% Enhanced</td>
</tr>
<tr>
<td>R = 3.5:1</td>
<td>346 nm</td>
<td>N/A</td>
</tr>
<tr>
<td>R = 2:1</td>
<td>346 nm</td>
<td></td>
</tr>
<tr>
<td>R = 1:1</td>
<td>346 nm</td>
<td></td>
</tr>
<tr>
<td>UW E3-AS</td>
<td>350 nm</td>
<td>23% Quenched</td>
</tr>
<tr>
<td>R = 1:1, 2:1</td>
<td>340 nm</td>
<td></td>
</tr>
<tr>
<td>K E3</td>
<td>349 nm</td>
<td>20% Quenched</td>
</tr>
<tr>
<td>R = 1:3</td>
<td>337 nm</td>
<td></td>
</tr>
<tr>
<td>UW E3</td>
<td>347 nm</td>
<td>22% Quenched</td>
</tr>
<tr>
<td>R = 1:1, 2:1</td>
<td>334 nm</td>
<td></td>
</tr>
<tr>
<td>K2 E3</td>
<td>350 nm</td>
<td>13% Quenched</td>
</tr>
<tr>
<td>R = 6:1</td>
<td>348 nm</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** R is the ratio of peptide:Cam.
Fluorescence intensities were taken at the maximum wavelength of each sample and compared.
Table 4.1 continued

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>$\lambda_{\text{MAX}}$</th>
<th>FLUORESCENCE INTENSITY CHANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>K E4</td>
<td>349 nm</td>
<td>349 nm</td>
</tr>
<tr>
<td>R = 1:3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UW E4</td>
<td>350 nm</td>
<td>344 nm</td>
</tr>
<tr>
<td>R = 1:2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R = 2.5:1</td>
<td>350 nm</td>
<td></td>
</tr>
<tr>
<td>K E5</td>
<td>360 nm</td>
<td>352 nm</td>
</tr>
<tr>
<td>R = 3.5:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UW E5</td>
<td>344 nm</td>
<td>341 nm</td>
</tr>
<tr>
<td>R = 1:1, 2:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K E6</td>
<td>349 nm</td>
<td>349 nm</td>
</tr>
<tr>
<td>R = 3.6:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UW E6</td>
<td>347 nm</td>
<td>347 nm</td>
</tr>
<tr>
<td>R = 1:1, 2:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R = 2.4:1</td>
<td>347 nm</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>349 nm</td>
<td>347 nm</td>
</tr>
<tr>
<td>R = 4:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E8</td>
<td>349 nm</td>
<td>324 nm</td>
</tr>
<tr>
<td>R = 1:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R = 3:1</td>
<td>339.5 nm</td>
<td></td>
</tr>
<tr>
<td>E9</td>
<td>349 nm</td>
<td>334.5 nm</td>
</tr>
<tr>
<td>R = 3.5:1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KirGem</td>
<td>350 nm</td>
<td>322 nm</td>
</tr>
<tr>
<td>R = 1:3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sm-MLCK</td>
<td>349 nm</td>
<td>325 nm</td>
</tr>
<tr>
<td>R = 1:3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine Brain Cam</td>
<td>303.5 nm</td>
<td></td>
</tr>
<tr>
<td>Neuro./Cam</td>
<td>303.5 nm</td>
<td></td>
</tr>
<tr>
<td>Octopus Cam</td>
<td>303.5 nm</td>
<td></td>
</tr>
<tr>
<td>Neuro./Cam</td>
<td>303.5 nm</td>
<td></td>
</tr>
</tbody>
</table>
344 nm. This may suggest a degree of secondary structure within the latter peptide that was affecting the local environment of the Trp.

For all of the UW peptides (except UW E6) and K E3, K E3-AS and K2 E3 when complexed to Cam, there was a marked blue shift and a quenching of fluorescence intensity. The blue shift is indicative of the Trp residue moving into a more hydrophobic environment where it is protected from solvent relaxation. The quenching of intensity may be due to the presence of a fluorescence quencher within the local environment of the Trp fluorophore in the complex as normally it has been reported that such a blue shift is accompanied by an enhancement in fluorescence intensity (70). A blue shift and enhancement was seen with the K E3-AS, sm-MLCK and KirGem peptides.

In the case of the UW E4 and UW E6 peptides complexed to Cam in ratios of excess peptide, there was no shift in wavelength but a fluorescence enhancement was observed. This is markedly different from the spectra seen for ratios with an excess of Cam and may suggest the formation of a 2:1 complex with an excess of peptide while the spectra with excess Cam are representative of the 1:1 complex. This indicates that the local environment of the Trp and hence the quantum yield are different for both complexes. The enhancement but lack of shift indicate that the fluorophore is buried to a degree but is more solvent exposed than those fluorophores which blue shift.

The spectra of UW E6 and K E4 with excess Cam indicated no shift in fluorescence maximum and a quenching of fluorescence intensity. This behavior is difficult to assess simply because the fluorophore may be buried to a small degree but in
close proximity to a quencher which would mask any enhancements. However, the lack of a blue shift indicates that if the fluorophore was buried to any degree it was small.

Comparison of spectra with excess peptide (K series) is difficult due to the large excess of peptide that may mask fluorescence shifts and changes associated with binding. Therefore, spectral data for the K series peptides will not be discussed further except to comment on the differences in spectral changes between the K series and the UW series. Due to this obvious discrepancy in behavior between the two series of peptides, the discrepancies in HPLC data and the lack of mass spectrometry data for the K series, the binding data and analysis of the K series vs the UW series will be discussed separately.

4.3 K Series

4.3.1 Cam/Peptide Titrations

The data for the titrations of peptide with Cam and Cam with peptide (K 12.1) for the K series of peptides are summarized in Table 4.2.

For selected peptides, the change in fluorescence as peptide was added to Cam was not consistent. For example with the K E3-AS peptide, there was no change in fluorescence during the first few additions of peptide followed by an enhancement at ratios past 0.5:1. There was no change in quantum yield during the first few additions of peptide suggesting either that there was no binding or that there was no effect on fluorescence as binding occurred. However, the subsequent fluorescence enhancement in further additions indicates that a 1:1 complex must have formed and there was no change in fluorescence as a result of 1:1 complex formation. The subsequent enhancement was due to an increased quantum yield of the 2:1 complex compared to that of the peptide
Table 4.2 -- Peptide/Cam Titrations of K Series Peptides.

<table>
<thead>
<tr>
<th>Peptide Sample</th>
<th>Cam → Peptide</th>
<th>Peptide → Cam</th>
</tr>
</thead>
<tbody>
<tr>
<td>K 12.1</td>
<td>Quenched</td>
<td>0.3:1 No Change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt; 0.3 Enhanced</td>
</tr>
<tr>
<td>K E3-LTV</td>
<td>N/A</td>
<td>Enhanced</td>
</tr>
<tr>
<td>K2 E3-LTV</td>
<td>N/A</td>
<td>Quenched</td>
</tr>
<tr>
<td>K E3-AS</td>
<td>N/A</td>
<td>0.5:1 No Change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;0.5:1 Enhanced</td>
</tr>
<tr>
<td>K E3</td>
<td>N/A</td>
<td>0.5:1 No Change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;0.5:1 Enhanced</td>
</tr>
<tr>
<td>K2 E3</td>
<td>N/A</td>
<td>Quenched</td>
</tr>
<tr>
<td>Monomer K E4</td>
<td>N/A</td>
<td>Enhanced</td>
</tr>
<tr>
<td>K E5</td>
<td>Quenched</td>
<td>Quenched</td>
</tr>
<tr>
<td>E2</td>
<td>N/A</td>
<td>Enhanced</td>
</tr>
<tr>
<td>E8</td>
<td>N/A</td>
<td>Enhanced</td>
</tr>
<tr>
<td>E9</td>
<td>N/A</td>
<td>Enhanced</td>
</tr>
</tbody>
</table>
alone or the 1:1 complex. This behavior was similar to changes found for K E3 and K 12.1 suggesting 2:1 complex formation in these cases.

Comparison between the Cam to peptide and peptide to Cam titrations of the K 12.1 peptide showed fluorescence quenching for the former and enhancement for the latter titration. In the case of the K 12.1 titration of Cam, the data suggested the formation of a 2:1 complex. This contradictory behavior for the same peptide cannot be rationalized at this time.

Data for the K E4 and K E6 peptides showed non-linearity in the curves in which peptide was added to buffer. This indicates a change in peptide quantum yield as the concentration of peptide increased. This non-linearity is not due to any inner filter effect since the maximum concentration of peptide was only 4 \( \mu \text{M} \). This was attributed to the formation of a dimer form of the peptide. Dimerization of small peptides has been observed previously (71, 72). For the K E6 peptide, the change in fluorescence of the dimer was much greater than the change in fluorescence due to binding to Cam. The change in fluorescence due to binding for this peptide could not properly be determined and K E6 data was not summarized in Table 4.3.

Data for K E3-LTV and K E5 indicated the formation of a 1:1 complex. Peptide titrations using E2, E8 and E9 were difficult to interpret due to the presence of more than one Trp fluorophore within the primary sequence of the peptide. Hence, it was impossible to distinguish between the two Trp’s and hence the local environment of the Cam/peptide complex as it was unknown which Trp (or both) was reporting quantum yield changes as binding to Cam occurred. However, based on the titration curves, in
Table 4.3 – Scatchard Analysis Summary of K Series Peptides.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_d$ (µM)</th>
<th>x-axis intercept</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>K 12.1 → Cam</td>
<td>0.20 ± 0.01</td>
<td>1</td>
<td>0.977</td>
</tr>
<tr>
<td>Cam → K12.1</td>
<td>0.20 ± 0.03</td>
<td>1</td>
<td>0.649</td>
</tr>
<tr>
<td>K E3-LTV → Cam</td>
<td>0.046 ± 0.002</td>
<td>1</td>
<td>0.964</td>
</tr>
<tr>
<td>K E3-AS → Cam</td>
<td>1.39 ± 0.08</td>
<td>1</td>
<td>0.943</td>
</tr>
<tr>
<td>K E3 → Sigma Cam</td>
<td>0.98 ± 0.08</td>
<td>.85</td>
<td>0.891</td>
</tr>
<tr>
<td>K E3 → Cam</td>
<td>0.46 ± 0.01</td>
<td>1</td>
<td>0.992</td>
</tr>
<tr>
<td>K E4 → Cam</td>
<td>0.043 ± 0.004</td>
<td>1</td>
<td>0.915</td>
</tr>
<tr>
<td>K E5 → Cam</td>
<td>0.58 ± 0.11</td>
<td>1.5</td>
<td>0.800</td>
</tr>
</tbody>
</table>
particular the plateaus or lack thereof, it is speculated that the binding affinity order was $E8 > E2 > E9$. This was chosen as the $E8$ titration curve plateaued at a 1:1 ratio indicating very strong binding while $E2$ plateaued at approximately 4 equivalents of peptide and $E9$ showed no evidence of reaching a plateau even after 3.5 equivalents of peptide were added. Examination of the sequences showed that the $E8$ and $E2$ peptides were homologous except for three residues in the C-terminus. The $E8$ C-terminus was much more hydrophilic than either of the $E2$ or $E9$ peptides and yet bound with higher affinity. This was surprising as it is contrary to evidence in the literature which suggests that hydrophobic residues within the C-terminus are important anchors in Cam binding (12, 19, 20, 28). However, the major band for this peptide was also seen to elute in 100% ammonium acetate from a cation exchange column, pH 7. Given that the net charge of the sequence was -1, this was also surprising and suggests that the peptide may not have been the sequence indicated.

The $E9$ peptide was found to bind to Cam with much lower affinity than the $E2$ peptide, the reason for this may reside in the choice of substitution of the Gly 13 Gly 14 residues. In all other peptides, this sequence was replaced with a hydrophilic-hydrophobic combination of residues. For $E9$, two hydrophobic residues were substituted which may disrupt the amphipathic nature of the peptide and account for its lower affinity. Due to the inability to distinguish between fluorophores and the questionable compositions, these three peptides will not be discussed further.

Both $K2 E3$ and $K2 E3-LTV$ appeared to bind with 1:1 stoichiometry. However, as their primary sequences are well characterized and the fluorescence changes upon
binding were similar to the UW peptides these peptides will be discussed with the UW series of peptides.

4.3.2 Binding Analysis and Interpretation

The results of Scatchard Analysis of all titrations where analysis was applicable are summarized in Table 4.3. It should be noted that the $K_d$'s can only be considered to be approximate estimates as peptide binding in most cases was quite strong and very little free peptide was present during the initial points of the titration. Therefore for most plots, these initial points were not included in the analysis which were usually the points at ratios $< 1:1$. The analysis was performed using only a portion of the binding curve. Secondly, the estimated $K_d$'s are based on estimates of $F_\infty$ or $\Delta F_\infty$. An added complication was 2:1 binding since the quantum yields of the 1:1 and 2:1 complexes were obviously different. The $K_d$ estimates for the 2:1 complexes are subject to the same uncertainties.

The x-axis intercepts, listed in Table 4.3, represent the number of binding sites on Cam and from the Table indicates that most of the resultant $K_d$'s are for 1:1 complexes. However, this may be misleading as in some of the peptide titrations of Cam, the data used may have represented only the formation of the Cam:P$_2$ complex from a 1:1 complex and hence the x-axis intercept would only show 1:1 binding.

Data for K 12.1, K E3-AS and K E3 indicate no change in fluorescence during the initial additions of peptide (Table 4.2). This indicates that the formation of the 1:1 complex was spectroscopically silent and that the $K_d$'s obtained are representative of the 2:1 complex.
The data for K E3-LTV, K E4 and K E5 showed no evidence of the formation of a 2:1 complex. Therefore, the $K_d$'s shown for K E3-LTV and K E5 represent the Cam:P complex and that of K E4 represents the monomer binding of the peptide to Cam.

The K 12.1 peptide, while showing evidence of 2:1 binding (Section 4.3.1), gave similar $K_d$'s for both titrations. However, the approximations detailed above makes it difficult to distinguish between them.

The binding affinities for each of the complexes as identified are summarized in Table 4.4 according to the assignments discussed. Since full mass spectrometry characterization of the composition of the K peptides was not available discussion of the meaning of this affinity data for this series will not be discussed further.

4.3.3 Acrylamide Quenching Data

Stern-Volmer constants for complexes of the K series peptides are summarized in Table 4.5. The data is compared with the assumption that the singlet lifetimes of the Trps in NATA and each of the peptides alone and complexed to Cam in both 1:1 and 2:1 complexes were the same. This is a serious assumption, but the fluorescence decay times of the peptides were not determined owing to uncertainties of the peptide purity and sequence. These experiments are discussed; however, to demonstrate the type of information that is available from them. Comparison of all of the peptide ratios with NATA indicate that the Trp's were buried to some extent within the Cam/Peptide complex. Comparison with the $K_{SV}$ of the K E3 peptide alone also indicated that the Trp in the N-terminus of this peptide was not fully solvent exposed. For the K E3, K E3-LTV, K E3-AS and E2 peptides in which two ratios of Cam:peptide were titrated, one to represent the 1:1; Cam:P complex and the other to represent the 1:2; Cam:P2 complex,
### Table 4.4 – $K_d$’s of Cam:$P$ and Cam:$P_2$ Complexes of K Series Peptides

<table>
<thead>
<tr>
<th>Peptide Sample</th>
<th>$K_d$ of Cam:$P$ (µM)</th>
<th>$K_d$ of Cam:$P_2$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K 12.1</td>
<td>0.20 ± 0.03</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>K E3-LTV</td>
<td>0.046 ± 0.002</td>
<td>N/A</td>
</tr>
<tr>
<td>K E3-AS</td>
<td>??</td>
<td>1.39 ± 0.08</td>
</tr>
<tr>
<td>K E3</td>
<td>??</td>
<td>0.98 ± 0.08/0.46 ± 0.01</td>
</tr>
<tr>
<td>K E4 monomer</td>
<td>0.043 ± 0.004</td>
<td>N/A</td>
</tr>
<tr>
<td>K E5</td>
<td>0.58 ± 0.11</td>
<td>N/A</td>
</tr>
</tbody>
</table>

### Table 4.5 – Stern-Volmer Constants of Various Peptide:Cam Ratios for K Series

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide:Cam</th>
<th>$K_{SV}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NATA</td>
<td>2 µM</td>
<td>15.3</td>
</tr>
<tr>
<td>K 12.1</td>
<td>2:1</td>
<td>8.2</td>
</tr>
<tr>
<td>K E3-LTV</td>
<td>1:4</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>1.4</td>
</tr>
<tr>
<td>K E3-AS</td>
<td>1:4</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>3:1</td>
<td>1.4</td>
</tr>
<tr>
<td>K E3</td>
<td>[Cam] = 0 µM</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>1:4</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>2.5:1</td>
<td>1.7</td>
</tr>
<tr>
<td>K E4</td>
<td>1:4</td>
<td>4.9, 2.2</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>6.7, 4.7</td>
</tr>
<tr>
<td>K E6</td>
<td>1:4</td>
<td>5.8, 4.8</td>
</tr>
<tr>
<td>E2</td>
<td>1:4</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>2.5:1</td>
<td>0.62</td>
</tr>
<tr>
<td>E9</td>
<td>1:4</td>
<td>1.9</td>
</tr>
</tbody>
</table>
data indicated that the $K_{SV}$'s of the latter complex showed that the Trp's were less solvent exposed than in the former complex. At the higher ratios of peptide:Cam, if the peptides were not binding 2:1, there would be a large excess of free peptide that should have a higher $K_{SV}$. The observation that lower $K_{SV}$'s were seen provides evidence for the formation of 2:1 complexes.

For the K E4 and K E6 peptides, two distinct slopes were observed for the Stern-Volmer plots. Regression of both indicated two $K_{SV}$ values. In this case the ratios used would indicate that of a 1:1 complex. Since the binding of K E4 and K E6 to Cam and the self-association of these peptides are competitive processes, the two $K_{SV}$'s may be assigned to the quenching of the Cam:P complex and the other to the peptide dimer.

4.3.4 Tb$^{3+}$ Energy Transfer Data

Terbium energy transfer experiments were performed in order to provide insight into the location of the peptide Trp with respect to the Ca$^{2+}$ binding loops of Cam. Excitation was at 295 nm to selectively excite the Trp and luminescence was monitored at 545 nm as aliquots of Tb$^{3+}$ were added. Luminescence enhancement during addition of Tb$^{3+}$ into each Ca$^{2+}$ binding loop indicates that the Trp was in close proximity to that loop. Data is summarized in Table 4.6 and indicated that for Cam:P complexes of K E3, K E4, K E6 and E2, luminescence was observed as Tb$^{3+}$ filled the 2nd and 3rd binding loops of Cam. This suggests that the N-terminal fluorophore of the peptide was in close proximity to Ca$^{2+}$ binding loops II and III provided that the order of fill did not change from that of free Cam. This could only occur if the central helix of Cam bent and
**Table 4.6 — Tb$^{3+}$ Energy Transfer Data for K series Peptides.**

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>ENERGY TRANSFER LOOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:3; K E3-LTV:Cam</td>
<td>no energy transfer</td>
</tr>
<tr>
<td>3:1; K E3-LTV:Cam</td>
<td>no energy transfer</td>
</tr>
<tr>
<td>3:1; K E3-AS:Cam</td>
<td>2nd and 3rd</td>
</tr>
<tr>
<td>1:3; K E3:Cam</td>
<td>2nd and 3rd</td>
</tr>
<tr>
<td>10:1; K E3:Cam</td>
<td>low 3rd and 4th</td>
</tr>
<tr>
<td>1:3; K E4:Cam</td>
<td>2nd and 3rd</td>
</tr>
<tr>
<td>1:3; K E6:Cam</td>
<td>2nd, 3rd and 4th</td>
</tr>
<tr>
<td>1:3; E2:Cam</td>
<td>2nd, 3rd and 4th</td>
</tr>
<tr>
<td>2.3:1; E2:Cam</td>
<td>3rd and 4th</td>
</tr>
<tr>
<td>3:1; E8:Cam</td>
<td>2nd and 3rd</td>
</tr>
<tr>
<td>3.5:1; E9:Cam</td>
<td>3rd and 4th</td>
</tr>
</tbody>
</table>
twisted in such a way as to allow binding loops from different lobes of Cam to interact with the Trp in the N-terminus of the peptide. At higher peptide:Cam ratios, the Trp in K E3-AS was in close proximity to these same loops while the Trp in K E3 appears to bind in proximity to the 3rd and 4th loops. In the 2:1 complex of the latter sample these results suggest that the second equivalent of peptide may have bound in such a manner that it displaced the first peptide equivalent slightly and both Trp's moved closer to the fourth loop. Comparison with the E2, E8 and E9 peptides was not possible because each peptide contained two Trp residues so that it was impossible to determine where they were.

The K E3-LTV peptide indicated no energy transfer regardless of ratio suggesting that the Trp of the Cam:P complex was not close to any of the binding loops and further that this peptide binds differently than the other peptides.

4.4 UW Series

4.4.1 Cam/Peptide Titrations

Since the UW peptides were characterized by mass spectrometry and had clean HPLC traces, greater significance was placed on the results obtained with these peptides than on those obtained with the K series. Hence, observations and affinity data for these peptides will be discussed in more detail. The data for the titrations of peptide with Cam and Cam with peptide are summarized in Table 4.7 for the UW series of peptides.

Similar to the K series of peptides, the UW series also showed evidence of 2:1 complex formation. In peptide titrations of UW 12.1, UW E3-LTV, UW E3-AS and UW E6, ΔF changed from a small degree of fluorescence quenching to a fluorescence
<table>
<thead>
<tr>
<th>Peptide Sample</th>
<th>Cam $\rightarrow$ Peptide</th>
<th>Peptide $\rightarrow$ Cam</th>
</tr>
</thead>
<tbody>
<tr>
<td>UW 12.1</td>
<td>Quenched</td>
<td>1:1 Quenched</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;1:1 Enhanced</td>
</tr>
<tr>
<td>UW E3-LTV</td>
<td>Quenched</td>
<td>1:1 Quenched</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;1:1 Enhanced</td>
</tr>
<tr>
<td>K2 E3-LTV</td>
<td>N/A</td>
<td>Quenched</td>
</tr>
<tr>
<td>UW E3-AS</td>
<td>0.2:1 No Change</td>
<td>2:1 Quenched</td>
</tr>
<tr>
<td></td>
<td>&gt;0.2 Quenched</td>
<td>&gt;2:1 Enhanced</td>
</tr>
<tr>
<td>UW E3</td>
<td>Quenched</td>
<td>Quenched</td>
</tr>
<tr>
<td>K2 E3</td>
<td>N/A</td>
<td>Quenched</td>
</tr>
<tr>
<td>UW E4</td>
<td>Quenched</td>
<td>Enhanced</td>
</tr>
<tr>
<td>UW E5</td>
<td>Quenched</td>
<td>Quenched</td>
</tr>
<tr>
<td>UW E6</td>
<td>Quenched</td>
<td>0.5:1 Quenched</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;0.5:1 Enhanced</td>
</tr>
</tbody>
</table>
enhancement midway through the titration. As with the K E3-AS peptide, this indicates first the formation of a 1:1 complex and 2:1, Cam:peptide complex and each has a different quantum yield. This biphasic behavior was also observed with the Cam titration of UW E3-AS. Similar to the K 12.1 peptide, UW E4 also showed a fluorescence quenching for the Cam titration of the peptide but an enhancement in the peptide titration of Cam. As with the K 12.1 peptide, this was interpreted as 2:1 complex formation where the Cam titration represents the 1:1 complex and the peptide titration represents the 2:1 complex. Lastly, only UW E3 and UW E5 were observed to bind with 1:1 stoichiometry.

4.4.2 Binding Analysis and Interpretation

As for the K series of peptides, the $K_d$'s obtained from the Scatchard plots should only be taken as rough estimates of the true binding affinities. The $K_d$'s obtained for the UW series of peptides are summarized in Table 4.8. Data for the K2 peptides were also summarized in this Table since these samples indicated similar binding curves to the UW peptides. As with the K series, the x-axis intercepts were all approximately 1 which again may be misleading due to the error in the estimation of $\Delta F_{\text{MAX}}$.

Results summarized in Table 4.7 show that many of the peptides bind to Cam in 2:1 complexes. This may explain the differences in $K_d$'s between titrations of peptide with Cam and Cam with peptide. For example, for the UW E4 peptide a $K_d$ value of 0.21 $\mu$M was obtained for the Cam titration of peptide. Since fluorescence data for this titration involves excess Cam, this value would be for the $K_d$ of the 1:1 complex. For the titration of Cam with peptide, a $K_d$ of 1.3 $\mu$M was estimated. Since peptide was in excess and there was other evidence supporting the formation of a 2:1 complex (Section 4.2),
Table 4.8 — Scatchard Analysis Summary of UW Series of Peptides.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_d$ (µM)</th>
<th>x-axis intercept</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cam $\rightarrow$ UW 12.1</td>
<td>$8.9 \pm 2.3$</td>
<td>3</td>
<td>0.533</td>
</tr>
<tr>
<td></td>
<td>$2.2 \pm 0.2$</td>
<td>1</td>
<td>0.942</td>
</tr>
<tr>
<td>UW E3-LTV $\rightarrow$ Cam</td>
<td>$0.54 \pm 0.10$</td>
<td>1.5</td>
<td>0.562</td>
</tr>
<tr>
<td>Cam $\rightarrow$ UW E3-LTV</td>
<td>$1.36 \pm 0.08$</td>
<td>1</td>
<td>0.953</td>
</tr>
<tr>
<td>K2 E3-LTV $\rightarrow$ Cam</td>
<td>$4.0 \pm 0.4$</td>
<td>2</td>
<td>0.848</td>
</tr>
<tr>
<td>UW E3-AS $\rightarrow$ Cam</td>
<td>$0.098 \pm 0.008$</td>
<td>0.6</td>
<td>0.890</td>
</tr>
<tr>
<td>Cam $\rightarrow$ UW E3</td>
<td>too strong</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>UW E3 $\rightarrow$ Cam</td>
<td>too strong</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>UW E4 $\rightarrow$ Cam</td>
<td>$1.3 \pm 0.2$</td>
<td>1</td>
<td>0.932</td>
</tr>
<tr>
<td>Cam $\rightarrow$ UW E4</td>
<td>$0.21 \pm 0.02$</td>
<td>1</td>
<td>0.888</td>
</tr>
<tr>
<td>UW E5 $\rightarrow$ Cam</td>
<td>$0.050 \pm 0.002$</td>
<td>1</td>
<td>0.688</td>
</tr>
<tr>
<td>Cam $\rightarrow$ UW E5</td>
<td>$0.13 \pm 0.02$</td>
<td>1</td>
<td>0.839</td>
</tr>
<tr>
<td>UW E6 $\rightarrow$ Cam</td>
<td>$5.9 \pm 1.4$</td>
<td>1.3</td>
<td>0.765</td>
</tr>
<tr>
<td>Cam $\rightarrow$ UW E6</td>
<td>$0.27 \pm 0.02$</td>
<td>1</td>
<td>0.969</td>
</tr>
</tbody>
</table>
this $K_d$ may be assumed to be that of the 2:1 complex. Results for UW E6 showed similar behavior.

However, not all titrations could be interpreted this way. For example, with UW 12.1, UW E3-LTV and UW E3-AS, the peptide titrations of Cam show a fluorescence quenching followed by an enhancement. Since the plateau value of the enhancement were not determined, $K_d$'s were estimated for the quenching part of the curve which represents the formation of a 1:1 complex. Similarly, since Cam was in excess for the titrations of peptide with Cam, $K_d$'s obtained for these titrations also represent the 1:1 complex. Therefore, discrepancies in these values are probably due to an inaccurate estimation of the plateau fluorescence (UW E3-LTV) and also strong 1:1 binding (UW 12.1, UW E3-AS).

Due to tight binding of the UW E3 peptide, Scatchard analysis could not be performed. The x-axis intercept of the K2 E3-LTV peptide shows the existence of two binding sites on Cam for this peptide. This may suggest that the $K_d$ determined was actually that of the 2:1 complex. This would be valid since the UW and K peptides of this type indicated much tighter binding of the 1:1 complex and the UW E3-LTV peptide showed evidence of 2:1 binding.

The plateau value of the 2:1 complex of the UW 12.1 peptide could not be estimated from the peptide titration of Cam. The Scatchard plot of the Cam titration of peptide showed two distinct slopes. Regression analysis of these curves were performed for two modes of binding the one being the 2:1 complex and the second $K_d$ of the 1:1 complex.
Binding affinities for each of the complexes are summarized in Table 4.9 according to the assignments discussed above. Comparison of the $K_d$'s for the UW series of 12.1, E3-LTV, E3-AS and E3 peptides, indicate that the order of affinity was UW E3 $>\text{UW E3-AS} > \text{UW E3-LTV} > \text{UW 12.1}$ suggesting that substitution of the Gly 13 Gly 14 helix breaker by Ala 13 Ser 14 did increase the affinity of the peptide for Cam. Further, substitution of the Thr 20, Arg 21 and Leu 22 by the more hydrophobic Leu 20, Thr 21 and Val 22 also increased the affinity of the peptide for Cam confirming the importance on affinity of long-chain hydrophobic residues in the C-terminus of the peptide. Comparison of the $K_d$ of the Cam:P$_2$ complex for the K2 E3-LTV peptide indicated that it bound tighter than the UW 12.1 peptide but could not be compared further due to the lack of $K_d$'s for the 2:1 complex of UW E3-AS peptide.

The UW E4, UW E5 and UW E6 peptides, for which the N-terminal Ser Thr had been removed, indicated $K_d$'s that were stronger than the UW 12.1 and UW E3-LTV peptides but weaker than the UW E3-AS and UW E3 peptides suggesting that the removal of the N-terminal Ser Thr had a small effect on the binding affinity but not as great as substitution of the Gly 13 Gly 14 helix breaker or within the C-terminus. The higher affinity of these peptides for Cam over the UW 12.1 and UW E3-LTV peptides confirms the importance of the Gly 13 Gly 14 substitution by other residues. The order of affinity for these peptides was UW E5 $> \text{UW E4} > \text{UW E6}$ such that again, as with the other group, the peptide with the long chain hydrophobic residues in the C-terminus (UW E5) bound with the highest affinity. The UW E4 and UW E6 peptides have similar
Table 4.9 -- $K_d$'s of Cam:P and Cam:P$_2$ Complexes of UW Series of Peptides.

<table>
<thead>
<tr>
<th>Peptide Sample</th>
<th>$K_d$ of Cam:P (µM)</th>
<th>$K_d$ of Cam:P$_2$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UW 12.1</td>
<td>2.2 ± 0.2</td>
<td>8.9 ± 2.3</td>
</tr>
<tr>
<td>UW E3-LTV</td>
<td>1.36 ± 0.08/0.54 ± 0.10</td>
<td>??</td>
</tr>
<tr>
<td>K2 E3-LTV</td>
<td>??</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>UW E3-AS</td>
<td>0.098 ± 0.008</td>
<td>??</td>
</tr>
<tr>
<td>UW E3</td>
<td>too strong</td>
<td>N/A</td>
</tr>
<tr>
<td>UW E4</td>
<td>0.21 ± 0.02</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>UW E5</td>
<td>0.050 ± 0.002/0.13 ± 0.02</td>
<td>N/A</td>
</tr>
<tr>
<td>UW E6</td>
<td>0.27 ± 0.02</td>
<td>5.9 ± 1.4</td>
</tr>
</tbody>
</table>
hydrophobic properties in their C-terminal regions and therefore the tighter binding properties of the UW E4 peptide could be due to the choice of residues for substitution of the Gly 13 Gly 14 helix breaker for which Gly 13 was substituted by Glu 13 for both peptides and Gly 14 was substituted by Leu 14 for UW E4 and Ala 14 for UW E6. The longer side chain of the Leu may stabilize helix formation and provide greater contacts to the Cam allowing the UW E4 peptide to bind with higher affinity than the UW E6 peptide. Next, the UW E3-AS and UW E3 peptides bind with higher affinity than UW E4, UW E5 and UW E6. This suggests that while the C-terminal hydrophobicity of the peptide was important, the choice of residues in the central Gly 13 Gly 14 was more important and that a hydrophobic residue followed by a hydrophilic residue replacement had a much larger effect on binding affinity than a hydrophilic-hydrophobic replacement (UW E4, UW E6) coupled with a more hydrophobic N-terminus (UW E5).

4.4.3 Hydrophobicity Plots and Helical Wheels

Hydrophobicity plots were generated for each of the peptides by averaging the hydrophobicities (73) for the ith residue. The average of the hydrophobicity of residues i - 2 to i + 2 for residues 3 to 23 for a 25 mer peptide were plotted versus the residue number i. This provided an indication of the hydrophobicity in each region of the sequence. Sample plots are shown in Figure 4.1 for the 12.1 and E4 peptides. Since only point mutations were made, the hydrophobicity plots of E3, E3-AS and E3-LTV are very similar to 12.1 and those of E5 and E6 are similar to that of E4. From each plot, the hydrophobicity appears to cycle from hydrophilic to hydrophobic in a 4 residue period.
Figure 4.1 – (A) Hydrophobicity plot of 12.1 peptide sequence. (B) Hydrophobicity plot of E4 peptide sequence.
Since α-helices are 3.6 cycle residues/helix (74), this demonstrates that the peptides are amphipathic. The sample plot for 12.1 indicates a lower degree of hydrophobicity in the sequence around residues 19 (17 in E4). This is due to the much higher hydrophilicity of the Arg 17 versus the substituted Thr 15 and the Arg 21 versus the substituted Ser 19 in this region. The E4 peptide also shows a higher degree of hydrophobicity at residue number 13 (15 of 12.1). This is due to the substitution of the highly hydrophobic Leu 12 for the less hydrophobic Gly 14. For E5 and E6, this was not seen as the Ala 12 substitution does not have as strong an effect on the hydrophobicity. The effect on affinity of this substitution is thought to be small compared to the substitutions of the central Gly Gly residues and the C-terminus. Other substitutions did not show such dramatic effects on the hydrophobicity plots.

Helical wheels were also constructed for each peptide sequence and are shown in Figure 4.2 for the 12.1 (lowest affinity), E3 (highest affinity), E4 and E5 sequences. From the helical wheel for the 12.1 sequence, the sequence is predominately amphiphilic. The E3 peptide was found to bind to Cam with the highest affinity. Comparing the helical wheel of this sequence with that of 12.1, this is surprising since the sequence of the E3 peptide places a highly hydrophobic Leu 20 on the hydrophilic face of the helix. However the sequence is still predominately amphipathic.

Comparison with the E4 and E5 helical wheels show that the peptide sequences are slightly more amphipathic than either the 12.1 or E3 sequences and yet bound with lower affinity than E3. Comparison of the E5 sequence with E4 show very similar amphipathic nature. These results show that the peptide sequences are all predominately
Figure 4.2 — Helical wheels of (A) 12.1; (B) E3; (C) E4 and (D) E5 peptide sequences.

**Note:** Changes from 12.1 peptide sequence are underlined.
amphipathic nature. These results show that the peptide sequences are all predominately amphipathic. Point mutations in the sequence did not significantly alter this pattern and hence comparison of these data does not provide much information. However, the sequences' amphipathic nature, as suggested by both the hydrophobicity plots and the helical wheels, does concur with the literature (Section 1.4) as a necessary requirement for Cam binding.

4.5 *KirGem, sm-MLCK and Neuromodulin*

4.5.1 Cam/Peptide Titrations

Data for the Cam titrations of peptide and peptide titrations of Cam for this series of peptides are summarized in Table 4.10.

Similar to the UW and K series peptides discussed earlier, sm-MLCK also indicated evidence of two distinct quantum yields as peptide bound to Cam suggesting 2:1 complex formation in titrations of Cam with peptide at 345 nm. Similar titrations at 325 nm and of peptide with Cam indicate only a 1:1 complex which is consistent with the literature (2, 19). This supposed discrepancy in the data may be due to the chosen emission wavelength in each case. For example, the fluorescence maximum of the 1:1 complex was 325 nm (Table 4.2). Hence, by monitoring the change in fluorescence at this point, only the formation of the 1:1 complex will be observed as the wavelength maximum of the 2:1 complex may not be shifted and would not have an effect at 325 nm. Emission at 345 nm allows for observation of binding of any complex whose quantum yield and wavelength maximum is not that of the solvent exposed fluorophore.

Data for the *KirGem* peptide at two different wavelengths indicated 1:1 complex formation for both. However, the Cam titration of peptide data indicated very strong
Table 4.10 — Peptide/Cam Titration Data for KirGem, sm-MLCK and Neuromodulin.

<table>
<thead>
<tr>
<th>Peptide Sample</th>
<th>Cam → Peptide</th>
<th>Peptide → Cam</th>
</tr>
</thead>
<tbody>
<tr>
<td>KirGem</td>
<td>@345 nm Enhanced</td>
<td>@345 nm Enhanced</td>
</tr>
<tr>
<td></td>
<td>@322 nm Enhanced</td>
<td>@322 nm Enhanced to 0.5:1 and then plateaus</td>
</tr>
<tr>
<td>sm-MLCK</td>
<td>@325 nm Enhanced</td>
<td>@345 nm 1:1 steep enhancement; &gt;1:1 less enhancement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>@325 nm enhancement</td>
</tr>
<tr>
<td>Neuromodulin</td>
<td>N/A</td>
<td>Enhanced</td>
</tr>
</tbody>
</table>
binding at both wavelengths while the peptide titration of Cam at 345 nm indicated very weak binding. The reason for this may be the same as for the K 12.1 and UW E4 peptides indicating 2:1 binding such that the Cam titrations best represents the 1:1 complex while the peptide titration at 345 nm represents the 2:1 complex. Data for the peptide titration of Cam at 322 nm indicated that the fluorescence intensity was enhanced up to a ratio of 0.5:1 where it plateaud. This behavior was not understood but may be indicating strong binding of a 1:1 complex with wavelength maximum at 322 nm.

Data for the titrations of both octopus and bovine brain Cam with neuromodulin in the presence of $\text{Ca}^{2+}$ indicated that the change in fluorescence was the same for both samples of Cam. This shows that only the Tyr in position 138 was affected as binding occurred and suggests that this Tyr may be involved in peptide binding while the Tyr in position 99 was unaffected by peptide binding. This confirms results found with studies using CD spectroscopy which have also indicated changes in the local environment of the Tyr 138 residue upon binding to peptides (75, 76). In the presence of EGTA, no change in fluorescence was observed as neuromodulin bound to bovine brain Cam. This suggested either that the interaction in the absence of $\text{Ca}^{2+}$ was spectroscopically silent in which case the interactions of neuromodulin with Apo and HoloCam were different or that the neuromodulin was not binding. In this case, the latter explanation was probably more likely as the experiment was conducted in 150 mM KCl and at this ionic strength neuromodulin has been found to bind with higher affinity in the presence of $\text{Ca}^{2+}$ than in the absence (56).
4.5.2 Binding Analysis and Interpretation

Similar to the UW 12.1 peptide, Scatchard plots of sm-MLCK indicated the presence of two distinct slopes. Selected curve fitting for two $K_d$'s was conducted where the first $K_d$ represented the 1:1 complex while the second represented the 2:1 complex. Also, x-axis intercepts for the sm-MLCK peptide indicated values of 0.2 for the Cam:P complex and 1 for the Cam:P$_2$ complex. This data is subject to the inaccuracy of estimating the value of $\Delta F_\infty$ used as the plateau value which was likely that of the Cam:P$_2$ complex.

Scatchard analysis of KirGem titrations of Cam indicated a weak 1:1 complex. However, titrations of KirGem with Cam indicated very strong binding. This data is very contradictory and therefore, it may be possible that the $K_d$ indicated for titrations of Cam with KirGem was that of the 2:1 complex leaving the 1:1 complex too strong for Scatchard analysis. Scatchard analysis with neuromodulin was not possible as the peptide bound too tightly and hence no free peptide was present at any time during the titration. Dissociation constants for KirGem and sm-MLCK are summarized in Table 4.11.

4.5.3 Acrylamide Quenching Data

Acrylamide quenching data for sm-MLCK and KirGem are summarized in Table 4.12 along with the $K_{SV}$ of NATA.

As with the K E4 and K E6 peptides, two distinct $K_{SV}$'s were found for the KirGem peptide. The presence of two $K_{SV}$'s for this peptide was surprising as the Cam:peptide ratio could only be that of the Cam:P complex.
### Table 4.11 -- Scatchard Analysis of KirGem & sm-MLCK Peptides.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_d$ (µM)</th>
<th>x-axis intercept</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>KirGem #1</td>
<td>7.3 ± 0.1</td>
<td>0.7</td>
<td>0.973</td>
</tr>
<tr>
<td>KirGem #2</td>
<td>7.2 ± 0.3</td>
<td>1.5</td>
<td>0.981</td>
</tr>
<tr>
<td>sm-MLCK #1</td>
<td>0.55 ± 0.09</td>
<td>0.2</td>
<td>0.741</td>
</tr>
<tr>
<td></td>
<td>14.2 ± 1.1</td>
<td>1</td>
<td>0.919</td>
</tr>
<tr>
<td>sm-MLCK #2</td>
<td>0.56 ± 0.02</td>
<td>0.15</td>
<td>0.935</td>
</tr>
<tr>
<td></td>
<td>17.7 ± 1.1</td>
<td>1</td>
<td>0.937</td>
</tr>
</tbody>
</table>

### Table 4.12 -- Stern-Volmer Constants of KirGem & sm-MLCK.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide:Cam</th>
<th>$K_{sv}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NATA</td>
<td>2 µM</td>
<td>15.3</td>
</tr>
<tr>
<td>sm-MLCK</td>
<td>1:3</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>5.7</td>
</tr>
<tr>
<td>KirGem</td>
<td>1:3</td>
<td>2.6, 1.8</td>
</tr>
</tbody>
</table>
The $K_{SV}$ of sm-MLCK in the presence of excess Cam indicated a very low value compared to NATA suggesting that in the Cam:P complex, the Trp was buried within the complex and was not exposed to solvent. At higher ratios where sm-MLCK was in excess, the $K_{SV}$ was much higher. From evidence suggesting the formation of a 2:1 complex, this suggests that the Trp’s of the Cam:P complex are much more exposed to solvent molecules than the Cam:P complex.

4.5.4 Tb$^{3+}$ Energy Transfer Data

Tb$^{3+}$ energy transfer data is summarized in Table 4.13 for KirGem and sm-MLCK. Sm-MLCK showed terbium luminescence when the 2nd and 3rd binding equivalents of Tb$^{3+}$ were added, a behavior similar to the K series of peptides.

The KirGem peptide indicated enhancement on addition of the first equivalent of Tb$^{3+}$ which would correspond to the first binding loop. This suggests that the KirGem peptide bound such that the Trp in the N-terminus of the KirGem interacted with the N-terminal domain of the Cam. This behavior was not observed with any of the other peptides.

Tb$^{3+}$ energy transfer experiments with neuromodulin support the assumption that the binding order of Tb$^{3+}$ to Cam was not altered when Cam was bound to a peptide.

4.5.5 Hydrophobicity Plots and Helical Wheels

Hydrophobicity plots of sm-MLCK and KirGem are shown in Figure 4.3, while the corresponding helical wheels are shown in Figure 4.4. The hydrophobicity plots showed an amphiphilic pattern. The helical wheels of sm-MLCK, KirGem and neuromodulin also indicated that the peptide sequences were predominately amphipathic.
These known Cam-regulated sequences confirm that amphiphilicity is an important requirement in Cam binding.
Table 4.13 — Tb\(^{3+}\) Energy Transfer Data for KirGem & sm-MLCK.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>ENERGY TRANSFER LOOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:3; sm-MLCK:Cam</td>
<td>2nd and 3rd</td>
</tr>
<tr>
<td>3:1; sm-MLCK:Cam</td>
<td>2nd and 3rd</td>
</tr>
<tr>
<td>1:3; KirGem:Cam</td>
<td>1st</td>
</tr>
</tbody>
</table>
Figure 4.3 – Hydrophobicity plots of (A) sm-MLCK and (B) KirGem.
Figure 4.4 — Helical wheels of (A) sm-MLCK, (B) KirGem and (C) Neuromodulin.
5. Conclusions

Mass spectrometry data and HPLC traces confirmed the purity and the composition of the peptides obtained from the University of Waterloo. Such data were available for select K peptides; however, these peptides were found to behave very differently from the UW series both in terms of fluorescence and binding affinity, suggesting that while the molecular weights of this series were correct, the sequences were not. Hence, more significance was placed on the UW peptide series.

From the fluorescence titrations and spectra of both series, the binding of more than one equivalent of peptide to Cam was clearly evident. Scatchard analysis of two different methods of peptide titrations allowed, in some cases, for the $K_d$'s of both the 1:1 and 2:1 complexes to be determined. The dissociation constants of the UW series indicated that the order of binding of the evolved series of peptides was $E3 > E3-AS > E5 > E4 > E6 > E3-LTV > 12.1$. This confirmed the influence of the long chain hydrophobic residues in the C-terminus on forming strong complexes with Cam. From the K series of peptides, the binding order of the E2, E8 and E9 peptides was $E8 > E2 > E9$. This was surprising as the E8 peptide has a very hydrophilic C-terminus; however, due to a lack of mass spectrometry data and from the HPLC trace of the E8 peptide, the exact sequence of this peptide was questioned. Helical wheel data and hydrophobicity plots indicated that the peptide sequences were all amphipathic. This confirmed the importance of amphiphilicity in Cam binding.

Studies with the peptide analogues of a known Cam-regulated enzyme, sm-MLCK, and a suspected Cam-regulated enzyme, KirGem, also showed evidence of 2:1
binding. In the case of sm-MLCK, the $K_d$'s of both the 1:1 and 2:1 complexes were estimated while for KirGem only that of the 2:1 complex could be estimated.

This apparent 2:1 binding, especially with sm-MLCK, casts some doubt on the validity of using peptide analogues as a model for enzyme binding to Cam. The peptide analogues are much less bulky structurally and this may be what permits the analogues to bind with greater than 1:1 stoichiometry. In vivo, the full enzyme would be much bulkier and steric hindrance alone may prevent the formation of a 2:1 complex. There is no evidence that sm-MLCK enzyme forms a 2:1 complex with Cam.
References


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