Thermodynamic and structural determinants of calcium-independent interactions of Calmodulin

Michael Dennis Feldkamp

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THERMODYNAMIC AND STRUCTURAL DETERMINANTS OF CALCIUM-INDEPENDENT INTERACTIONS OF CALMODULIN

by

Michael Dennis Feldkamp

An Abstract

Of a thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biochemistry in the Graduate College of The University of Iowa

July 2010

Thesis Supervisor: Professor Madeline A. Shea
ABSTRACT

Calmodulin (CaM) is an essential protein found in all eukaryotes ranging from vertebrates to unicellular organisms such as Paramecia. CaM is a calcium sensor protein composed of two domains (N and C) responsible for the regulation of numerous calcium-mediated signaling pathways. Four calcium ions bind to CaM, changing its conformation and determining how it recognizes and regulates its cellular targets. Since the discovery of CaM, most studies have focused on the role of its calcium-saturated form.

However, an increasing number of target proteins have been discovered that preferentially bind apo (calcium-depleted) CaM. My study focused on understanding how apo CaM recognizes drugs and protein sequences, and how those interactions differ from those of calcium-saturated CaM. I have used spectroscopic methods to explore CaM binding the drug Trifluoperazine (TFP) and the IQ-motif of the type 2 Voltage-Dependent Sodium Channel (Na1.2IQp). These studies have shown that both TFP and Na1.2IQp preferentially bind to the “semi-open” conformation of apo CaM.

TFP was shown to be an unusual allosteric effector of calcium binding to CaM. Using 15N-HSQC NMR spectroscopy, I determined the stoichiometry of TFP binding to apo Cam to be 2:1 and to (Ca2+)4-CaM to be 4:1 TFP:CaM. That difference in stoichiometry determined whether TFP decreased or increased the affinity of CaM for calcium. Analysis of residue-specific chemical shift differences indicated that TFP binding to apo and (Ca2+)4-CaM perturbed the C-domain more than the N-domain, prompting high-resolution structural studies of the isolated C-domain of CaM.

Crystallographic studies of TFP bound to a calcium-saturated C-domain fragment of CaM (CaM76-148) revealed that CaM adopted an “open” tertiary conformation. The unit cell contained two protein and 4 drug molecules. The orientation of TFP revealed that its trifluoromethyl group was found in two alternative positions (one in each protein in the unit cell), and that Met 144 acted as a gatekeeper to select the orientation of TFP.
In contrast to TFP binding to the “open” conformation of calcium-saturated CaM_{76-148}, my NMR studies showed that TFP bound the “semi-open” conformation of apo CaM_{76-148}. TFP interacted with CaM residues near the perimeter of the hydrophobic pocket, but did not contact residues that are solvent-accessible only in the “open” form. Allosteric effects due to TFP binding were observed in the calcium-binding loops of apo CaM_{76-148}. These properties suggest that TFP may antagonize interactions between apo CaM and target proteins such as ion channels that preferentially bind apo CaM.

Na_{v}1.2, is responsible for the passage of Na^{+} ion across cellular membranes. Apo binding of CaM to Na_{v}1.2 poises it for action upon calcium release in the cell. My NMR studies of CaM binding to the Na_{v}1.2 IQ-motif sequence (Na_{v}1.2IQp) showed that the C-domain of apo CaM was necessary and sufficient for binding. My high-resolution structure of the isolated C-domain of CaM bound to Na_{v}1.2IQp revealed that the domain adopted a “semi-open” conformation. At the interface between the IQ-motif and CaM, the highly conserved I and two Y residues of Na_{v}1.2IQp interacted with hydrophobic residues of CaM, while the invariant Q residue interacted with residues in the loop between helices F and G of CaM. This is the first CaM-IQ complex to be determined by NMR; the only other available structure of apo CaM bound to an IQ-motif was determined crystallographically.

To accomplish its regulatory roles in response to cellular Ca^{2+} fluxes, CaM has evolved multiple binding interfaces that are allosterically linked to its Ca^{2+}-ligation state. My studies of CaM binding to TFP and Na_{v}1.2 demonstrate the versatility of CaM functioning as a regulatory protein comprised of domains having separable functions.
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by

Michael Dennis Feldkamp

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biochemistry in the Graduate College of The University of Iowa

July 2010

Thesis Supervisor: Professor Madeline A. Shea
CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

Michael Dennis Feldkamp

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in Biochemistry at the July 2010 graduation.

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Ernesto Fuentes

Lei Geng

Shahram Khademi

Daniel Weeks
For those who value truth more than dogma
To laugh often and much; to win the respect of intelligent people and the affection of children; to earn the appreciation of honest critics and endure the betrayal of false friends; to appreciate beauty; to find the best in others; to leave the world a bit better, whether by a healthy child, a garden patch or a redeemed social condition; to know even one life has breathed easier because you have lived. This is to have succeeded.

-Ralph Waldo Emerson
ACKNOWLEDGMENTS

I have often heard the saying that “science is done by standing on top of the shoulders of giants”. The further I have progressed in my Ph.D. studies, I have found a deeper and deeper appreciation for this phrase. Science is truly a team sport and there are many people I would like to thank for their personal or professional support over the course of obtaining my Ph.D.

I would like to first and foremost thank my advisor Madeline Shea, whose support and guidance has shaped me as a scientist. She provided me the opportunity to pursue studies in x-ray crystallography and NMR, and allowed me to attend multiple local and national conferences to present my work. She has always kept my best interests at heart and made numerous sacrifices to ensure that my training in her lab was second to none. Above all I thank her for her compassion and understanding, the door to her office was always open to discuss problems I had science related or not. I would like to thank the past and present members of the Shea lab, all of whom made coming to work everyday an enjoyable and positive experience. I would especially like to thank Susan O’Donnell, I am glad to have had the benefit of progressing through graduate school with someone as energetic and skilled as her. I would like to thank my thesis committee Adrian Elcock, Ernie Fuentes, Lei Geng, Sharham Khademi, and Dan Weeks. I can honestly say that each of you has individually taken the time to help me outside the committee meeting environment. Your input, and encouragement throughout this process is greatly appreciated. To my unofficial 6th committee member, Liping Yu. I’m grateful for your help and guidance in my NMR based studies as well as encouragement over the course of what seemed like a never ending onslaught of peak assignments to be made. Your dedication to helping others succeed using NMR is unmatched. I would like to thank Rams and Lokesh Gakhar for their help in collection and processing of x-ray diffraction data.
I’m honored to have met the many friends that I’ve made in Iowa, you have helped make my time here so much more enjoyable. Our conversations about science initially brought us together, but it is the non-science conversations that I will miss most dearly when I leave.

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LIST OF ABBREVIATIONS

APBS    Adaptive Poisson-Boltzmann Solver
BAA     Basic Amphipathic Alpha-helix
CaMBD   Calmodulin binding domain
CaMKII  CaM Kinase II (CaMKII)
DPD     N-(3,3,-Diphenylpropyl-N'-[1-R-(2 3,4-Bis-Butoxyphenyl)-Ethyl]-Propylenediamine
EGTA    Ethylene Glycol bis2 (aminoethylether)=N,N,N',N'
         Tetraacetic Acid
FRET    Fluorescence Resonance Energy Transfer
HPLC    High Pressure Liquid Chromotography
KAR-2   3"-(Beta-Chloroethyl)-2" ,4"-Dioxo-3, 5"-Spiro-Oxazolidino-4-
         Deacetoxy-Vinblastine
LPBE    Linearized Poisson-Boltzmann Equation
mCaM    Mammalian Calmodulin
MLCK    Myosin Light Chain Kinase
Na$_v$1.2 Voltage-Dependent Sodium Channel type 2
Nav1.2BAA BAA-motif of Nav1.2
Nav1.2$_{v1.2iQp}$ Voltage-Dependent Sodium Channel type 2 IQ-motif
NOE     Nuclear Overhauser Effect
NOESY   Nuclear Overhauser Effect Spectroscopy
PCaM    Paramecium Calmodulin
RMSD    Root Mean Standard Deviation
SAXS    Small Angle x-ray Scattering
SPAN    Value determined by subtracting minimum data value from maximum data value, for use in normalization of data
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<tr>
<td>T2</td>
<td>Transverse Relaxation</td>
</tr>
<tr>
<td>TFP</td>
<td>10-[3-(4-Methyl-Piperazin-1-yl)-Propyl]-2-Trifluoromethyl-10H-Phenothiazine</td>
</tr>
<tr>
<td>TFP</td>
<td>Trifluoperazine</td>
</tr>
<tr>
<td>TOCSY</td>
<td>Total Correlation Spectroscopy</td>
</tr>
<tr>
<td>W-7</td>
<td>N-(6-Aminoethyl)-5-Chloro-1-Naphthalenesulfonamide</td>
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CHAPTER I

INTRODUCTION

The Ca^{2+} ion plays a vital role as a secondary messenger where its spatial and temporal location can be tightly regulated to perform a myriad of regulatory functions. Upon signaling, Ca^{2+} can be released into the cytosol from both intracellular and extracellular stores where it is then sensed by a variety of Ca^{2+}-binding proteins. The most prominent of these Ca^{2+}-binding proteins is Calmodulin, which regulates over 300 target proteins in a Ca^{2+}-dependent manner (Yap et al., 2000). To accomplish its regulatory functions, CaM interacts with its protein targets using a variety of Ca^{2+} ligation states. The first CaM-dependent proteins identified were Ca^{2+}-dependent, and the conclusion was drawn (prematurely) that Ca^{2+} was required for target binding by CaM. Targets have since been identified that interact with either apo or partially Ca^{2+}-saturated CaM, representing new classes of proteins whose interaction with one or both domains of CaM is Ca^{2+}-independent. This thesis seeks to examine the structural and thermodynamic properties of apo CaM when interacting with the anti-psychotic drug Trifluoperazine (TFP) as well as a peptide that represents the CaM binding domain of the voltage dependent sodium channel 1.2 isoform (Na_{v}1.2).

**CaM Background**

CaM is a Ca^{2+} sensor protein that is essential to many eukaryotic signal transduction pathways. The CaM sequence (148 a.a.) is highly acidic (pI of 4) and divided between two homologous domains which are connected by a flexible 5-residue linker (**Figure 1.1**). Each domain binds two Ca^{2+} ions cooperatively in neighboring EF-Hand motifs, giving rise to a total of 4 bound Ca^{2+} ions per molecule of CaM (Chattopadhyaya et al., 1992; Crouch and Klee, 1980; Pedigo and Shea, 1995; Rao et al., 1993). Sequence comparison of EF-hand Ca^{2+}-binding sites reveals a high level of sequence conservation among residues that coordinate the Ca^{2+} ion via pentagonal
bipyramidal geometry (Strynadka and James, 1989; Wilson and Brunger, 2000; Yang et al., 2002) (Figure 1.2). The primary sequence of CaM is highly conserved among eukaryotes. Paramecium CaM (PCaM) is 88% identical to all mammalian CaM (mCaM) sequences, with only four differences in the N-domain (residues 1-80, sites I and II) and 13 differences in the C-domain (residues 76-148, sites III and IV) (Kung et al., 1992; VanScyoc et al., 2006) (Figure 1.3). Although the two domains are similar in sequence and structure, the N-domain binds Ca$^{2+}$ with a 10-fold lower affinity than the C-domain (Linse and Chazin, 1995; VanScyoc et al., 2002).

CaM has been observed to adopt multiple conformations (closed, semi-open, and open) dependent upon its ligation state (Figure 1.4) (Ataman et al., 2007; Chattopadhyaya et al., 1992; Houdusse et al., 2006; Kuboniwa et al., 1995; Meador et al., 1992). These conformations are defined by the inter-helical angles adopted by the EF-hand motifs within each domain. The “open” domain conformation of CaM is adopted by the N- and C-domains of CaM when Ca$^{2+}$ binds to them either in the absence or presence of a target (Ataman et al., 2007; Swindells and Ikura, 1996; Wilson and Brunger, 2000). The “semi-open” domain conformation of CaM, which has only been observed in the C-domain of CaM, is adopted when binding to targets in the absence of Ca$^{2+}$ (Houdusse et al., 2006; Swindells and Ikura, 1996). More commonly the absence of Ca$^{2+}$ results in a “closed” conformation for both the N- and C-domains (Kuboniwa et al., 1995).

**Interaction of (Ca$^{2+}$)$_4$-CaM with Protein Targets**

Changes in intracellular Ca$^{2+}$ levels are linked to cellular events by the effect of Ca$^{2+}$ on CaM: it triggers conformational changes that expose hydrophobic surfaces in both domains, altering its binding affinity for many target proteins (Bayley et al., 1996; Colbran, 1992; Klee, 1980). The Ca$^{2+}$ binding affinity of CaM is “tuned” dependent upon which target it is bound to, allowing CaM to regulate numerous cellular processes
dependent upon \([\text{intra-cellular Ca}^{2+}]\) (Evans and Shea, 2006; Peersen et al., 1997). The canonical mode of interaction of \((\text{Ca}^{2+})_4\)-CaM with intra-cellular protein targets such as metabolic enzymes, cyclases, kinases, phosphatases, and ion channels is a compact ellipsoidal conformation (Hoeflich and Ikura, 2002; Meador et al., 1993; Mori et al., 2000). The protein target sequence recognized by CaM is often a Basic Amphipathic Alpha-helix (BAA) motif where CaM typically binds in a \(\text{Ca}^{2+}\)-saturated manner (O'Neil and DeGrado, 1990). When bound to \(\text{Ca}^{2+}\)-dependent targets such as CaM Kinase II (CaMKII), or myosin light chain kinase (MLCK), the domains of CaM adopt an “open” conformation that exposes hydrophobic patches used for target binding resulting in \(\text{Ca}^{2+}\)-dependent regulation as shown previously in Figure 1.4 (O'Neil et al., 1987).

**Interaction of apo CaM with Protein Targets**

Historically, activation of target proteins by CaM was thought to occur in a strictly \(\text{Ca}^{2+}\)-dependent manner where target binding always increased the \(\text{Ca}^{2+}\) affinity of CaM (Prozialeck and Weiss, 1982; Roberts and Harmon, 1992; Wang and Sharma, 1980; Weiss and Wallace, 1980). An emerging class of \(\text{Ca}^{2+}\)-independent CaM targets, typically found with ion channels and myosin motor proteins, interact more favorably with apo CaM instead of \((\text{Ca}^{2+})_4\)-CaM. These targets typically contain IQ-motifs, although other non IQ-motif apo CaM Binding Domains (apo CaMBD’s) have been observed (Fanger et al., 1999; Gerendasy et al., 1994; Liu and Storm, 1990; Martin and Bayley, 2004; Swindells and Ikura, 1996). Compared to structural studies of \((\text{Ca}^{2+})_4\)-CaM, structural studies of apo-CaM interacting with targets are much less common. The disparity in representation of targets bound to \((\text{Ca}^{2+})_4\)-CaM compared to apo-CaM is likely due to the mobility of the N-domain relative to the C-domain in the absence of \(\text{Ca}^{2+}\), as well as the lack of an ordered structure within the \(\text{Ca}^{2+}\) binding loops of CaM in the absence of \(\text{Ca}^{2+}\). The two high-resolution structures available of an apo C-domain
CaM interacting with peptides derived from either Myosin V (IQ-motif) or the SK-channel (non IQ-motif), depict CaM in an extended conformation where its C-domain interacts with each target through a “semi-open” conformation (Figure 1.5) (Houdusse et al., 2006; Schumacher et al., 2001).

The IQ-motif was first discovered and characterized from neuromodulin, a neurospecific apo CaM binding protein (Chapman et al., 1991; Liu and Storm, 1990). The IQ-motif is approximately 11 amino acids in length and distributed across multiple protein families ranging from myosins and ion channels, to Ras exchange and neuronal growth proteins (Bähler and Rhoads, 2002). The consensus sequence of this motif is (IQxxxBGxxxB, B=Lys or Arg), which forms an amphiphilic α-helix characterized as capable of binding to calmodulin in a Ca$^{2+}$-independent manner (Figure 1.6) (Swindells and Ikura, 1996). Analysis of genomic sequencing results indicate that there are at least 208 IQ-motifs in 108 proteins within the human genome (Venter et al., 2001). Examination of IQ-motifs across all eukaryotes reveals that of the IQ-motif defining residue Gln at position 1 is invariant, while IQ-motif position 6 (Gly) is the most variable (Figure 1.6). As indicated by the higher number of IQ-motifs than number of proteins which contain them (208 IQ-motifs per 108 proteins), several proteins contain multiple IQ-motifs (Houdusse et al., 2006; Martin and Bayley, 2004; Trybus et al., 2007). These proteins are mainly found in the myosin family, which depending upon the myosin variant, possess between 1 and 7 IQ-motifs typically separated by 9-16 residues (Koide et al., 2006).

The Voltage-Dependent Sodium Channel, 1.2

The voltage-dependent sodium channel, 1.2 (Nav1.2) is an integral membrane protein comprised of one pore-forming α–subunit (2005 aa, 260 kDa), and one or more β–subunits (215 aa, 33–36 kDa each) which control the kinetics and gating of the channel as shown in (Figure 1.7) (Yu and Catterall, 2003). The physiological role of this channel
is to selectively regulate the flow of \( \text{Na}^+ \) ions across the cell membrane of central and peripheral neurons, allowing for the creation of action potentials (Catterall, 2000b). The IQ–motif containing CaM binding region of \( \text{Na}_\text{V}1.2 \) is located near its C-terminus and has a high degree of sequence identity to corresponding regions of all 10 known human sodium channel isoforms (Figure 1.8) (Mori et al., 2003; Theoharis et al., 2008; Yu and Catterall, 2003).

The \( \alpha \)-subunit is comprised of four domains formed from six transmembrane helices, where the fourth helix contains a voltage sensor, responsible for activation of the \( \text{Na}_\text{V}1.2 \) upon depolarization of the cell membrane (Cormier et al., 2002; Mantegazza et al., 2001). Inactivation of \( \text{Na}_\text{V}1.2 \) is achieved via an intracellular loop between domains III and IV which contains the inactivation gate and the C–terminal tail of the \( \alpha \)-subunit (Figure 1.7) (Chin and Means, 2000; Herzog et al., 2003; Mantegazza et al., 2001; Yu and Catterall, 2003). The mechanism of inactivation is hypothesized to occur when the inactivation gate physically blocks the channel pore via a CaM mediated interaction with C-terminal tail of \( \text{Na}_\text{V}1.2 \) (Chin and Means, 2000; Herzog et al., 2003; Mantegazza et al., 2001; Yu and Catterall, 2003). Among the ten known sodium channel isoforms the C–terminal tail is responsible for the different rates of inactivation of the \( \alpha \)-subunit (Deschênes et al., 2001; Mantegazza et al., 2001). The membrane proximal half of the intracellular C–terminal tail has been modeled to contain six \( \alpha \)-helices, where deletion of the putative sixth \( \alpha \)-helix region slows recovery from inactivation, maintaining \( \text{Na}_\text{V}1.2 \) in a closed inactivated state (Cormier et al., 2002). In this proposed sixth \( \alpha \)-helix of the C–terminal region, between residues 1901–1927 of the \( \text{Na}_\text{V}1.2 \), is located a CaM binding IQ-motif (Mori et al., 2000; Theoharis et al., 2008). Studies of \( \text{Na}_\text{V}1.2 \), as well as other sodium channel isoforms have shown that CaM binding is necessary for functional sodium currents, indicating a regulatory role for CaM on the sodium channel (Mori et al., 2003; Yu and Catterall, 2003).
The two CaM domains (N and C) may have distinct regulatory roles in sodium channel modulation. Separable roles for the CaM domains were first shown physiologically with in vivo studies by Kung and associates (Gustin et al., 1986). A genetic screen of mutagenized but viable Paramecium identified two classes of mutants that under- or over-reacted to chemical stimuli (Preston et al., 1992). These mutants were found to have defective regulation of their Ca\(^{2+}\)-dependent sodium and potassium channel currents, and the mutations were located in CaM. Under-reacting mutations that occurred between sites I and II and in site II of the N-domain of CaM were shown to affect only sodium conductance. In contrast, over-reacting mutations occurred within sites III and IV and the fourth helix of the C-domain and only affected potassium conductance.

**Interaction of CaM with Drugs**

In addition to interacting with naturally occurring protein targets, (Ca\(^{2+}\))\(_4\)-CaM has also been shown to interact with small molecule compounds such as N-(6-Aminohexyl)-5-Chloro-1-Naphthalenesulfonamide (W-7), N-(3,3,-Diphenylpropyl-N'-[1-R-(2 3,4-Bis-Butoxyphenyl)-Ethyl]-Propylenediamine (DPD), 3"-(Beta-Chloroethyl)-2",4"-Dioxo-3, 5"-Spiro-Oxazolidino-4-Deacetoxy-Vinblastine (KAR-2), and 10-[3-(4-Methyl-Piperazin-1-yl)-Propyl]-2-Trifluoromethyl-10H-Phenothiazine (Trifluoperazine, TFP) (Figure 1.9) (Cook et al., 1994; Harmat et al., 2000a; Hennessey and Kung, 1984; Horvath et al., 2005; Matsushima et al., 2007; Osawa et al., 1998; Tang et al., 2006; Vandonselaar et al., 1994a; Vertessy et al., 1998b). The compounds W-7 and DPD have primarily been used in vitro as CaM antagonists, while KAR-2 (a potent anti-microtubular agent), and TFP (antipsychotic agent) have been used in the clinical setting (Cook et al., 1994; Harmat et al., 2000a; Hennessey and Kung, 1984; Horvath et al., 2005; Matsushima et al., 2007; Osawa et al., 1998; Tang et al., 2006; Vandonselaar et al., 1994a; Vertessy et al., 1998b). In structures of these drugs bound to (Ca\(^{2+}\))\(_4\)-CaM, their
aromatic moieties insert into the hydrophobic pockets of CaM, mimicking CaM-peptide interactions which employ similar interactions to insert aromatic groups into CaM. In all of these structures the drug bound to the “open” conformation and like peptide targets all crystallographically determined structures of these drugs bound to (Ca^{2+})_{4}\text{-CaM} (with the exception of W-7 determined via NMR) depict CaM in a collapsed conformation as seen with peptides (Figure 1.10) (Cook et al., 1994; Harmat et al., 2000b; Horvath et al., 2005; Osawa et al., 1998; Vandonselaar et al., 1994a; Vertessy et al., 1998a). Although CaM has been observed to bind drugs in vitro, it is not the intended primary in vivo target of the previously mentioned compounds making in vivo CaM interactions with these drugs a secondary or off target effect (Sheets et al., 2006).

**TFP**

Trifluoperazine (TFP) is a phenothiazine class antipsychotic drug primarily used in the treatment of schizophrenia and related mental disorders (Abuzzahab, 1977; Oybir, 1962). Its first clinical trial for use in human patients with mental disorders was in 1958; more recently TFP has been indicated to reduce levels of opioid addiction (Tang et al., 2006; Wallis, 1958). Its primary function is that of a dopamine antagonist where it binds to, but does not activate, the dopamine receptor, thus blocking the action of dopamine or exogenous agonists (Clow et al., 1980; Kerwin et al., 1984; Roudebush et al., 1991). Clinically, TFP can be administered orally in solid pill form, as a liquid, or as an intramuscular injection where the daily amount administered is typically 15-20 mg per day (Carscallen et al., 1968; Gauron and Rowley, 1970; Hodes, 1960). The clinical use of TFP as an anti-psychotic has been discontinued in favor of newer formulations that do not carry the often irreversible side-effect of tardive dyskinesia typical of TFP and other first generation anti-psychotics like it (Lahti et al., 1993).

TFP has been shown in vitro to be a potent CaM antagonist, where it is often added to cell cultures to disrupt CaM interactions with its protein targets (Lydan and
O'Day, 1988; Pelech et al., 1983). The interaction of TFP with CaM is the most studied of small molecule CaM antagonists. However, the results of many of these studies which focused on the stoichiometry of TFP binding, its effect on Ca$^{2+}$-binding affinity, as well as how it alters the structure of CaM have been inconclusive (Cook et al., 1994; Massom et al., 1990b; Matsushima et al., 2000; Matsushima et al., 2007; Vogel et al., 1984; Yamaotsu et al., 2001). An example of this can be found in the stoichiometry of TFP bound to (Ca$^{2+}$)$_4$-CaM in the three structures of that have been determined, where depending upon the structure examined, either 1, 2, or 4 TFP are bound to (Ca$^{2+}$)$_4$-CaM as shown previously in (Figure 1.10).

**Electrostatic Interactions of CaM with Targets**

In addition to hydrophobic interactions, electrostatic interactions between CaM and its protein targets have a significant role in recognition and binding (Linse et al., 1991; Noguchi et al., 2004; Ogawa and Tanokura, 1984). At pH 7.4, CaM is highly acidic (pI = 4) and carries a net charge of -24 (apo) or -16 (Ca$^{2+}$-saturated), while the CaMBD’s of target proteins contain basic residues arginine or lysine, resulting in electrostatic attraction between the two molecules. The opening and closing of ion channels necessary for cell signaling results in a constant flux of Ca$^{2+}$, K$^+$, and Na$^+$ ions, altering the strength of electrostatic interactions between CaM and its targets. K$^+$ and Na$^+$ also reduce the Ca$^{2+}$-binding affinity of CaM, making it intriguing to learn the functional consequences of how they affect CaM-protein interactions.

**Description of Thesis Content**

**Chapter II** describes studies conducted to explore how the Ca$^{2+}$-binding properties of CaM are altered upon binding TFP, as well as the binding stoichiometry of TFP to apo and (Ca$^{2+}$)$_4$-CaM. In this chapter fluorescence-monitored Ca$^{2+}$ titrations demonstrate that dependent upon the [TFP] examined that the Ca$^{2+}$-binding affinity of CaM can either increase or decrease relative to values observed in the absence of TFP.
Additionally using $^{15}$N-HSQC NMR spectroscopy the stoichiometry of TFP binding was dependent upon the Ca$^{2+}$ ligation state of CaM where 2 and 4 TFP were found to bind to apo and (Ca$^{2+}$)$_4$-CaM respectively. The major finding of experiments conducted in Chapter II were reported in a manuscript titled “Allosteric Effects of the Anti-Psychotic Drug Trifluoperazine on the Energetics of Calcium Binding by Calmodulin” that has been published in Proteins: Structure, Function, and Bioinformatics.

Chapter III addresses how TFP alters the structures of apo and (Ca$^{2+}$)$_4$-CaM at the atomic level. In this chapter I show, using x-ray crystallography, that TFP binds (Ca$^{2+}$)$_2$-CaM$_{76-148}$ in two different orientations, unifying what were otherwise conflicting observations within the field. I will also show, using NMR spectroscopy, that the chemical environment of specific atoms of apo CaM$_{76-148}$ are perturbed upon TFP addition. The major findings of experiments conducted in Chapter III will be reported in a manuscript that is in preparation for submission.

Chapter IV discusses how binding of Na$_v$1.2IQp alters the structure of apo CaM$_{76-148}$. In this chapter I will show using NMR spectroscopy how apo CaM$_{76-148}$ adopts a “semi-open” conformation upon binding Na$_v$1.2IQp. I will also demonstrate, using NMR spectroscopy, that when Na$_v$1.2IQp binds to apo CaM$_{1-148}$, the N- and C-domains adopt “closed” and “semi-open” domain conformations respectively. The major findings of experiments conducted in Chapter IV will be reported in a manuscript that is in preparation for submission.

Chapter V presents preliminary studies of how changing the solution ionic strength via KCl or NaCl alters the strength of electrostatic interaction between CaM and peptides (melittin, CaMKII, and Na$_v$1.2IQp). In this chapter I show using fluorescence anisotropy the effect that varied [NaCl] and [KCl] salts have on the binding affinity of CaM for the peptides Melittin, CaMKII, and Na$_v$1.2IQp. Poisson-Boltzman calculations were performed to theoretically examine how varying [NaCl] or [KCl] altered the electrostatic attraction between (Ca$^{2+}$)$_4$-CaM and CaMKII. These studies indicated that
increasing [salt] lowered the electrostatic attraction of CaM for all peptides tested. Significant differences were observed though between experimental and theoretical studies involving \((\text{Ca}^{2+})_4\)-CaM and CaMKII suggesting that another factor, possibly conformational change by CaM and/or CaMKII influence the electrostatic interaction between these two molecules.

**Chapter VI** discusses the results of the prior chapters and their contribution to our understanding of the role of target interactions with CaM under apo conditions. While the work described in this thesis presents a high-resolution structure of apo CaM bound to \(\text{Na}_v1.2\text{IQp}\), the structural basis of how \((\text{Ca}^{2+})_4\)-CaM interacts with \(\text{Na}_v1.2\text{IQp}\) or another proposed \(\text{Ca}^{2+}\)-dependent CaM binding site (\(\text{Na}_v1.2\text{BAA}\)) is not clear. Future experiments well determine how \((\text{Ca}^{2+})_4\)-CaM interacts with its binding domains (\(\text{Na}_v1.2\text{IQp}\) and \(\text{Na}_v1.2\text{BAA}\)) of \(\text{Na}_v1.2\). Experiments are proposed to resolve the role of the N-domain in \(\text{Na}_v1.2\) regulation under \(\text{Ca}^{2+}\)-saturating conditions where it has been implicated in interacting with the \(\text{Na}_v1.2\text{BAA}\) region of \(\text{Na}_v1.2\).

**Appendices** provide Fortran functions used in nonlinear squares analysis, and chemical shift assignments of apo CaM\(_{76}\), apo CaM\(_{76-148}\) when bound to TFP, and apo CaM\(_{76-148}\) when bound to \(\text{Na}_v1.2\text{IQp}\).
Figure 1.1: Structure of (Ca²⁺)₄-CaM. Ca²⁺-binding sites (green) I and II are located in the N-domain (blue, helices A-D), while Ca²⁺-binding sites III and IV are located in the C-domain (red, helices E-H), allowing CaM to bind 4 Ca²⁺ ions (yellow spheres). The N- and C-domains of CaM are connected by a 5 residue linker (black).

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The EF-hand consists of two α-helices connected by a 12-residue loop. Residues 1, 3, 5, 7, 9 and 12 (sticks) of the loop contribute a side chain or backbone oxygen (red) atom necessary for Ca\textsuperscript{2+} (yellow sphere) binding. Residue 12, which is a Glu, contributes both oxygens from its side chain carboxylic acid group. Residue 9 of the Ca\textsuperscript{2+}-binding loop does not directly bind the Ca\textsuperscript{2+} ion but instead coordinates a water (red sphere) at position -X. Below are aligned Paramecium and mammalian CaM sequences where the identity of X, Y, Z, -X, -Y, and –Z are in each of the 4 Ca\textsuperscript{2+}-binding loops are boxed. Differences in primary sequence of the Ca\textsuperscript{2+}-binding loops are highlighted in gray.

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Figure 1.3: Comparison of *Paramecium* (PCaM) and Mammalian (mCaM) sequences. Differences are highlighted in gray, while Ca²⁺-binding sites are highlighted in yellow.

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Figure 1.4: Superposition of experimentally observed conformations of CaM. Examples of the “closed” (1CFC.pdb orange), “semi-open” (2IX7.pdb green) and “open” (1CDM.pdb aqua and 1CLL.pdb magenta) conformations of the C-domain are aligned according to the positions of the F and G (first and fourth) helices of the domain.

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Figure 1.5: Structures of apo CaM or partially Ca\(^{2+}\)-ligated CaM interacting with a target.

A. Apo CaM (“closed” N-domain blue, “semi-open” C-domain red) bound to a peptide containing tandem IQ-motifs derived from Myosin V (green).  
B. Partially Ca\(^{2+}\)-ligated CaM (“open” N-domain blue, “semi-open” C-domain red) where its N-domain is Ca\(^{2+}\)-saturated (Ca\(^{2+}\) yellow sphere), and apo C-domain are bound to peptide derived from the small-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels (SK-channel) in green.  
C. Superposition of apo C-domains of CaM from myosin V (red and green) and SK-channel (orange and dark green).

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Figure 1.6: IQ-motif amino acid sequence conservation from 208 sequences derived from 108 human proteins, where Q at position 1 is almost invariant.

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Figure 1.7: Domain structure of Na$_2$1.2.

A. Na$_2$1.2 is composed of a single 4-domain $\alpha$-subunit, and multiple noncovalently attached $\beta$-subunits.

B. The $\alpha$-subunit of Na$_2$1.2 is composed of 4 domains, each of which is made up of 6 helices, where the 4$^{th}$ helix (green) is part of the Na$^+$ channel pore. The loop between domains III and IV, contains the inactivation gate (red sphere) which is responsible for blocking the inside of the channel shortly after it has been activated. The CaM-binding IQ-motif (purple cylinder) is located near the C-terminus of the channel.
Figure 1.8: Sequence alignment of all ten human Na\textsuperscript{+} channel isoforms. The locations of IQ-motif defining residues have been highlighted in red.

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Figure 1.9: Chemical structures of CaM-binding drugs whose binding interface with CaM has been determined structurally.
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Figure 1.10: Structures of $(\text{Ca}^{2+})_4$-CaM bound to drugs.

X-ray (Kar-2, DPD, TFP) or NMR (W-7) determined structures of $(\text{Ca}^{2+})_4$-CaM bound to drugs where the N- and C-domain of $(\text{Ca}^{2+})_4$-CaM are colored blue and red respectively, drug molecules are shown in sticks, while Ca$^{2+}$ ions are represented by yellow spheres.

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CHAPTER II
ALLOSTERIC EFFECTS OF THE ANTI-PSYCHOTIC DRUG TRIFLUOPERAZINE ON THE ENERGETICS OF Ca\(^{2+}\) BINDING BY CAM

Introduction

Calmodulin (CaM) is a small (148 a.a.), essential, and highly conserved eukaryotic protein that is required for many calcium-sensitive signal transduction pathways (Hidaka and Ishikawa, 1992; Newman, 2008). It is composed of two homologous domains (N and C). Each domain consists of a pair of EF-hands (a helix-loop-helix motif) that forms a 4-helix bundle, and binds two calcium ions cooperatively. The domains are connected by a linker that plays a regulatory role in determining calcium affinity, and permits the domains to adopt multiple relative orientations to optimize interactions with target proteins (Figure 2.1a) (Sorensen et al., 2002b; Sorensen and Shea, 1998; Zhang et al., 1995).

Although the two domains are similar in sequence and structure, the affinity of the N-domain for calcium is an order of magnitude lower than that of the C-domain (Seamon, 1980; VanScyoc et al., 2002). As the concentration of intracellular calcium increases, calcium binding to 12-residue sites in CaM triggers conformational changes, causing the pairs of helices in each 4-helix bundle to separate. This structural change associated with the transition from apo (calcium-depleted) to \((\text{Ca}^{2+})_4\)-CaM exposes hydrophobic residues that alter the affinity of CaM for target proteins (Chattopadhyaya et al., 1992; Kuboniwa et al., 1995; Meador et al., 1992; Wilson and Brunger, 2000). \((\text{Ca}^{2+})_4\)-CaM (4 bound calcium ions) is shown in Figure 2.1b.

In a CaM-target complex, the protein-protein interface is determined by the number and location of occupied calcium-binding sites of CaM, conformational change propagated from those binding sites, and the surface of the target protein. Based
on the interhelical angles adopted by the paired helices of each domain, the CaM C-domain has been categorized as adopting three distinct conformations: “closed”, “semi-open”, and “open” (Chattopadhyaya et al., 1992; Kuboniwa et al., 1995; Meador et al., 1992; Wilson and Brunger, 2000). Any of these may be adopted by free CaM (Figure 2.2), consistent with the hypothesis that changes in the distribution of pre-existing conformational states occur upon binding to Ca\(^{2+}\) or a target protein (Yap et al., 1999). In high-resolution structures, only apo CaM has been observed to adopt the “closed” (Figure 2.2B) and “semi-open” forms (Figure 2.2C), while only (Ca\(^{2+}\))\(_4\)-CaM has been observed in the “open” form (Figure 2.2D-E). Binding of a target protein stabilizes the “semi-open” or “open” conformation of CaM by burying hydrophobic surface area that would otherwise be exposed to solvent. For example, a CaMKII peptide bound to calcium-saturated “open” CaM (1CM1.pdb) buries 1226 Å\(^2\) of the surface area of CaM; of that, 990 Å\(^2\) (81%) is hydrophobic (Wall et al., 1997).

For many years, activation of CaM-regulated target proteins such as metabolic enzymes, kinases, and phosphatases was thought to occur in a strictly calcium-dependent manner, such that the extent of binding of a target protein to apo CaM was negligible, and binding to (Ca\(^{2+}\))\(_4\)-CaM always increased its calcium affinity (Cox, 1988). However, there are subclasses of CaM-regulated target proteins, including some ion channels, that contain IQ-motifs that reduce the calcium affinity of CaM (Martin and Bayley, 2004; Theoharis et al., 2008). These targets interact preferentially with apo CaM. The only high resolution structure of apo CaM interacting with an IQ-motif (2IX7.pdb) is the crystallographic observation of two apo CaM molecules bound to a peptide containing two adjacent IQ-motifs derived from Myosin V (Houdusse et al., 2006) (see Figure 2.2c). In this complex, both N-domains were “closed” whereas the C-domains were “semi-open”. Analysis of the CaM-peptide interface showed that hydrophobic residues of CaM accounted for most (71% and 75%) of the buried surface of the two CaM C-domains.
Drugs with aromatic moieties bind to CaM in a manner similar to protein targets that bury a phenylalanine or tryptophan residue in the hydrophobic pockets of CaM (Cook et al., 1994; Vandonselaar et al., 1994b; Vertessy et al., 1998b). Trifluoperazine (TFP) (Figure 2.1c) is a CaM antagonist historically used in the treatment of mental illness because of its interaction with the dopamine receptor; recently, it was implicated in the disruption of opioid tolerance (Tang et al., 2006). TFP is membrane-permeable and is commonly added to cell culture media to disrupt CaM-mediated processes (Barrington and Majewski, 1994; Chen et al., 2008; Frankfurt et al., 1995). Structures of three TFP-CaM complexes (Cook et al., 1994; Vandonselaar et al., 1994b; Vertessy et al., 1998b) have been determined crystallographically. In these, 1, 2, or 4 molecules of TFP are bound to (Ca\(^{2+}\))\(_4\)-CaM; all of them share a TFP-binding site in the C-domain but only one structure (1LIN.pdb) has a TFP-binding site in the N-domain. Superposition of these structures showed that the backbone of CaM adopts indistinguishable conformations in all of them, despite differences in the number and location of TFP molecules bound (Figure 2.3D). The relative abundance of these three ligation states of TFP bound to CaM in solution is not known. In all three complexes, the tertiary structure of CaM mimics that of (Ca\(^{2+}\))\(_4\)-CaM when bound to CaMKII, and other kinases that increase its calcium affinity (Clapperton et al., 2002; Heidorn et al., 1989; Ikura et al., 1992). The CaM-binding domain in those enzymes is a BAA (basic amphipathic alpha helical) motif.

The conflicting structural evidence regarding a preferred binding stoichiometry for TFP binding to CaM, as well as disagreement on the effects of TFP on the calcium-binding properties of CaM (Cook et al., 1994; Craven et al., 1996; Massom et al., 1990c; Tanokura and Yamada, 1985; Vandonselaar et al., 1994b; Vertessy et al., 1998b), motivated this study of the thermodynamic and structural properties of intermediate ligation states. These are critical for understanding how the highly homologous N- and C-domains of CaM exert different physiological effects on target proteins, and exploring
whether exogenous, pharmaceutical applications of TFP and related drugs truly target only the calcium-saturated form of CaM, as has been assumed.

TFP titrations of CaM monitored by $^{15}$N-HSQC spectroscopy showed that TFP saturated apo CaM at a ratio of 2:1, but saturated $(Ca^{2+})_{4}$-CaM at a ratio of 4:1. Equilibrium calcium titrations monitored by steady-state fluorescence spectroscopy demonstrated that, unlike the majority of effectors (e.g., peptides and proteins) whose binding to CaM has been examined in detail, TFP reduced the calcium affinity of CaM at low stoichiometries. Thus, thermodynamic linkage requires that TFP have a higher affinity for apo CaM than for $(Ca^{2+})_{4}$-CaM; this is similar to the preferential binding of most IQ-motifs to apo CaM.

However, the multiplicity of binding stoichiometries allows for reversal of this effect. At higher ratios (8:1) of TFP:CaM, the calcium affinity of CaM reversed, and was more favorable than that in the absence of TFP. These effects were found to be similar, but not identical, in each domain (N and C) of CaM and were compared to the effects of TFP on isolated domain fragments. On this basis, a model is proposed in which the “semi-open” conformation offers a “pocket” for binding of aromatic moieties that is unlike the FLMM pocket (Ataman et al., 2007) of the “open” conformation where aromatic side chains of many peptides are known to bind to CaM. TFP recognizes this site on the “semi-open” conformation of an apo domain that is not available when CaM adopts the “open” conformation.

The binding preference of a single TFP molecule for apo CaM is significant because, to the best of our knowledge, it is the only drug identified to reduce calcium affinity. This motivates a review of studies in which TFP antagonizes CaM-dependent cellular phenomena. The interference observed in those studies has been interpreted as arising from the effects of the drug on $(Ca^{2+})_{4}$-CaM and its regulation of enzymes or channels. However, the findings presented here show that TFP can act as an antagonist...
of apo CaM, which is critical for regulating pathways that are distinct from those modulated by (Ca\(^{2+}\))\(_4\)-CaM.

**Materials and Methods**

**Protein over-expression and purification**

IPTG-induced over-expression of CaM was performed using transformed in the *E. coli* strains BL21 DE3 or BL21 DE3-pLysS cells containing the recombinant pT7-7 vector of interest: full-length mammalian CaM\(_{1-148}\), CaM\(_{1-80}\), and CaM\(_{76-148}\) (Pedigo et al., 1992). For \(^{15}\)N-labeled proteins used in the NMR studies, *Paramecium* CaM\(_{1-148}\) (PCaM) was over-expressed in minimal medium with \(^{15}\)N-NH\(_4\)Cl (Cambridge Isotopes) as the sole nitrogen source. The proteins were then purified as described by Putkey (Putkey et al., 1985). The recombinant proteins were 97-99% pure as judged by silver-stained SDS-PAGE and reversed-phase HPLC. Protein concentrations were calculated from UV absorbance in 0.1 N NaOH, using the extinction coefficients for Phe and Tyr reported by Beaven and Holiday (Beaven and Holiday, 1952).

**Equilibrium calcium titrations monitored by intrinsic protein fluorescence**

Calcium titrations were monitored at 22 °C with a PTI-QM4 Fluorimeter (Photon Technology International, Birmingham, NJ) using bandpasses of 4 nm (for excitation) and 6 nm (for emission). CaM (CaM\(_{1-148}\), CaM\(_{1-80}\), or CaM\(_{76-148}\) at 6 µM) solutions, containing 0, 6, 12, 18, 24, or 48 µM TFP (Sigma-Aldrich, St. Louis, MO), were prepared in 50 mM HEPES, 100 mM KCl, 5 mM NTA, 0.05 mM EGTA, 1 mM MgCl\(_2\), and 5.75 nM Oregon Green (pH 7.4). The concentration of TFP was determined using an \(\varepsilon_{305.5}\) of 3540 M\(^{-1}\) cm\(^{-1}\) (Hart et al., 1983). CaM was titrated using a microburet (Micro-Metric Instrument Co., Cleveland, OH) fitted with a 250 µL Hamilton syringe (Hamilton Co., Reno, NV) containing a concentrated CaCl\(_2\) solution prepared in a matching buffer.
Binding of calcium to sites I and II in the N-domain was monitored with $\lambda_{\text{ex}}$ of 250 nm, and $\lambda_{\text{em}}$ of 280 nm, based on the intrinsic phenylalanine fluorescence. Binding to sites III and IV in the C-domain was monitored with $\lambda_{\text{ex}}$ of 277 nm, and $\lambda_{\text{em}}$ of 320 nm, based on the intrinsic tyrosine fluorescence as previously described (VanSeyoc et al., 2002). The fluorescent calcium indicator dye Oregon Green 488 BAPTA-5N (Oregon Green, 5.75 nM) (Molecular Probes, Eugene, OR) whose increase in fluorescence intensity was linearly proportional the [Calcium]$_{\text{free}}$ was used to determine the free calcium concentration at each point in the titration according to Equation 2.1, described previously by (VanSeyoc et al., 2002)

$$[\text{calcium}]_{\text{free}} = K_d \frac{f_{\text{high}} - f_{\text{low}}}{f_{\text{high}} - f_{\text{[X]}}}$$ (2.1)

where $f_{\text{high}}$ and $f_{\text{low}}$ are the highest and lowest observed fluorescence intensity signals, respectively, observed for Oregon Green during the titration. The $K_d$ of calcium binding to Oregon Green was determined previously to be 34.24 µM in 50 mM HEPES, 100 mM KCl, and 1 mM MgCl$_2$ (pH 7.4) at 22 C ($\lambda_{\text{ex}}$ of 494 nm, $\lambda_{\text{em}}$ of 521 nm) (VanSeyoc et al., 2002). Each titration was repeated at least three times.

Free energies of calcium binding to the pair of sites in each domain were determined by fitting the titrations to a model-independent two-site (Adair) function (Equation 2.2), as described previously (VanSeyoc et al., 2002),

$$\dot{Y} = \frac{K_1[X] + 2K_2[X]^2}{2(1 + K_1[X] + K_2[X]^2)}$$ (2.2)

where $[X]$ is free calcium, and the macroscopic association constant $K_1$ is the sum of intrinsic microscopic equilibrium constants ($k_1 + k_2$) for two sites: either sites I and II in the N-domain, or sites III and IV in the C-domain. This formulation allows the microscopic binding constants ($k_1$ and $k_2$) to be nonequivalent. The second macroscopic equilibrium constant $K_2$ ($k_1 k_2 \kappa_c$) is the product of the intrinsic microscopic equilibrium
constants \((k_1, k_2)\) and the cooperativity constant \((k_c)\). The parameters \(\Delta G_1\) and \(\Delta G_2\) are macroscopic binding free energies, with \(\Delta G_i = -RT \ln K_i\). The parameter \(\Delta G_2\) is thus the total free energy of saturating both calcium-binding sites in a domain.

Changes in fluorescence intensity for the calcium titrations were normalized to the highest and lowest experimentally determined signals. To account for experimental variations in the asymptotes of replicate titrations, we performed nonlinear least-squares analysis of the fluorescence intensity signal using the function \(f(X)\), as given by Equation 2.3, as described previously (VanScyoc et al., 2002).

\[
f(X) = Y_{[X]low} + \bar{Y} \cdot \text{Span}
\]

where \(\bar{Y}\) refers to the average fractional saturation as described by Equation 2.2, and \(Y_{[X]low}\) corresponds to the value of the fluorescence intensity in the absence of calcium. \(\text{Span}\) refers the normalized range \((0-1, \text{ or } -1-0)\) of the data signal. The parameter \(\text{Span}\) was negative in the case of a decreasing signal and positive in the case of an increasing signal. For monotonic titrations with well defined asymptotes, values for all parameters \((\Delta G_1, \Delta G_2, Y_{[X]low} \text{ and Span})\) were fit simultaneously using NONLIN (Johnson et al., 1981; Johnson and Frasier, 1985).

Note that a ratio of N:1 TFP:CaM does not indicate that all CaM molecules in the solution have N TFP molecules bound. Because it was not possible to determine the population distribution of TFP:CaM species in solution from an independent, experimentally observable property, the values of \(\Delta G_2\) determined in the presence of TFP are apparent values (i.e., \(\Delta G_2^{\text{app}}\)).

NONLIN provides several measures of the goodness-of-fit for the parameters that minimize the variance of each fit. These error statistics include (a) the value of the square root of variance, (b) the values of asymmetric 65% confidence intervals, (c) the systematic trends in the distribution of residuals, (d) the magnitude of the span of residuals, and (e) the absolute value of elements of the correlation matrix. From these,
best-fit values were selected after testing multiple sets of initial guesses for parameters to probe for the presence of local minima. Free energies determined from at least three replicate titrations were averaged; those values and standard deviations are reported in Table 2.1.

In some titrations, the value determined for ΔG₁ was sensitive to starting guesses; in those cases, a manual grid search was conducted to obtain the lowest square root of variance. For titrations (Figure 2.9) that exhibited alternating increasing and decreasing calcium-dependent changes in fluorescence intensity, it was necessary to fix the values of both ΔG₁, and Span, as described in the Results section. Estimates of the apparent total free energy corresponding to each transition are reported separately in Table 2.1.

NMR Spectra

15N-HSQC spectra were acquired at 25 °C on a Bruker Avance II 800 MHz US² spectrometer with a 5 mm TXI ¹H (¹⁵N/¹³C/D) probe featuring XYZ gradients. All spectra were processed in NMRPipe/NMRDraw (Delaglio et al., 1995b), while peak-picking and analysis were performed using SPARKY (Goddard and Kneller). TFP titrations of 15N-PCaM₁-148 under apo conditions were carried out in 10% D₂O, 10 mM imidazole, 100 mM KCl, 50 µM EDTA, pH 6.5 at 22°C; in the case of (Ca²⁺)₄-PCaM₁-148 TFP titration studies, 10 mM CaCl₂ was included. Starting volumes were 500 µL.

TFP titration of CaM Monitored by NMR

15N-HSQC spectra of apo ¹⁵N-PCaM₁-148 were acquired at incrementally increasing concentrations of TFP. The initial concentration of both apo- and (Ca²⁺)₄-¹⁵N-PCaM₁-148 was 617 µM. In the TFP titration series performed under apo conditions, the [TFP]_total was 0, 0.15, 0.29, 0.44, 0.61, 0.75, 0.90, 1.04, 1.21, 1.35, 1.49, 1.63, 1.79, 1.93, 2.07, and 2.37 mM (16 spectra). In the (Ca²⁺)₄-¹⁵N-PCaM₁-148 TFP titration series, the [TFP]_total was 0, 0.14, 0.28, 0.41, 0.58, 0.71, 0.85, 0.99, 1.15, 1.28, 1.41, 1.55, 1.70, 1.83, 1.96, 2.25, and 2.75 mM (17 spectra). The amide assignments for apo and (Ca²⁺)₄-¹⁵N-
PCaM₁₋₁₄₈ in the absence of TFP were reported previously (Jaren et al., 2002). To determine the change in chemical shift upon TFP binding to apo and (Ca²⁺)₄-PCaM₁₋₁₄₈, chemical-shift changes in both the ¹H and ¹⁵N dimensions were quantified using the modified Pythagorean theorem shown in Equation 2.4, described previously (Jaren et al., 2002).

\[
\Delta \text{ppm} = \sqrt{(\Delta^{1}H \text{ppm})^2 + (0.10134 \cdot \Delta^{15}N \text{ppm})^2}
\]

In this equation, \(\Delta \text{ppm}\) refers to the linear change of a specific resonance peak from its initial starting position as TFP is titrated into solution, as done previously (Jaren et al., 2002).

**Computational Modeling of TFP Binding**

*AutoDock Vina 1.0.3* (Trott and Olson, 2009) was used to simulate the binding of a single molecule of TFP to a fragment of CaM corresponding to the apo C-domain in three different tertiary conformations: “closed”, “semi-open” and “open”. Coordinates for residues 82-146 were extracted from these structures: 1DMO.pdb (apo, “closed”), 2IX7.pdb (apo, “semi-open”), 2HQW.pdb (calcium-saturated, “open”, bound to NR1C1 peptide), and 1LIN.pdb (calcium-saturated, “open”, 4 TFP molecules bound). To approximate an apo “open” structure (which has not been observed experimentally), calcium ions were removed from 2HQW and 1LIN. Each of the four protein fragments were placed in a cubic (45 Å³) search space with implicit water. The exhaustiveness parameter (number of times calculation was re-run) was 128. In Figure 2.11A, the 20 models that had the most favorable (lowest) free energies are depicted by PyMol™ v.1.2r2 (DeLano Scientific), using a gradient of green (most favorable) to white (least favorable) for the position of the unique sulfur atom in TFP. The remainder of each drug molecule is shown in light gray sticks. For each of the four CaM structures, CaM-TFP complexes calculated to have identical free energies are shown in the same color.
Results

The major aims of this study were to understand the allosteric effects of TFP on the domains of CaM by comparing the stoichiometry of TFP binding to apo and calcium-saturated domains of CaM, and determining thermodynamic effects of TFP on calcium-binding affinity.

TFP Titration of apo $^{15}$N-PCaM

The stoichiometry of TFP binding to apo PCaM was determined using $^{15}$N-HSQC spectra to examine changes in the local chemical environment of individual amide resonances as TFP was titrated into a solution of uniformly $^{15}$N-labeled PCaM. The sample had been depleted of calcium via extensive dialysis against metal chelators.

In the absence of TFP, 126 resonances were identified for PCaM$_{1-148}$. TFP addition resulted in residue-specific perturbations of almost all of these resonances. Individual peaks found to be in fast exchange were tracked over the course of the TFP titration (a subset are shown in Figure 2.4a-c). This analysis revealed that TFP saturated apo PCaM at a stoichiometry of 2:1. At saturation by TFP, resonances corresponding to the C-domain of PCaM showed a greater average degree of chemical shift perturbation ($\Delta$ppm of 0.044) than those of the N-domain ($\Delta$ppm of 0.030) (Figure 2.4c). As shown in Figure 2.4d, residues having significant backbone amide chemical shifts ($\Delta$ppm $\geq$ 0.05 a value used previously by Jaren et al., 2002 (Jaren et al., 2002)) are mapped onto corresponding residues of a high resolution solution structure of apo CaM in its extended form (Kuboniwa et al., 1995; Zhang et al., 1995).

The drug was observed to bind sequentially to the two domains of apo PCaM. The largest change in most resonances of the C-domain occurred in the range of 0 to 1 molar equivalents of TFP, whereas the largest change in most resonances of the N-domain occurred in the range between 1 and 2 molar equivalents of TFP. This indicated that TFP bound preferentially to the C-domain, despite the extensive similarity of the N-
and C-domains in sequence and structure. A distinct subset of residues (~30%) responded continuously over the range of zero to 2 molar equivalents of TFP. That group included Phe16, Ile86, Thr110, and Gly113; their response is shown in Figure 2.4b.

TFP Titration of (Ca\(^{2+}\))\(_4\)\(^{15}\)N-PCaM

For calcium-saturated PCaM, 133 resonances were resolved. Saturation with TFP was reached at a ratio of 4:1 TFP:PCaM (Figure 2.5). Over the course of the titration, 17 resonances experienced slow or intermediate exchange, with the majority (13) of these located in the C-domain of (Ca\(^{2+}\))\(_4\)-PCaM. Final chemical shift values due to TFP addition for these residues were unable to be determined, because only \(^{15}\)N-HSQC spectra were collected for this study. Therefore, it was not possible to determine \(\Delta\)ppm. Their positions are represented by the absence of a bar in Figure 2.5c.

Of the 116 resonances that were observed to be in fast exchange upon TFP addition, 98 were classified as being perturbed significantly (\(\Delta\)ppm \(\geq\) 0.05). They correlated closely with the location of TFP-binding sites observed in the crystallographic structure (1LIN.pdb) that showed 4 TFP bound to (Ca\(^{2+}\))\(_4\)-CaM (Figure 2.5d). Although the calcium-binding sites of CaM are distant from the TFP-binding sites observed in all three of the crystal structures shown in Figure 2.1, some of their resonances were perturbed also.

Part of each \(^{15}\)N-HSQC spectrum collected for the first and last point of the titration is shown overlapped in Figure 2.5a; representative titrations of individual residues are shown in Figure 2.5b. Of the 98 peaks that could be tracked throughout the titration, there were 74 that shifted monotonically; approximately half of those were in each domain of CaM (35 in the N-domain vs. 39 in the C-domain). The remaining 24 resonances exhibited a biphasic response to TFP addition. Some examples are shown in Figure 2.6a-c. Many of these residues were located at the interface between the N- and C-domains of CaM (Figure 2.6d).
This analysis of TFP-induced chemical shifts in (Ca\(^{2+}\))\(^4\)\(^{15}\)N-PCaM indicated that the TFP-binding sites were non-equivalent, and that some residues responded to TFP binding at more than one of its sites. Notably, some residues in the N-domain (e.g., Glu11 and Glu14) were among this group, even though the N-domain of calcium-saturated CaM has only been observed to have a single TFP-binding site.

**Equilibrium Calcium Titration of CaM\(_{1-148}\)**

To determine the effect of TFP on the affinity of calcium for CaM, equilibrium calcium titrations of CaM\(_{1-148}\) were conducted in the presence of discrete molar ratios of TFP:CaM ranging from zero to eight. In the absence of TFP, calcium binding to sites I and II in the N-domain of CaM\(_{1-148}\) was monitored by observing a decrease in intrinsic phenylalanine fluorescence intensity (Figure 2.7a, blue) as described in Materials and Methods. Nonlinear least squares analysis according to a model-independent two-site (Adair) function (Eq 2) established a reference total free energy (\(\Delta G_2\)) of \(-13.05 \pm 0.06\) kcal/mol (Table 2.1). An increase in intrinsic tyrosine fluorescence intensity was used to monitor calcium binding to sites III and IV in the C-domain of CaM\(_{1-148}\) (Figure 2. b, blue). In the absence of TFP, the total free energy was \(-15.00 \pm 0.06\) kcal/mol (Table 2.1).

**Effect of TFP on Calcium Binding to CaM\(_{1-148}\)**

Calcium titrations of CaM\(_{1-148}\) were conducted at molar ratios of 1:1 (green), 2:1 (red), 3:1 (black), 4:1 (cyan), and 8:1 (purple) TFP:CaM\(_{1-148}\) (Figure 2.7, 2.8). In these titrations, there is no experimental signal that reports directly on the number of TFP molecules bound to CaM or the fractional population of the possible ligation states of TFP bound to apo and calcium-saturated CaM. Therefore, each set of titrations will be referred to by the known independent variable: the ratio of the final mols of TFP to mols of CaM.
The calcium affinity of sites I and II of CaM1-148 decreased or increased depending upon the concentration of TFP (Figure 2.7a). Of the TFP:CaM1-148 ratios examined, a 1:1 ratio caused the largest decrease (2.08 kcal/mol) in calcium affinity at sites I and II (apparent free energy of -10.97 kcal/mol; green bar in inset). Calcium affinity was diminished at ratios of 2:1 (red) and 3:1 (black), but the effects were less severe than the ratio of 1:1. The smallest decrease (0.58 kcal/mol) in apparent free energy of binding at sites I and II occurred at a ratio of 4:1 TFP:CaM1-148 (-12.47 kcal/mol, turquoise). In contrast, an 8:1 ratio reversed the effect and made calcium binding to sites I and II more favorable by -0.54 kcal/mol (relative to the binding affinity observed in the absence of TFP). This effect is represented by the bar graph inset in Figure 2.7a showing values of ΔΔG2. Although small in absolute magnitude, this reversal is considered significant because the standard deviation of replicate measurements for all of these titrations ranged from 0.04 to 0.16 kcal/mol, and was much smaller than 0.54 kcal/mol.

The effects of TFP on calcium sites III and IV of CaM1-148 shared several features of its effects on sites in the N-domain. At all levels tested, TFP made calcium binding to sites III and IV of CaM1-148 less favorable. The pattern of effects (Figure 2.7b) in response to an increasing ratio of TFP:CaM1-148 was similar to that observed for sites I and II (Figure 2.7a). The bar graph inset shows that the free energy of -12.39 kcal/mol at a 1:1 TFP:CaM1-148 ratio represented the maximum change in ΔG2 of 2.61 kcal/mol. At a ratio of 2:1, the effect was slightly smaller; ratios of 3:1 and 4:1 both caused a decrease of ~1.8 kcal/mol in the calcium affinity of sites III and IV. A TFP:CaM1-148 ratio of 8:1 had the smallest effect; the apparent ΔG2 was -13.99 kcal/mol, representing a change of only 1 kcal/mol relative to the absence of TFP. In this set of titrations, the Span was positive for ratios of 0, 1:1, 2:1 TFP:CaM. At ratios above 2:1, the fluorescence intensity decreased in response to an increase in calcium. For ease of comparison of medians and
slopes of the titration, the signal for the titrations conducted at ratios of 3:1, 4:1 and 8:1 TFP:CaM are shown inverted.

The inversion of the C-domain Tyr signal may be attributed to multiple factors linked to TFP binding to CaM. One possible scenario that may alter the fluorescence properties of the C-domain Tyr signal may be TFP increasing the exposure of Tyr residues to the solvent in a Ca$^{2+}$-dependent manner resulting in quenching of the Tyr signal. Another possibility may be that dipole-dipole interactions are occur between TFP and Tyr as well as between neighboring TFP that result in quenching of the Tyr signal upon Ca$^{2+}$ addition. These possibilities are complicated due the stoichiometry of TFP binding to CaM, as each ratio of TFP:CaM carries the potential to exhibit a unique fluorescence signal. Even further complicating the nature of the Tyr signal is the heterogeneous mix of CaM bound to TFP at varied ratios at the intermediate [TFP] examined. In these cases the fluorescent signal of CaM likely is the result of an ensemble of CaM in complex with TFP at various ratios based on the 6 µM [CaM] and 1-5 µM $K_d$ of TFP previously reported (Massom et al., 1990b).

The domain-specific effects of TFP on calcium binding to CaM$_{1-148}$ were complex, and suggested that the domains had intrinsic differences in affinity for TFP, and possibly stoichiometry of TFP binding. The NMR-monitored TFP titrations of CaM$_{1-148}$ suggested that TFP might bind to an interface between domains, as well as a hydrophobic cleft in each domain. To attempt to simplify these linked binding processes, each half-CaM domain fragment (CaM$_{1-80}$ and CaM$_{76-148}$) was studied independently. Each one contains a pair of EF-hands that retain (a) cooperative calcium binding energetics, and (b) secondary and tertiary structure nearly identical to that of full-length CaM.

**Effect of TFP on Calcium Binding to CaM$_{1-80}$**

Equilibrium calcium titrations of the CaM$_{1-80}$ fragment (N-domain) were performed to examine the effect that TFP has on the calcium affinity of sites I and II in
the absence of the C-domain. Analysis of a calcium titration in the absence of TFP (Figure 2.8a, blue) showed that the total free energy of ΔG₂ of calcium binding to sites I and II was -12.91 kcal/mol (Table 2.1).

As was observed for CaM₁-₁₄₈, the effect of TFP on the apparent free energy of calcium binding to sites I and II changed in magnitude in a nonlinear manner between ratios of 1:1 and 8:1 TFP:CaM (Figure 2.8a). At a ratio of 1:1, the apparent ΔG₂ was -11.12 kcal/mol, almost 2 kcal/mol less favorable than for CaM alone. This ratio of TFP:CaM₁-₈₀ induced a smaller change than had been observed for calcium binding to sites I and II of CaM₁-₁₄₈. A TFP:CaM₁-₈₀ ratio of 2:1 reduced the calcium affinity further, such that ΔG₂ was -10.70 kcal/mol; this was the largest effect that TFP was observed to have on CaM₁-₈₀, as shown in the bar graph inset of ΔΔG₂ values in Figure 2.8a. A TFP:CaM₁-₈₀ ratio of 3:1 had a slightly greater, but nearly identical effect, to a ratio of 4:1, consistent with its effect on sites I and II in CaM₁-₁₄₈ at these ratios. The most striking difference was observed at the ratio of 8:1 TFP:CaM. Calcium binding to sites I and II remained less favorable by 0.63 kcal/mol for CaM₁-₈₀. This indicated that the C-domain was necessary for the favorable effect (-0.54 kcal/mol) of TFP on sites I and II in CaM₁-₁₄₈ that had been observed at an 8:1 TFP:CaM ratio.

**Effect of TFP on Calcium Binding to CaM₇₆-₁₄₈**

To examine the effect that TFP had on the calcium affinity of sites III and IV in the absence of the N-domain, the free energy of calcium binding to the pair of sites in CaM₇₆-₁₄₈ was determined. In the absence of TFP, ΔG₂ was determined to be -14.47 kcal/mol (Table 2.1) (Figure 2.8b). A ratio of 1:1 TFP:CaM₇₆-₁₄₈ led to a decrease in affinity (the apparent ΔG₂ was less favorable by 1.67 kcal/mol). This was smaller than the change (2.56 kcal/mol) observed for calcium binding to the same sites in CaM₁-₁₄₈. The difference of almost 1 kcal/mol is greater than the largest standard deviation (0.26 kcal/mol) observed for a single condition. The calcium-dependent change in
fluorescence intensity was positive, as it was in the absence of TFP. However, the absolute magnitude of the intensity was lower (data not shown).

At ratios of 2:1 (red) and 3:1 (black) TFP:CaM76-148, non-monotonic calcium-dependent changes in fluorescence intensity signals were observed. The first inflection was an increase in intensity, like that observed for calcium titrations conducted at a ratio of 1:1 TFP:CaM. Representative normalized data sets are shown in Figure 2.8b. The second inflection was a decrease in intensity; both the first and second transitions are shown in Figure 2.9 for the ratios of 2:1 and 3:1. Apparent free energies were estimated using piece-wise analysis of the two transitions, as described below.

As shown in Figure 2.8b, at ratios of 4:1 and 8:1 TFP:CaM76-148, a greater decrease in the calcium affinity of sites III and IV was observed than had been seen at the same ratio of TFP:CaM for these sites in CaM1-148. Presumably this relates to the absence of the N-domain and interdomain sites as locations for TFP binding. Also, as had been observed for CaM1-148, the Span observed for the calcium-dependent change in fluorescence intensity was negative. For ease of comparing medians and slopes of the titrations, the normalized titrations at these two conditions were inverted in Figure 2.8b. The slope of the calcium titration at a ratio of 8:1 TFP:CaM was notably more shallow than those at other molar ratios of TFP:CaM. This may arise from a change in cooperativity and/or may represent a mixed population of ligation states: CaM76-148 saturated with varying numbers of TFP.

**Piecewise Analysis of Biphasic Calcium Titrations of CaM76-148**

The calcium titrations conducted at ratios of 2:1 and 3:1 TFP:CaM (Figure 2.9) are comprised of two phases with a sharp transition between them. Because the asymptotes for each phase were not well defined, it was not possible to determine an
independent maximum for the upward-trending signal, or minimum for the downward-trending signal by fitting the data to Equation 2.3.

Instead, to estimate the apparent free energy of calcium binding, the fluorescence signal was normalized to the maximal observed intensity, and the value of \( \text{Span} \) was set equal to 1.0. Using that approach, the apparent free energies of Ca\(^{2+}\) binding were -12.78 kcal/mol (at ratio of 2:1) and -13.02 kcal/mol at a ratio of 3:1. The corresponding estimates of \( \Delta \Delta G \) for calcium binding to sites III and IV are shown in the solid bars in the inset of Figure 2.8b. These values were similar to what had been observed at 1:1 TFP:CaM. (The maximal fluorescence intensity for the increasing phase must be at least as high the value observed, but could be higher. If it were under-estimated, this approach would also under-estimate the effect of TFP by estimating a median calcium concentration lower than the actual value and therefore closer to the value in the absence of TFP.)

A similar approach was applied to analysis of the decreasing signal recorded at ratios of 2:1 (red) and 3:1 (black) TFP:CaM\(\text{76-148}\). The net downward deflection was fixed to be as large as that for the increasing phase. Using this approach, the apparent free energies were -9.55 (a ratio of 2:1) and -10.65 kcal/mol (at 3:1). The dashed bars shown in the inset of Figure 2.8b represent the value of \( \Delta \Delta G \) values obtained assuming that the net change in affinity is equal to the effect represented by the decreasing fluorescent intensity. If the value of the \( \text{Span} \) of this transition were not as large as the increasing phase, this assumption would err on the side of reporting a weaker calcium-binding affinity (i.e., a median concentration for the titration that would be higher than the actual one).

The presence of multi-phasic fluorescence signals, changes in direction of calcium-depending response of steady-state fluorescence, and differing free energies of Ca\(^{2+}\) binding as a function of [TFP] provide strong evidence for the existence of populated intermediates that have different fluorescence signals. All estimates of calcium...
binding affinity in the presence of TFP are denoted as apparent free energies to draw attention to the complexity of analysis of multiple, partial ligation states.

**Discussion**

The studies presented here address the nature of TFP binding to apo and calcium-saturated CaM, and the allosteric effects of TFP on calcium binding to the non-equivalent domains of CaM. Their combined effects on conformational switching of this essential regulatory protein are of interest because of the ubiquitous practice of applying drugs to cell cultures to disrupt CaM-mediated pathways of calcium-dependent signal transduction.

**Two TFP molecules bind to apo CaM**

Although some reports have suggested that TFP binds to apo CaM (Matsushima et al., 2000) (Matsushima et al., 2007) most have not supported this premise (Massom et al., 1990a; Massom et al., 1991; Tanokura and Yamada, 1985). However, stoichiometric TFP titrations of apo CaM1-148 (460 µM) monitored by NMR showed it to be saturated by two TFP molecules, with preferential binding to the C-domain; studies of calcium binding to TFP-saturated apo CaM demonstrated that TFP reduced calcium affinity. This was similar to the effect of peptides derived from individual protein targets, such as those containing IQ-motifs, that bind preferentially to apo CaM (Martin and Bayley, 2004; Theoharis et al., 2008). They also have the thermodynamic property of decreasing the calcium-binding affinity of the EF-hand sites of CaM. However, to our knowledge, this is the first time such behavior has been reported for a drug binding to CaM.

In a unique high resolution study of apo CaM bound to a peptide representing an IQ-motif (from myosin V (Houdusse et al., 2006)), the C-domain of CaM adopted the “semi-open” tertiary conformation (Figure 2.2). The interface between the peptide and the C-domain buries more surface area than does the peptide interaction with N-domain which is in the “closed” conformation. In other structures of CaM:peptide complexes,
the C-domain has been observed to adopt multiple conformations, depending on the nature and number of ligand(s) (calcium and/or protein) bound (Figure 2.2). Solution studies of apo CaM alone have shown that the C-domain has a lower fraction of ordered secondary structure and is less thermodynamically stable than the N-domain (Masino et al., 2000; Sorensen and Shea, 1998). These findings indicate that, under apo conditions, fluctuation between a “closed” and “semi-open” conformation is more energetically favorable for the C-domain than for the N-domain, consistent with TFP binding preferentially to the C-domain. It is also possible for either apo N- or C-domain to sample the “open” conformation. However, favorable tertiary constraints within each domain provide an energetic barrier for this transition. Thus, the population of this conformation of apo CaM will be low.

Small-angle x-ray scattering (SAXS) data indicate that TFP binds to both apo and \((\text{Ca}^{2+})_4\text{-CaM}\). However, the radius of gyration of each ensemble is different (\((\text{Ca}^{2+}\text{-CaM}:\text{TFP} = 20.5 \pm 0.3 \text{ Å, versus apo CaM 20.5 \pm 0.3 Å ) (Matsushima et al., 2000; Matsushima et al., 2007)\), suggesting that the dominant tertiary structure and stoichiometry of TFP binding are not identical for both apo and \((\text{Ca}^{2+})_4\text{-CaM}\). Because both of these differ from apo CaM alone (radius of gyration = 21.5 \pm 0.3 \text{ Å}) which preferentially samples the “closed” conformation, we hypothesize that TFP binds preferentially to the “semi-open” conformation of the 4-helix bundle domains of apo CaM. At any specific level of TFP, the fraction of apo CaM having TFP bound to a “semi-open” domain will be determined by the energy of isomerization reactions needed for conformational rearrangements, the energy of TFP binding to CaM and concentration of TFP.

Additional evidence that TFP recognizes different sites within apo and \((\text{Ca}^{2+})_4\text{-CaM}\) comes from comparing \(^{15}\text{N}\)-HSQC spectra of TFP-saturated apo and \((\text{Ca}^{2+})_4\text{-CaM} (\text{Figure 2.10})\). The spectra differ at most positions, meaning that the local chemical environments of most amide bonds in the CaM backbone are dissimilar. Note that the
changes observed in this study appear to be considerably larger than those observed by Matsushima et al. (Matsushima et al., 2007); although a direct comparison cannot be made because chemical shifts due to TFP binding were not quantified in that study.

It would be attractive to determine a high-resolution structural model of TFP bound to apo CaM. This would allow us to determine residues participating in the drug-protein interfaces and interhelical angles of each 4-helix bundle domain. However, it is beyond the scope of this study. Instead, a computational approach (AutoDock Vina) (Trott and Olson, 2010) was used to identify an ensemble of preferred binding sites for TFP on the apo C-domain of CaM in a “closed”, “semi-open” and “open” conformation (Figure 2.11a).

For each tertiary structure, an overlay of the 20 models that were most favorable energetically are shown. The single sulfur atom in each computationally docked TFP molecule is shown as an enlarged sphere. The color of that sphere corresponds to the predicted free energy of binding (darkest green corresponds to most favorable positions); the range of predicted energies of the models is shown in the bar below. The docking results of AutoDock Vina have been validated in control experiments in which a ligand was extracted from a known complex and then successfully re-docked in a similar orientation by AutoDock Vina as was observed experimentally (Trott and Olson, 2010). AutoDock Vina was also validated for use in TFP binding to CaM, by extracting TFP from the x-ray structure of (Ca²⁺₄)-CaM bound to 1 TFP and then allowing AutoDock Vina to determine where the extracted TFP molecule would bind. Upon completion of docking simulation, it was observed that the predicted TFP binding site was less than Å away from the experimentally determined TFP binding site (Figure 2.11a).

For the “closed” C-domain (based on 1DMO), the preferred binding locations of TFP were on the exterior surface near the first and second helix of the domain, and near the highly acidic calcium-binding sites III and IV; predicted free energies for this set of models ranged from -6 to -5.1 kcal/mol. For the “semi-open” C-domain (based on 2IX7),
there were two preferred binding locations: one was in the shallow cleft between the pairs of helices in the 4-helix bundle and the other was near site III. Free energies of TFP binding to the “semi-open” domain ranged from -6.8 to -6.0 kcal/mol.

The “open” C-domain has only been observed in structures of calcium-saturated CaM. However, it may be sampled at a very low frequency by apo CaM. Therefore, TFP binding to an apo “open” C-domain was modeled by removing the calcium ions from two “open” tertiary structures of calcium-saturated CaM that differed in their side chain orientations. One set of coordinates was taken from a structure of (Ca\(^{2+}\))\(_4\)-CaM bound to a peptide (2HQW) and another was from a structure of (Ca\(^{2+}\))\(_4\)-CaM bound to 4 TFP (1LIN). In both cases, the most favorable binding site for TFP was located deep in the hydrophobic pocket between the pairs of helices.

The “open” conformation is the only one for which there are high resolution structures showing the location(s) of TFP bound to the C-domain. The position of TFP at site A of 1LIN.pdb (see Figure 2.3) is shown in magenta for comparison to the models. This is the site that is occupied in all three of the crystallographic structures of TFP bound to (Ca\(^{2+}\))\(_4\)-CaM. For the 20 models having the lowest energy, the sulfur atom in each computationally docked TFP molecule was within 1 Å of the location where it had been observed experimentally in 1LIN, suggesting that the interhelical angles and surface residues are necessary and sufficient to provide a binding site for TFP in the absence of calcium.

This prediction of sites of TFP binding to the “closed” and “semi-open” conformations of a single domain does not explore additional possible sites that might exist in full-length CaM in pockets created by the juxtaposition of the two domains. However, the models suggest that TFP binding to the “semi-open” form has the potential of interfering with calcium binding. Note that all of these calculations have CaM account only for TFP binding, and not for the energy required for conformational isomerization. That barrier exists, in part, because the “open” form of each 4-helix bundle domain of
(Ca\(^{2+}\))\(_4\)-CaM exposes more hydrophobic surface to solvent than the “closed” or “semi-open” conformations (Houdusse et al., 2006; Kuboniwa et al., 1995).

**Four TFP bind to (Ca\(^{2+}\))\(_4\)-CaM**

Residue-specific titrations monitored by NMR (Figure 2.5) showed the stoichiometry of TFP binding to (Ca\(^{2+}\))\(_4\)-CaM was 4:1 in agreement with a SAXS study (Matsushima et al., 2000), an HPLC study (Massom et al., 1991)(Massom et al., 1990c), and one of the three crystallographic structures 1LIN.pdb (Vandonselaar et al., 1994a). The stoichiometry of 4 contrasts with two other crystallographic structures of CaM:TFP (1CTR.pdb, 1A29.pdb, see Figure 2.3), and a recent computational study that concluded that TFP binds only to the C-domain of (Ca\(^{2+}\))\(_4\)-CaM (Kovesi et al., 2008). Although NMR is a powerful method for precisely determining the stoichiometry of binding, the observed spectral changes report on changes in chemical environment that may arise from local binding, or a global conformational change. Thus, it is challenging to determine the location of individual binding sites when multiple ligands bind. It was evident that TFP binding perturbed amide resonances in both domains of (Ca\(^{2+}\))\(_4\)-CaM, consistent with TFP binding to each, as depicted in the superposition shown in Figure 2.3d (sites A, B, C, and D). The majority of residues in slow exchange mapped to the C-domain, indicating that this domain of (Ca\(^{2+}\))\(_4\)-CaM contained the site with highest affinity for TFP. This observation, coupled with the locations of residues in both domains that undergo fast exchange, indicates that a hierarchy of 4 TFP-binding sites is present in (Ca\(^{2+}\))\(_4\)-CaM. Interpreted according to the positions of TFP in 1LIN.pdb, it appeared that two TFP binding sites with different affinities exist in the C-domain, that a third low-affinity site is present in the N-domain, and that a fourth site (also of low affinity) bridges the two domains (Figure 2.3). Although a hierarchy of TFP binding sites was identified in this work, it was not possible to distinguish a preferential order of binding order to the low-affinity sites.
Interdomain Interactions

For any protein binding 4 ligands, there are 5 macroscopic ligation states (0, 1, 2, 3, 4 ligand:protein). Thus, in principle, it might be possible to titrate (Ca$^{2+}$)$_4$-CaM with TFP and monitor 4 independent transitions corresponding to individual TFP-binding sites as has been done for calcium binding to 4 sites in CaM (Jaren et al., 2002), (Martin et al., 1986; Starovasnik et al., 1992). For residues of CaM affected by a single TFP molecule, a monotonic transition between a “free” and “bound” state might be observed. For each residue that experienced only those two chemical environments, a stoichiometric titration would show (a) a linear transition, if in fast exchange, or (b) reciprocal changes in intensity for pairs of peaks (one diminishing, one increasing), if in slow exchange. Similarly, if there were 4 sites with identical affinity, all residues affected by TFP binding would titrate identically over the range of 0 to 4 equivalents of TFP added.

However, it is also possible that intermediate ligation states adopt distinct conformers with unique biophysical properties. A residue that responds to TFP binding at multiple sites has the potential to experience a different environment in each, and therefore show a nonlinear response to TFP binding as monitored by NMR or fluorescence. In HSQC spectra, this was observed for a subset of residues (Figure 2.6b) that experienced at least three chemical environments and sampled at least one intermediate conformation. These residues responding to multiple TFP-binding sites are most likely located at the interface between the N- and C-domains, or between TFP binding sites within a single domain (Figure 2.6d). The crystallographic structure of 4 TFP molecules bound to (Ca$^{2+}$)$_4$-CaM shows that, at their closest approach, TFP binding sites A, B, and C are in close proximity (~4 Å) to each other, while the TFP molecule bound at site D is ~9 Å away from site C (Figure 2.3d). This constellation would allow for unique chemical environments to be sampled as TFP sequentially fills its 4 binding sites, and would lead to changes in the chemical environment of adjacent TFP binding sites.
This type of biphasic response of CaM resonances was observed previously in calcium titrations monitored by $^{15}$N-HSQC that showed that several residues within the linker region between domains of CaM experienced three distinct chemical environments (Jaren et al., 2002). These residues were in slow exchange between 0 and 2 equivalents of calcium (i.e., during saturation of the C-domain), and were in fast exchange between 2 and 4 (i.e., during saturation of the N-domain). Similar biphasic responses were observed in drug titrations of cardiac Troponin C (a related calcium-binding EF-hand protein) (Kleerekoper et al., 1998). Nonlinear peak shifts due to the significant population of an intermediate state have also been observed for other proteins such as the phosphorylated kinase-inducible activation domain (pKID) of the transcription factor camp response element-binding protein (CREB) binding to subdomain of CREB Binding Protien (CBP) termed KIX (Sugase et al., 2007).

Further evidence for domain interactions was provided by the behavior of residues Glu11 and Glu14, located in the first helix of the N-domain of $(\text{Ca}^{2+})_4$-CaM. It was expected that these residues would respond to TFP binding to the N-domain itself based on their location and proximity to a target peptide or drug observed in 17 $(\text{Ca}^{2+})_4$-CaM-peptide or drug complexes (Ataman et al., 2007). In those, both Glu11 and Glu14 were within 4.5 Å of the peptide or drug interacting with $(\text{Ca}^{2+})_4$-CaM. Over the full range of 0 to 4 TFP molecules binding to $(\text{Ca}^{2+})_4$-CaM, these residues exhibited a biphasic response to TFP binding (Figure 2.6d), initially increasing with a maximum at 2 TFP:CaM. As observed in TFP-CaM structures shown in Figure 2.2, these residues are located between TFP-binding sites in the N- and C-domain of $(\text{Ca}^{2+})_4$-CaM which positions them to respond to saturation of all TFP binding sites. The highest affinity TFP-binding site in $(\text{Ca}^{2+})_4$-CaM is in the C-domain, assumed to be TFP-site A (Figure 2.3d, Figure 2.1a). Glu11 and Glu14 are also < 4 Å from TFP-site B which is comprised primarily of C-domain residues. This hints at the possibility that the response of Glu11 and Glu14 from 0 to 2 relates to occupancy of sites A and B, and the response
from 2 to 4 indicates occupancy of sites C and D. But, other models of hierarchical binding are also consistent with the titrations.

**Effects of TFP on the Calcium Affinity of CaM**

Most proteins known to be regulated by CaM contain a BAA motif (basic amphipathic alpha-helix) that binds to CaM, and causes an increase in the calcium affinity of CaM. Thermodynamic linkage requires that the BAA motif bind to (Ca$^{2+}$)$_4$-CaM with higher affinity than it binds to apo CaM in order to increase the Ca$^{2+}$-binding affinity of CaM. A subset of CaM-target interactions—typically those between CaM and targets bearing IQ-motifs—lead to a reduction in the calcium affinity of CaM, due to the higher affinity of these targets for apo CaM (Bahler and Rhoads, 2002; Cui et al., 2003; Martin and Bayley, 2004; Mori et al., 2003; Putkey et al., 2003). A peptide (Na$_v$1.2 IQp) representing the IQ-motif from the Voltage-Dependent Sodium Channel Na$_v$1.2 has been shown to have a negligible effect on calcium binding to the N-domain of CaM, while significantly lowering calcium-binding affinity of the C-domain (Theoharis et al., 2008). To satisfy thermodynamic constraints, an IQ-motif with this property has a higher affinity for the apo C-domain than the (Ca$^{2+}$)$_2$-C-domain.

Like an IQ-motif, at most concentrations studied in this study, TFP diminished the calcium-binding affinity of both domains of CaM. TFP binds with lower absolute affinity to apo CaM (Massom et al., 1990a) than does Na$_v$1.2 IQp (Theoharis et al., 2008). However, like Na$_v$1.2 IQp, it has a higher relative affinity for the C-domain of apo CaM (Figure 2.5) than for the N-domain. This is consistent with the observation that the C-domain exhibited a larger TFP-induced decrease in calcium affinity than the N-domain (Figure 2. b).

If (Ca$^{2+}$)$_4$-CaM had not bound TFP at all, or bound TFP more weakly than apo CaM but at the same sites, then the major allosteric effect of TFP would be to decrease calcium affinity by binding preferentially to apo CaM. The magnitude of the TFP effect
would increase monotonically until CaM was saturated with TFP. In this way, its effect on CaM would be analogous to that of 2,3-BPG reducing oxygen binding affinity by binding preferentially to deoxy hemoglobin (Ackers, 1979; Arnone, 1972; Benesch and Benesch, 1967). Mammalian adaptation to high altitudes depends on this mechanism of promoting oxygen release from hemoglobin under the low-oxygen conditions of human tissues (Martin et al., 1975). However, this mechanism of negative allosteric regulation would not explain how the effect of TFP on calcium binding reversed direction (Figures 2.7 and 2.8) when the ratio of TFP:CaM increased from 1 to 8. Several possible explanations were considered.

The reversal of the initially negative allosteric effect of TFP on calcium binding by CaM might be explained if higher levels of total added TFP did not actually represent higher soluble concentrations. For example, the effective concentration of TFP might drop if it formed micelles that would compete with CaM as a sink for additional TFP. However, that micelle-sink model contradicts several observations in this study (Caetano et al., 2003; Caetano and Tabak, 2000). For example, a prediction of that model is that increasing TFP would ameliorate the initially negative effect until all of it was drawn into micelles and the calcium-binding affinity of CaM returned to that observed in the absence of TFP. Instead, an increase in TFP ultimately increased the calcium-binding affinity of N-domain of CaM, rather than returning it to the values in the absence of TFP. An additional contrary observation was that the direction of calcium-dependent changes in fluorescence signal changed over the course of the TFP titration: monotonically increasing in the absence of TFP, and monotonically decreasing at the 8:1 ratio, showing that TFP was still associated with CaM at the 8:1 ratio. Finally, in all CaM-TFP samples (including the millimolar CaM samples used in NMR studies), there was no visual evidence of turbidity that would indicate the formation of a significant population of micelles.
Another possibility is that the mechanism of allosteric reversal depends on TFP changing the relative populations of “closed”, “semi-open”, and “open” tertiary structures of apo CaM. Given that both calcium and TFP have micromolar affinity for CaM, and that two calcium ions are needed to drive each domain to adopt the “open” state in the absence of TFP, it may be that 2 TFP molecules are needed to drive the conformational change of opening each domain. The NMR-monitored TFP titrations showed only two TFP bound to apo CaM1-148, but they did so sequentially with higher affinity for the C-domain. In contrast, multiple TFP may bind cooperatively to “open” calcium-saturated domains. Thus, as the level of TFP increases above 1:1, there is a chance for more than 1 to bind to a single domain. TFP may promote the “open” conformation of apo CaM in a manner similar to that of BAA-motif peptides that bind with high affinity to the hydrophobic surfaces exposed upon Ca\(^{2+}\) binding (Ataman et al., 2007; Meador et al., 1993). That would then increase calcium-binding affinity. This biphasic binding shares some features with that observed for an IQ-motif from neuromodulin studied by Persechini and colleagues (Black et al., 2006).

Comparison of the calcium-binding free energies for sites I and II in CaM1-148 and CaM1-80 at a ratio of 8:1 TFP:CaM revealed that the calcium affinity does not become more favorable than that in the absence of TFP. This difference may result from the loss of a TFP-binding site that bridges the N- and C-domains of CaM1-148 with contributions from both (Figure 2.12). Calcium titrations of CaM76-148 at ratios of 2:1 and 3:1 TFP:CaM76-148 resulted in multiphasic fluorescence signals attributed to a mix of different TFP-bound CaM76-148, complexes that each have unique properties. This is consistent with kinetic studies of calcium release from (Ca\(^{2+}\))4-CaM mixed with TFP (Martin et al., 1985). The different species in solution are sufficiently populated at ratios of 2:1 and 3:1 TFP:CaM76-148 to exhibit multiple signals, but are not abundant at the ratios of 1:1, 4:1, and 8:1. For the ratios of 2:1 and 3:1, the maximum value of raw fluorescent intensity was approximately a third of that observed for the calcium titrations conducted at 1:1 and
4:1 ratios of TFP:CaM_{76-148}. All of these observations are consistent with a mechanism of drug action that specifies that the apo C-domain has a higher affinity for TFP than does the apo N-domain, and that TFP binding interferes with calcium binding by inhibiting the conformational switch from “semi-open” to “open” conformation.

Unlike Ca^{2+}-titration curves at 1, 2, 3, or 4:1 TFP:CaM_{76-148}, a significant decrease in cooperativity of Ca^{2+} binding to CaM_{76-148} was observed at a ratio of 8:1 (Figure 2.8). This decrease in cooperativity may be a result of multiple factors such as TFP uncoupling Ca^{2+}-binding sites III and IV such that one site has a more favorable Ca^{2+}-binding affinity than the other and/or a heterogeneous mix of TFP bound CaM_{76-148} species in solution.

These interpretations are summarized in a simplified isomerization and binding model shown in Figure 2.11b. Distinct tertiary conformations of CaM provide unique TFP binding interfaces with different relative affinities for TFP. A “closed” domain of apo CaM is depicted as having no interaction with TFP. A “semi-open” apo domain has hydrophobic residues located sufficiently near the perimeter of the canonical target binding pocket (blue patches) to interact with TFP. With TFP bound at these positions, the domain responds as it does when interacting with the Ile-Gln dipeptide found in a canonical IQ-motif of ion channels. Calcium binding is sufficient to switch the tertiary structure from the “semi-open” to “open” conformation, exposing hydrophobic residues (indicated by blue patches) located deep in the hydrophobic cleft. The “open” conformation may also be sampled by apo CaM, allowing TFP at high concentrations to bind there. In either case, TFP bound at the blue sites may have an allosteric effect on calcium affinity more like that of a BAA motif peptide which consistently buries an aromatic group in the FLMM pocket of the C-domain (Ataman et al., 2007; Yamniuk and Vogel, 2004).
Summary

This study of the allosteric regulation of calcium binding to CaM by TFP demonstrates that there is considerable complexity in the interactions between these two ligands of CaM. TFP interacts with distinct interfaces available in the dominant tertiary conformations of apo and (Ca$^{2+}$)$_4$-CaM. These are likely to be primarily a “semi-open” state in the ensemble of conformations that are energetically sampled by apo CaM, and the “open” conformation for (Ca$^{2+}$)$_4$-CaM. TFP lowered the calcium binding affinity of sites III and IV in the C-domain of CaM$_{1-148}$ more than sites I and II in the N-domain, indicating that the apo C-domain has a higher affinity for TFP. TFP titrations monitored by NMR showed differences in the stoichiometry and location of binding of TFP to apo and (Ca$^{2+}$)$_4$-CaM; this is the driving force behind the non-monotonic allosteric effect of TFP on the calcium affinity of CaM.

This analysis suggests that despite the significant number of calcium-dependent processes regulated by (Ca$^{2+}$)$_4$-CaM, it is equally important to consider interactions of target proteins with apo CaM when testing drugs similar to TFP. This broadens the interpretation of a widely used approach of bathing cells in vitro in a TFP-containing solution with the goal of disrupting pathways that are regulated by (Ca$^{2+}$)$_4$-CaM.

Like other anti-psychotic drugs, TFP can cause the debilitating side-effect of tardive dyskinesia (Lahti et al., 1993). The etiology of this is not completely understood, but believed to originate from hypersensitive dopamine receptors, which have been shown to be regulated by CaM under both apo and calcium-saturating conditions (Liu et al., 2007; Woods et al., 2008). Apo and (Ca$^{2+}$)$_4$-CaM have also been shown to regulate numerous ion channels responsible for the propagation of nerve impulses (Ataman et al., 2007; Schumacher et al., 2004; Shah et al., 2006; van Petegem et al., 2005). It is possible that TFP alters physiological processes by disrupting interactions of apo CaM with these receptors.
Given the large number of signaling pathways that CaM regulates, CaM itself is not a promising target for drug design. However, an interface between CaM and a particular target may offer more selectivity. This study of allostERIC interactions between calcium and TFP suggests that interactions between channels and the “semi-open” form of CaM may be an especially attractive target for future drug testing.
Table 2.1 TFP Effects on Calcium Binding to CaM

<table>
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<th>[TFP]</th>
<th>Ratio</th>
<th>ΔG&lt;sup&gt;2&lt;/sup&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ΔΔG&lt;sup&gt;2&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ΔG&lt;sup&gt;2&lt;/sup&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ΔΔG&lt;sup&gt;2&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>-</td>
<td>-</td>
<td>-13.05 ± 0.06</td>
<td>-</td>
<td>-15.00 ± 0.06</td>
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<td>-12.39 ± 0.07</td>
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<td>0.58</td>
<td>-13.07 ± 0.09</td>
<td>1.83</td>
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<th>[TFP]</th>
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<sup>a</sup>ΔG<sup>2</sup> (kcal/mol) represents apparent total free energy indicated TFP]<sub>total</sub>/<CaM]<sub>total</sub> ratio

<sup>b</sup>ΔΔG<sup>2</sup> = ΔG<sup>2</sup><sup>app</sup> (TFP Added) - ΔG<sup>2</sup> (TFP Absent)

<sup>c</sup>Values determined from initial phase of increasing fluorescent signal.

<sup>d</sup>Values determined from decreasing fluorescent signal at higher [calcium].
Figure 2.1: Structures of apo CaM, (Ca^{2+})_4-CaM, and Trifluoperazine.
A: Superposition of solution structure models of CaM (1CFC.pdb) determined by NMR. Alignment minimized the difference between models with respect to the N-domain (residues 1-75 in blue), illustrating flexibility of interdomain linker (resides 76-80 in black) and range of positions adopted by C-domain (residues 81-148 in red). A single model is highlighted to reveal the tertiary structures of the apo N- and C-domains B: (Ca^{2+})_4-CaM structure determined crystallographically (1CLL.PDB); backbone colored as in panel A. Ca^{2+} ions (yellow) are bound at sites I and II in the N-domain, and at sites III and IV in the C-domain. C: Chemical structure of the antipsychotic drug Trifluoperazine (TFP; green), with sulfur atom in yellow and fluorine atoms in light blue.
Figure 2.2: Superposition of 3 Tertiary structures of the C-domain of CaM. Examples of the “closed” (1CFC.pdb–orange), “semi-open” (2IX7.pdb–green) and “open” (1CDM.pdb–aqua and 1CLL.pdb–magenta) conformations of the C-domain of CaM are aligned according to the positions of the F and G (second and third) helices of the domain. Structures of the corresponding full-length CaM is shown below. Users/nmr_mike/Thesis/Chapter_II/Figure2_2.jpg
Figure 2.3: Structures of (Ca\textsuperscript{2+})\textsubscript{4}-CaM bound to TFP. Individual panels show crystallographically derived structures of TFP:(Ca\textsuperscript{2+})\textsubscript{4}-CaM complexes, with drug:protein ratios of 1:1 (1CTR.pdb), 2:1 (1A29.pdb), and 4:1 (1LIN.pdb), as well as a structural superposition of these three structures, with the TFP-binding sites labeled A (green), B (magenta), C (brown) and D (orange). TFP-binding sites A and B are located in the C-domain (backbone red), site C bridges the two domains, and site D is located in the N-domain (backbone blue).

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Figure 2.4: $^{15}$N-HSQC-monitored TFP titration of uniformly $^{15}$N-labeled apo PCaM
A: Comparison of subset of $^{15}$N-HSQC spectra for apo PCaM (black) and TFP-saturated apo PCaM (red); arrows indicate change in resonance positions over the course of the TFP titration. B: Normalized TFP-induced chemical shifts of individual representative residues of apo CaM. C: Bar graph of net chemical shift per residue caused by TFP saturation of CaM. D: Location of each apo CaM residue whose chemical shift was perturbed > 0.05 ppm by TFP saturation (white spheres); backbone modeled as that of apo CaM (1DMO.pdb). Solution conditions: 10% D$_2$O, 10 mM imidazole, 100 mM KCl, 50 µM EDTA, 5mM, pH 6.5 at 22°C.

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Figure 2.5: $^{15}$N-HSQC-monitored TFP titration of uniformly $^{15}$N-labeled (Ca$^{2+}$)$_4$-CaM. A: Comparison of (Ca$^{2+}$)$_4$-CaM (black) and TFP-saturated (Ca$^{2+}$)$_4$-CaM (red) $^{15}$N-HSQC spectra, arrows indicate change in resonance positions over the course of the TFP titration. B: Normalized TFP-induced chemical shifts of individual representative residues of (Ca$^{2+}$)$_4$-CaM. C: Bar graph of net chemical shift per residue caused by TFP saturation of CaM. D: Location of each (Ca$^{2+}$)$_4$-CaM residue whose chemical shift was perturbed > 0.05 ppm by TFP saturation (white spheres); backbone modeled according to the structure of TFP bound to (Ca$^{2+}$)$_4$-CaM at a 4:1 ratio (1LIN.pdb). Solution conditions: 10% D$_2$O, 10 mM imidazole, 100 mM KCl, 50 µM EDTA, 5 mM CaCl$_2$, pH 6.5 at 22°C. Users/nmr_mike/Thesis/Chapter_II/Figure2_5.jpg
Figure 2.6: Multiple chemical environments observed upon TFP titration of (Ca$^{2+}$)$_4$-CaM
A: $^{15}$N-HSQC spectrum of uniformly $^{15}$N labeled (Ca$^{2+}$)$_4$-CaM titrated with TFP, where
arrows represent the movement of each resonance from its initial position.  
B: Schematic diagram of quantitative criterion for classification of biphasic chemical shift.  
C: Locations of select residues that underwent a biphasic response upon TFP addition, 
mapped onto the structure of TFP bound to (Ca$^{2+}$)$_4$-CaM at a 4:1 ratio (1LIN.pdb).  
D: Normalized chemical shift plots for select individual residues deemed to undergo a 
biphasic response upon TFP titration of (Ca$^{2+}$)$_4$-CaM.  
Solution conditions: 10% D$_2$O, 10 mM imidazole, 100 mM KCl, 50 µM EDTA, 5mM CaCl$_2$, pH 6.5 at 22°C.  
Users/nmr_mike/Thesis/Chapter_II/Figure2_6.jpg
Equilibrium calcium titrations of CaM (6 µM) were conducted in the presence of 0 (blue), 6 (green, 1:1), 12 (red, 2:1), 18 (black, 3:1), 24 (cyan, 4:1), or 48 µM (purple, 8:1 TFP:CaM) TFP, and were monitored using the intrinsic fluorescence of CaM. A: phenylalanine fluorescence (250 nm<sub>ex</sub> and 280 nm<sub>em</sub>). B: tyrosine fluorescence (277 nm<sub>ex</sub> and 320 nm<sub>em</sub>). In B, for 3:1, 4:1 and 8:1 TFP:CaM, the raw signal decreased; it is shown inverted to facilitate comparisons. Solid curves were simulated according to Equation 2.3 and free energies in Table 1; bar graph insets represent ΔΔG<sub>2</sub> values in Table 1.

Solution conditions: 50 mM HEPES, 100 mM KCl, 5 mM KCl, 0.05 mM EGTA, 1 mM MgCl<sub>2</sub>, and 6 nM Oregon Green (pH 7.4) at 22°C.
Figure 2.8: Effect of TFP on the calcium binding affinity of CaM_{1-80} and CaM_{76-148}
Equilibrium calcium titrations of CaM (6 µM) were conducted in the presence of 0 (blue), 6 (green, 1:1), 12 (red, 2:1), 18 (black, 3:1), 24 (cyan, 4:1), or 48 µM (purple, 8:1 TFP:CaM) TFP, and were monitored using the intrinsic fluorescence of CaM. A: phenylalanine fluorescence (250 nm_{ex} and 280 nm_{em}). B: tyrosine fluorescence (277 nm_{ex} and 320 nm_{em}). In B, for 2:1 and 3:1 TFP:CaM, only the first transition is shown. In B, for 4:1 and 8:1 TFP:CaM, the raw signal decreased; it is shown inverted to facilitate comparisons. Solid curves were simulated according to Equation 2.3 and free energies in Table 1; bar graph insets represent ΔΔG_2 values in Table 1. Solution conditions were 50 mM HEPES, 100 mM KCl, 5 mM KCl, 0.05 mM EGTA, 1 mM MgCl_2, and 6 nM Oregon Green (pH 7.4) at 22°C.

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Figure 2.9: Biphasic fluorescence response to calcium binding at intermediate TFP
Effect of TFP on calcium titration of CaM$_{76-148}$ at 12 µM (red, 2:1 TFP:CaM) and 18 µM
(black, 3:1 TFP:CaM) monitored using the intrinsic tyrosine fluorescence of CaM (277
nm$_{ex}$ and 320 nm$_{em}$). Evidence for multiple species, and piecewise analysis described in
Results. Solid curves for calcium-dependent increase in fluorescence intensity were
simulated according to Equation 2.3 and free energies in Table 1; dashed curves
correspond to decrease in fluorescence intensity.

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Figure 2.10: Comparison of TFP-saturated apo CaN \((\text{Ca}^{2+})_4\text{-CaM}_{1-148}\) Overlay of $^{15}\text{N}$-HSQC spectra of apo CaM (black, 2 TFP:CaM) and \((\text{Ca}^{2+})_4\text{-CaM}\) (red, 4 TFP:CaM). Few peaks overlap, indicating significantly different chemical environments for backbone amides in the structures of apo and \((\text{Ca}^{2+})_4\text{-CaM}\) saturated by TFP.

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Figure 2.11: Docking and models of TFP binding to the C-domain of CaM

A. TFP Docking to alternative tertiary conformations of apo CaM. Ribbon diagrams of C-domain fragments (residues 82 to 146) represent the “closed” (1DMO.pdb), “semi-open” (2IX7.pdb) and “open” (2HQW, 1LIN.pdb) conformations. Calcium was removed from 2HQW and 1LIN. AutoDock Vina 1.0.3 predicted positions of TFP binding; 20 models having lowest free energy are shown as sticks. The single sulfur atom of each TFP is shown as a sphere; green corresponds to the most favorable free energy of binding; white is the least favorable. Color thermometer below each set of models indicates the range of energies predicted. The TFP molecule observed at site A of 1LIN.pdb is shown in magenta. Residues in calcium-binding sites are yellow; arrows are included only to orient the viewer to chain direction. B. Model of conformational transition of apo C-domain in equilibrium between a “closed” and “semi-open” conformation. Binding of TFP to the blue patches accessible in the “semi-open” conformation is energetically more favorable than binding to “closed” form. TFP binding to the blue occludes hydrophobic patches show in purple of the apo C-domain that are otherwise exposed to solvent. An “open” conformation is adopted upon calcium binding, whether alone or also bound to a drug or protein target exposing hydrophobic patches shown in purple.

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Figure 2.12: Interdomain interactions mediated by TFP
Based on the crystal structure with 4:1 TFP:(Ca\(^{2+}\))\_4-CaM (1LIN.pdb), the trifluoperazine molecule shown in ball-and-stick (green) with fluorine, sulphur, and nitrogen atoms in cyan, yellow, and blue respectively) interacts with residues in both the calcium-saturated N-domain (blue) and C-domain (red). Those within 4 Å were residues 8, 11, 72, 92, 144, 145, TFP 1, and TFP 2. (Ca\(^{2+}\))\_4-CaM backbone (gray), 4 calcium ions (yellow spheres), and three other TFP (gray sticks) are shown.
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CHAPTER III
BINDING OF TRIFLUOPERAZINE TO THE C-DOMAIN OF CAM

Introduction

A difficulty encountered in Chapter II studies involving TFP binding to apo and (Ca\(^{2+}\))\(_4\)-CaM was found in the added complexity of TFP binding to CaM at stoichiometries greater than 1:1. Binding of TFP at ratios greater than 1:1 complicates structural analysis of TFP induced effects upon CaM as it is difficult to attribute observed changes in CaM to TFP binding at one site, as opposed to another. Further complicating analysis are the allosteric linkages that exist between Ca\(^{2+}\) and TFP binding sites if structural changes within CaM can be induced by either direct TFP binding to an area of CaM, or allosterically via propagated change. These complicating factors prompted us to examine how TFP interacted with an isolated C-domain fragment of CaM (CaM\(_{76-148}\)).

CaM\(_{76-148}\) was appealing to us as studies performed in Chapter II showed that TFP preferentially interacted with the C-domain of apo and (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\). Choosing to work with isolated CaM\(_{76-148}\) reduces that complexity of allosteric interactions that occur between domains of CaM\(_{1-148}\) upon TFP binding while still retaining similar Ca\(^{2+}\)-binding properties as found in the C-domain of CaM\(_{1-48}\). There is also a common TFP binding site within the C-domain of crystal structures of TFP bound to (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) where a significant difference is observed in TFP binding orientation that we would like to resolve why it exists.

The crystal structures of TFP bound to (Ca\(^{2+}\))\(_4\)-CaM differ in the stoichiometry of TFP per molecule of (Ca\(^{2+}\))\(_4\)-CaM (Figure 2.1c) (Cook et al., 1994; Vandonselaar et al., 1994a; Vertessy et al., 1998b). These structures also differ in the orientation of the trifluoromethyl group of the TFP molecules common to all 3 TFP bound (Ca\(^{2+}\))\(_4\)-CaM structures. In the structures of TFP bound (Ca\(^{2+}\))\(_4\)-CaM at ratios of 2:1 and 4:1 the
trifluoromethyl group is inserted into the hydrophobic pocket of the C-domain, while in the 1:1 structure it is flipped 180° (Figure 3.1). There is no clear explanation as to why the trifluoromethyl would adopt one orientation over the other, as the backbone and side chain conformations of all 3 TFP bound \((\text{Ca}^{2+})_4\text{CaM}\) structures are nearly indistinguishable (Figure 3.2).

Chapter III builds upon thermodynamic studies performed in Chapter II to more closely examine the molecular constraints required for TFP binding to apo and \((\text{Ca}^{2+})_2\text{CaM}_{76-148}\). This chapter presents structural studies of apo and \((\text{Ca}^{2+})_2\text{CaM}_{76-148}\) using NMR and X-ray crystallography respectively. Using NMR spectroscopy we have assigned backbone and side chain nuclei of apo CaM_{76-148} in the absence and presence of a 1:1 ratio of TFP, allowing for quantification of the residue specific changes in chemical shift. X-ray crystallography was used to determine the structure of TFP bound to \((\text{Ca}^{2+})_2\text{CaM}_{76-148}\). Findings reported here reveal residue specific changes and interactions associated with TFP binding to apo and \((\text{Ca}^{2+})_2\text{CaM}_{76-148}\).

Materials and Methods

Protein Overexpression

IPTG-induced CaM overexpression was performed using transformed \(E.\ coli\) BL21(DE3) cells containing the recombinant pT7-7 vector expressing the C-domain of \(Rattus\ Norvegicus\) CaM. Proteins were overexpressed in Luria-Bertani broth. CaM was then purified as previously described by Putkey et al. (Putkey et al., 1985). The recombinant proteins were 97-99% pure as judged by silver-stained SDS-PAGE gels. Protein concentrations were determined by UV spectroscopy of protein denatured with NaOH or native at pH 7.4 (Crouch and Klee, 1980).
Crystallography Materials and Methods

Crystallization of TFP-bound to (Ca$^{2+}$)$_2$-CaM$_{76-148}$ was performed by adding a 10-fold molar excess of TFP to 500µl of ~10mg/ml of CaM$_{76-148}$ in 50mM HEPES, 100mM KCl, 1mM MgCl$_2$, 5mM NTA, 50µM EGTA, pH 7.4, with 500µl of 200mM potassium thiocyanate, 20% polyethylene glycol 3350, pH 6.64 (Solution PEG 62 Qiagen) as a hanging drop in a 96-well tray. The tray was incubated at 15° C for ~8 months, at which time a single rod shaped crystal was observed. The crystal was cryo-protected with mother liquor containing 10% ethylene glycol prior to being flash-frozen at 100 K. Data were collected on this crystal at 100 K at the 4.2.2 synchrotron beamline at the Advanced Light Source at the Ernest Orlando Lawrence Berkeley National Laboratory, with a 150 mm crystal-to-detector distance and the assistance of Jay Nixx (beam-line manager). The program d*TREK was used to analyze and scale the data (Pflugrath, 1999). The monoclinic crystals diffracted to a resolution of 1.9 Å and were of the space group P2$_1$. Molecular replacement was performed using the extracted C-domain of TFP bound (Ca$^{2+}$)$_4$-CaM$_{1-148}$ (1LIN.pdb) as a template with the program Phaser (Read, 2001). TFP and Ca$^{2+}$ were removed from the template prior to use in molecular replacement. Refinement was performed using the program Refmac5 of the CCP4 program suite (Murshudov et al., 1997). Coot was used for molecular visualization and model building (Emsley and Cowtan, 2004). Ca$^{2+}$ and TFP were modeled into clearly visible electron density, water molecules were finally added to the structure using Coot, followed by manual editing. Structure validation was performed using the WhatIf Web Server (http://swift.cmbi.ru.nl/servers/html/index.html).

Overexpression and Purification of Isotope Enriched CaM

All isotopes were obtained from Cambridge Isotope Laboratories (Andover, MA). IPTG-induced CaM overexpression was performed using transformed BL21(DE3) cells
containing the recombinant pET vector expressing the C-domain of *Paramecium* CaM (a gene generously provided by C. Kung, University of Wisconsin, Madison, WI). $^{15}$N-labeled proteins were overexpressed in minimal medium, using 2 g/L unlabeled glucose as a carbon source and 1 g/L $^{15}$NH$_4$Cl as the sole nitrogen source. Double labeled ($^{13}$C- and $^{15}$N-) proteins were produced using 2 g/L $^{13}$C-glucose as the sole carbon source and 1 g/L $^{15}$NH$_4$Cl as the sole nitrogen source. CaM was then purified as previously described by Putkey et al. The recombinant proteins were 97-99% pure as judged by silver-stained SDS-PAGE. Protein concentrations were determined by UV spectroscopy of protein denatured with NaOH or native at pH 7.4 (Beaven and Holiday, 1952).

**Assignment of Backbone and Side chain Resonances**

The NMR spectra apo CaM$_{76-148}$ ± TFP were collected at 25 °C on a Bruker Avance II 500 or 800 NMR spectrometer. The $^1$H, $^{15}$N, and $^{13}$C resonances of the backbone were assigned using triple resonance experiments (HNCA, HN(CO)CA, HNCACB, HN(CO)CACB, HNCO, and HN(CA)CO) (Yamazaki et al., 1994) with the uniformly $^{15}$N and $^{13}$C-labeled CaM in complex with unlabeled TFP. $^1$H$_a$ resonances were assigned from an $^{15}$N-edited TOCSY spectrum using an uniformly $^{15}$N-labeled protein (Clore and Gronenborn, 1994) and from HA(CACO)NH experiment using an uniformly $^{15}$N and $^{13}$C-labeled sample. The side chain signals were assigned from 3D H(CCO)NH-TOCSY, C(CO)NH-TOCSY, HCCH-TOCSY, $^{15}$N-edited TOCSY, and $^{15}$N or $^{13}$C-edited NOESY spectra (Clore and Gronenborn, 1994; Fesik and Zuiderweg, 1988).

**Quantification of $^{15}$N-apo CaM$_{76-148}$ Chemical Shifts due to TFP Addition**

To determine the change in chemical shift upon TFP binding to apo CaM$_{76-148}$, chemical-shift changes in both the $^1$H and $^{15}$N dimensions were quantified using the modified Pythagorean theorem previously described by Jaren et al., 2002 shown in Equation 3.1.
$\Delta ppm = \sqrt{(\Delta 1H ppm)^2 + (0.10134 \cdot \Delta 15N ppm)^2}$  \hspace{1cm} (3.1)

In this equation, $\Delta ppm$ refers to the linear change of a specific resonance peak from its initial starting position in apo CaM$_{76-148}$.

**Results**

**Structure of TFP Bound (Ca$^{2+}$)$_2$-CaM$_{76-148}$**

X-ray crystallography studies of TFP bound to (Ca$^{2+}$)$_2$-CaM$_{76-148}$ were refined to 2.1 Å resolution with statistical measures of the goodness-of-fit listed in Table 1. It is important to point out that the % completeness value (80.92%) reported in this table is not ideal. The reason for this lower than expected value is due to the presence of an ice ring in the data that needed to be removed to properly index and scale the dataset resulting in the loss of some of the diffraction data. Although this value is not ideal, it was sufficient to provide a usable electron density map for model building.

The structure revealed that there were 2 (Ca$^{2+}$)-CaM$_{76-148}$ and 4 TFP molecules per asymmetric unit (Figure 3.3a). The conformations adopted by the 2 (Ca$^{2+}$)$_2$-CaM$_{76-148}$ chains were very similar to each other with an all atom RMSD of 0.46 Å (Figure 3.3b). The interhelical angles adopted between helices E-F of TFP bound (Ca$^{2+}$)$_2$-CaM$_{76-148}$ chains A and B were 79.0° and 82.9° respectively, while interhelical angles of 89.7° (chain A) and 88.4°(chain B) were observed for helices G-H. Consistent with other structures of (Ca$^{2+}$)$_4$-CaM$_{1-148}$ either with or without a bound target, both chains of TFP bound (Ca$^{2+}$)$_4$-CaM$_{76-148}$ adopt an “open” domain conformation.

As previously stated and shown in Figure 3.3b, 4 TFP molecules were found within the asymmetric unit. Both (Ca$^{2+}$)$_2$-CaM$_{76-148}$ chains share a common TFP-binding site located within each of their hydrophobic pockets. Although each (Ca$^{2+}$)-CaM$_{76-148}$ chain has a TFP molecule bound at a common position, the orientation of TFP within the hydrophobic pocket is different dependent upon the (Ca$^{2+}$)$_2$-CaM$_{76-148}$ chain examined (Figure 3.4). Examination of residues within hydrophobic pockets of (Ca$^{2+}$)$_2$-CaM$_{76-148}$
that are within 4 Å of TFP that may account for the 180° flip of TFP between (Ca²⁺)₂-CaM₇⁶-₁₄₈ chains indicate that most of the residues are unchanged with the exception of M₁₄₄ (Figure 3.5).

In addition to the 2 TFP molecules that were observed to bind in the hydrophobic pockets of (Ca²⁺)₂-CaM₇⁶-₁₄₈, 2 additional TFP molecules were observed within the asymmetric unit. Analysis of contacts within 4 Å of these TFP molecules indicate that they largely interact with other TFP molecules and made few interactions with (Ca²⁺)₂-CaM₇⁶-₁₄₈, compared to TFP molecules found within the hydrophobic clefts of (Ca²⁺)₂-CaM₇⁶-₁₄₈ (Figure 3.6).

This structural study unequivocally shows that TFP-binding to (Ca²⁺)₂-CaM₇⁶-₁₄₈ does not alter the “open” backbone conformation of (Ca²⁺)₂-CaM₇⁶-₁₄₈ observed in the absence of TFP. Previous studies conducted in Chapter II, indicated that a binding interface used by the D-domain of apo CaM₁-₁₄₈ for TFP binding was distinct from those of apo CaM₁-₁₄₈ alone and that of TFP-bound (Ca²⁺)₂-CaM₇⁶-₁₄₈. To more closely examine the apo CaM₇⁶-₁₄₈ TFP binding interface in-depth solution NMR experiments were required.

**TFP-Induced Changes of apo CaM₇⁶-₁₄₈ as Monitored by **¹⁵**N-HSQC Spectroscopy**

Due to the inherent flexibility found within the Ca²⁺-binding loops of apo CaM₇⁶-₁₄₈, solution NMR methods were used to examine how TFP interacts with apo CaM₇⁶-₁₄₈ at the structural level. Figure 3.7 shows an overlay of spectra of apo CaM₇⁶-₁₄₈ without and with TFP at a 1:1 apo CaM₇⁶-₁₄₈:TFP ratio. TFP binding to apo CaM₇⁶-₁₄₈ was observed to be in fast exchange on the NMR time scale due to its weak (~1 µM) binding affinity. TFP binding induced significant chemical shift perturbations of apo CaM₇⁶-₁₄₈ amide resonances, as shown in Figure 3.7. ¹⁵N-HSQC peak assignments of apo CaM₇⁶-₁₄₈ in the presence and absence of TFP were determined via 3-dimensional NMR
experiments described in the materials and methods resulting in ~95% assignment of backbone resonances.

Quantification of chemical shifts of apo CaM\textsubscript{76-148} amide resonances due to TFP binding resulted in an average chemical shift of 0.047 ppm. Individual residue chemical shift values are shown in Figure 3.8 where it can be observed that TFP binding did not shift apo CaM\textsubscript{76-148} resonances in a uniform manner, but rather causes shifts at unique positions. Of the 73 amino acids that comprise CaM\textsubscript{76-148}, 21 were observed to have a chemical shift greater that 0.05 ppm upon TFP addition. Although the structure of TFP-bound apo CaM\textsubscript{76-148} is unknown, it is likely that TFP binds to an exposed hydrophobic patch of apo CaM\textsubscript{76-148} comprised of hydrophobic residues identified in Figure 3.8. Computational docking of TFP to the C-domain of CaM described in Chapter II predicted that TFP bound within the shallow hydrophobic cleft of a “semi-open” conformation composed of similar hydrophobic residues identified here.

The location and magnitude of these chemical shifts have been mapped onto the solution structure (1F71.pdb) of apo CaM\textsubscript{76-148} (Figure 3.8), where it can be observed that although sequentially distant in primary sequence, many of the TFP perturbed residues are located near each other spatially. As shown in Figure 3.8, apo CaM\textsubscript{76-148} helices F-H as well as the Ca\textsuperscript{2+} binding loops contain resonances that are significantly (>0.05ppm) perturbed, indicating that these regions are either directly or allosterically perturbed upon TFP binding.

**Dynamics of TFP Bound apo CaM\textsubscript{76-148} Monitored with T\textsubscript{2} Relaxation Spectroscopy**

The change in overall size of apo CaM\textsubscript{76-148} upon TFP binding was investigated using T\textsubscript{2} NMR relaxation experiments. Comparison of average amide T\textsubscript{2} relaxation times of apo CaM\textsubscript{76-148} with (147 ± 79.90 msec) and without TFP (161 ± 59.46 msec), indicate that TFP binding to apo CaM\textsubscript{76-148} causes an increase in hydrodynamic radius. Shown in
**Figure 3.9**, are the calculated individual $T_2$ amide relaxation times for free and TFP bound apo CaM$_{76-148}$. As expected the N- and C-termini of both TFP free and TFP bound apo CaM$_{76-148}$ have significantly longer $T_2$ relaxation times indicative of their lack of defined secondary structure. Consistent with the analysis of $^{15}$N-HSQC spectra of TFP binding to apo CaM$_{76-148}$ presented in this chapter, the $T_2$ relaxation times apo CaM$_{76-148}$ of residues within helices F-G, and $Ca^{2+}$ binding loops of CaM are increased upon TFP addition (**Figure 3.10**).

**Discussion**

The C-domains of apo and $(Ca^{2+})_4$-CaM can bind to their targets using a variety of conformations described previously in Chapter II. To simplify structural studies of TFP interacting with the C-domain of apo and $(Ca^{2+})_4$-CaM$_{1-148}$, the C-domain fragment (CaM$_{76-148}$) was used. NMR studies presented in this chapter address residue-specific changes in apo CaM$_{76-148}$ upon TFP addition. The crystal structure of TFP bound $(Ca^{2+})_2$-CaM$_{76-148}$ consolidates differing observations of the location of the trifluoromethyl group of the TFP common in all observed structures, as well as provides for the first time a molecular basis for the trifluoromethyl group location.

**Protein Crystallography of $(Ca^{2+})_2$-CaM$_{76-148}$-TFP Complex**

Crystallization of TFP bound to $(Ca^{2+})_2$-CaM$_{76-148}$ revealed that TFP binds within the hydrophobic cleft of the isolated C-domain in a similar manner to TFP binding to $(Ca^{2+})_4$-CaM$_{1-148}$. In the asymmetric unit, 4 TFP molecules were found. The TFP molecules were numbered based on their order from left to right when chain A when Chain A is position on the left and chain B on the right as depicted in **Figure 3.3**. TFP #2 and TFP #3 are likely crystallization artifacts, as both of these TFP molecules made few interactions with either chain A or B compared to TFP bound within the clefts of $(Ca^{2+})_2$-CaM$_{76-148}$ (**Figure 3.6**). Examination of the asymmetric unit reveals that these bound
TFP molecules function to create a continuous array of TFP molecules between chains A and B (Figure 3.3a). This array is similar to the way TFP molecules are positioned in the 4:1 TFP-(Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) structure, although the (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\) domains are rotated 180° relative to each other (Figure 2.1c, Figure 3.3). This rotation is likely the result of the lack of covalent linkage between the C-domain fragments which would otherwise be present in (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\).

Protein chains A and B have a common TFP molecule found in each of their hydrophobic pockets although the location of the TFP’s trifluoromethyl groups are flipped 180° relative to each other (Figure 3.4). Previous structures of TFP bound (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) report that the difference in location of the trifluoromethyl group at the common TFP binding site was reported as improperly assigned electron density (Cook et al., 1994; Vandonselaar et al., 1994a; Vertessy et al., 1998a). These structures were determined at 2.45 (1:1), 2.74 (2:1), and 2.0 Å (4:1) resolution, and only contained one molecule of (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) per unit cell. The electron density of the TFP binding site in our 1.9 Å resolution structure which contains two molecules of (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\) per asymmetric unit clearly indicates that the trifluoromethyl group at the common TFP binding site can occupy both orientations (Figure 3.11).

Superposition of chain A or B of (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\) with the TFP bound C-domain of (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) (1CTR.pdb and 1LIN.pdb) shows that the backbone of the C-domain of CaM in CaM\(_{76-148}\), and CaM\(_{1-148}\) are similar (RMSD < 0.9Å). However, differences were observed between TFP-bound (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\), and (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) at the location of TFP binding, and location of the trifluoromethyl groups (Chain A vs. 1:1 CaM\(_{1-148}\):TFP and Chain B vs. 4:1 CaM\(_{1-48}\):TFP) (Figure 3.12). Superposition of chains A and B revealed that nearly identical conformations were adopted by both chains as well as the residues within 4Å of the common TFP binding sites in both chains (Figure 3.4, 3.5). Slight differences in the structure of (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\) formed by chains A and B were seen outside of the hydrophobic cleft. Shown in Figure 3.13 is a Chimera
(Pettersen et al., 2004) generated all-atom morph between chains A and B which depicts that magnitude of change between chains.

The most significant difference between chain A and B is the orientation of the side chain of M144 which appears to act as a selection gate to control whether the trifluoromethyl group can be accommodated within the hydrophobic pocket of (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\) (Figure 3.5). Variability in the side chain conformation of M144 is consistent with other structures of (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) bound to drugs or peptides. Side chain methyl dynamics measurements of M144 indicate that it is highly dynamic with respect to other CaM Met residues (Chen et al., 1993; Ehrhardt et al., 1995). In a comparison of 17 compact CaM-drug or CaM-peptide complexes, a tetrad of CaM of residues termed FLMM (L92, L105, M124, M144) was found to consistently contact the ligand for the C-domain in all complexes examined (Ataman et al., 2007). Of the FLMM tetrad residues, the side chain of M144 was the most variable in its closest approach distance when interacting with the hydrophobic anchor residue of the ligand, as well as most variable in terms of amino acid identity in 102 CaM sequences (Ataman et al., 2007). Similar variability conservation of a Met residue in the FLMM tetrad at position 144 was observed in a computational study in which the hydrophobic cleft of CaM was redesigned to improve target binding affinity (Shifman and Mayo, 2002; Shifman and Mayo, 2003). The binding site for TFP within the hydrophobic cleft of the C-domain of CaM is not well defined based on the dynamic nature of Met 144 as its conformation solely selects the orientation of TFP that can be accommodated by the hydrophobic cleft. These observations indicate that the dynamic nature of Met144 results in multiple modes of TFP binding that are used by the C-domain of (Ca\(^{2+}\))\(_4\)-CaM.

This structure helps to resolve controversy within the field when comparing the location of the common TFP binding site found in each of the full length structures of TFP bound to (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) at 1:1, 2:1, and 4:1 ratios. It shows that TFP employs 2 modes of binding to the C-domain of CaM, as both orientations of the trifluoromethyl
group are observed within the same unit cell. Previous studies with (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) have shown 2 different TFP binding orientations in separate protein crystals obtained from differing crystallization solutions, whereas our findings are from a single crystal and therefore cannot be a result of difference in solution conditions. This structure also indicates that both conformations of TFP have similar binding affinities for the C-domain as protein crystallization requires molecules to adopt low energy conformations to form an ordered crystal lattice. If one of the CaM conformations observed had a significantly more favorable energy than the other, it would be unlikely for both a high and low energy conformation to co-crystallize together.

**TFP-Induced Changes of apo CaM\(_{76-148}\) as Monitored by \(^{15}\)N-HSQC Spectroscopy**

To examine the effect of TFP upon individual residues of apo CaM\(_{76-148}\) we have used \(^{15}\)N-HSQC chemical shift mapping to determine TFP induced changes in the chemical environments of residues within apo CaM\(_{76-148}\). **Figure 3.7** shows an overlay of \(^{15}\)N-HSQC spectra of apo CaM\(_{76-148}\) recorded in the absence and presence of TFP, where the quantified average change in chemical shift upon TFP addition to apo CaM\(_{76-148}\) was 0.047 ppm. If TFP interacted in a nonspecific manner with apo CaM\(_{76-148}\), all residues would have individual Δppm values of ~0.047, but comparison of the average Δppm with individual residue values indicates that TFP selectively interacts with specific residues of apo CaM\(_{76-148}\) (**Figure 3.8**). This observation is in agreement with observations made in **Chapter II** that TFP interacts with a selective set of apo CaM C-domain residues. TFP binding to apo CaM\(_{76-148}\) perturbs residues located near the hydrophobic cleft of apo CaM\(_{76-148}\) as well as residues within the Ca\(^{2+}\)-binding loops of sites III and IV of apo CaM\(_{76-148}\) (**Figure 3.8**). This result provides direct evidence of allosteric linkage between TFP and Ca\(^{2+}\) binding to CaM\(_{76-148}\), consistent with Ca\(^{2+}\) titration data in **Chapter II**,
which indicated that TFP at a 1:1 ratio lowered the Ca\(^{2+}\)-binding affinity of CaM\(_{76-148}\) by 2.56 kcal/mol.

**TFP-Induced Changes of apo CaM\(_{76-148}\) as Monitored by T\(_2\) Relaxation**

Comparison of average T\(_2\) relaxation times of apo CaM\(_{76-148}\) and TFP bound apo CaM\(_{76-148}\) (161 ± 59 and 147 ± 79 msec, respectively) indicates that TFP binding to apo CaM\(_{76-148}\) induces a conformational change within apo CaM\(_{76-148}\). Due to the inverse relationship between T\(_2\) time and molecular weight (↑ T\(_2\) time = ↓ molecular tumbling), TFP binding causes apo CaM\(_{76-148}\) to tumble at a slower rate in solution (Palmer, 2001). This conformational change is interpreted to be due to TFP binding resulting in a “semi-open” conformation of apo CaM\(_{76-148}\) that was previously in a “closed” conformation. The average T\(_2\) values of apo CaM\(_{76-148}\) and TFP bound apo CaM\(_{76-148}\) also indicated that both molecules are found as monomers in solution. It may also possibly however unlikely that the change in T\(_2\) time after TFP addition could be a result of aggregation, though visual inspection of the sample indicated that no precipitate was present. An orthogonal approach proposed for future studies to verify the absence of precipitate in the NMR sample would be to use dynamic light scattering or analytical ultracentrifugation to verify
Table 3.1: Data collection and refinement statistics of (Ca^{2+})_2-CaM_{76-148}:TFP complex

<table>
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<th>Data Collection Parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (K)</td>
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</tr>
<tr>
<td>Wavelength (Å)</td>
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</tr>
<tr>
<td>Space Group</td>
<td>P 21</td>
</tr>
<tr>
<td>Unit cell parameters</td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>24.59, 85.54, 35.35</td>
</tr>
<tr>
<td>α, β, δ (Å)</td>
<td>90.00, 93.03, 90.00</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>22.181 – 2.1</td>
</tr>
<tr>
<td>I/σ</td>
<td>6.0</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>80.92</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>0.091</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.53</td>
</tr>
</tbody>
</table>

| Refinement Details          |     |
| Resolution (Å)              | 2.1 |
| R_work/R_free (%)           | 1 |
| Number of atoms             | 2194 |
| Protein                     | 1969 |
| Ligand                      | 139 |
| Water                       | 86 |
| B-factor average (Å^2)      |     |
| Protein (main chain)        | 28.55 |
| TFP                         | 33.00 |
| Water                       | 31.08 |
| RMS deviation from ideal geometry |     |
| Bond lengths (Å)            | 0.009 |
| Bond angles (°)             | 1.277 |
| Dihedral angles (°)         | 22.020 |
| Planarity (°)               | 0.004 |
| Chirality (°)               | 0.100 |
| Ramachandran plot (% residues) |     |
| Most Favored                | 100 |
| Additionally allowed        | 0 |
| Disallowed                  | 0 |
Figure 3.1: Superposition of TFP:(Ca$^{2+}$)$_4$-CaM$_{1-148}$ structures
Superposition of crystallographically derived structures of TFP:(Ca$^{2+}$)$_4$-CaM$_{1-148}$ with
drug:protein ratios of 1:1 (1CTR.pdb), 2:1 (1A29.pdf), and 4:1 (1LIN.pdb). The TFP-
binding sites are labeled A (green), B (magenta), C (brown) and D (orange). TFP-
binding sites A and B are located in the C-domain (backbone red), site C bridges the two
domains, and site D is located in the N-domain (backbone blue). The 180° flip of TFP
molecules at TFP binding site A (dashed square) results in the trifluoromethyl group of
TFP being found in 2 locations (dashed ovals).
Users/nmr_mike/Thesis/Chapter_III/Figure3_1.jpg
Figure 3.2: Side chain Orientations of $(\text{Ca}^{2+})_4\text{CaM}_{1-148}$ when binding TFP at site A

A-C: Isolated residues of $(\text{Ca}^{2+})_4\text{CaM}_{1-148}$ C-domain (red) bound to TFP within 4 Å of TFP bound at site A (transparent sticks) with Ca$^{2+}$ ions shown as yellow spheres. D: Superposition of isolated C-domains of 1:1, 2:1, and 4:1 TFP:$(\text{Ca}^{2+})_4\text{CaM}_{1-148}$ shown in panels A-C to illustrate that nearly identical binding interfaces and side chain conformations are used when binding TFP at site A.

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Figure 3.3: Crystal structure of TFP bound (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\)
A: (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\) chains A and B are shown in red, and orange respectively while TFP molecules is depicted in sticks, Ca\(^{2+}\) are yellow spheres. B: Superposition of (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\) chains A and B shown in red and orange respectively.

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Figure 3.4: Comparison of common TFP binding sites within \((\text{Ca}^{2+})_{2}\text{-CaM}_{76-148}\)
Superposition of TFP (sticks) bound to \((\text{Ca}^{2+})_{2}\text{-CaM}_{76-148}\) chains A and B colored red and orange respectively. The TFP molecules associated with chains A and B are colored green and magenta respectively where the 180° flip in the location of the trifluoromethyl of TFP is indicated by dashed circles.
Users/nmr_mike/Thesis/Chapter_III/Figure3_4.jpg
Figure 3.5: Met 144 gating determines TFP orientation
A: Superposition of (Ca²⁺)₂-CaM₇₆-1₄₈ chains A and B colored red and orange respectively, where residues within 4 Å of TFP are shown as sticks. Shown in ball and stick are the conformations adopted by Met 144 of chains A and B colored green and slate respectively. B: Space-filling representation of Met 144 conformations in relation to TFP. Modeled steric clash between Met 144 side chain conformation found in chain B when TFP #1 from chain A is superimposed into the binding site of TFP 4.

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Figure 3.6: Contacts made by TFP molecules with (Ca$^{2+}$)$_2$-CaM$_{76-148}$

The molecular surface of (Ca$^{2+}$)$_2$-CaM$_{76-148}$ chains A and B are shown as red and orange respectively, where portions are colored according to the respective TFP examined to indicate areas of (Ca$^{2+}$)$_2$-CaM$_{1-148}$ that are within 4 Å.

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Figure 3.7: $^{15}$N-HSQC spectrum of apo CaM$_{76-148}$ showing the effect of TFP binding. Overlaid $^{15}$N-HSQC spectra of apo CaM$_{76-148}$ alone (blue) and in the presence of TFP (red). Users/nmr_mike/Thesis/Chapter_III/Figure3_7.jpg
Figure 3.8: Change in amide chemical shift of apo CaM76-148 upon TFP addition

The difference in chemical shift value ($\Delta \text{ppm}$) is plotted for each assigned apo CaM76-148 residue (absent bar represents unassigned residue), dashed line represents average value of TFP induced change in chemical shift of 0.047 ppm. The apo CaM76-148 structure is shown as a cartoon with the C$\alpha$ atoms of residues whose quantified chemical shift upon TFP addition was greater than 0.05 ppm shown as spheres.

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Figure 3.9: $T_2$ Values for apo CaM$_{76-148}$ in the absence and presence of TFP

<table>
<thead>
<tr>
<th></th>
<th>w/out TFP</th>
<th>w/ TFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average $T_2$</td>
<td>161 ± 59</td>
<td>147 ± 79</td>
</tr>
<tr>
<td>Median $T_2$</td>
<td>152</td>
<td>135</td>
</tr>
</tbody>
</table>

$\downarrow T_2$ time = $\uparrow$ Hydrodynamic radius

TFP addition decreases $T_2$ time

Figure 3.9: $T_2$ Values for apo CaM$_{76-148}$ in the absence and presence of TFP
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Figure 3.10: Change in $T_2$ values of apo CaM$_{76-148}$ upon TFP addition mapped upon the structure of apo CaM$_{76-148}$
Apo CaM$_{76-148}$ is shown as a cartoon with labeled helices and Ca$^{2+}$ binding sites.
Users/nmr_mike/Thesis/Chapter_III/Figure3_10.jpg
Figure 3.11: Electron density of TFP binding sites in (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\)

The asymmetric unit of (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\) bound to TFP where chains A and B are shown in red and orange respectively, while TFP are shown as sticks. The electron density for TFP molecules in upper panel is displayed at 2\(\sigma\) over each modeled TFP (lower panel).

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Figure 3.12: Superposition of common TFP binding sites in (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\) and (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\)
Superpositions of Isolated C-domains of TFP:(Ca\(^{2+}\))-CaM\(_{1-148}\) at 1:1 and 4:1 ratios shown in cyan and light green respectively onto TFP bound (Ca\(^{2+}\))-CaM\(_{76-148}\) chains A and B colored red and orange respectively.
Users/nmr_mike/Thesis/Chapter_III/Figure3_12.jpg
Figure 3.13: Structural morph between chain A and B or TFP-bound (Ca$^{2+}$)$_2$-CaM$_{76-148}$
UCSF Chimera generated morph between chain A (red) and chain B (blue), indicating areas of similarity and divergence between chains.

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CHAPTER IV
BINDING OF NAV1.2IQP TO THE C-DOMAIN OF APO CAM

Introduction

The voltage-dependent sodium channel type II (Na, 1.2) is necessary for the propagation of action potentials along unmyelinated axons that are found primarily in neuronal and muscle tissues (Schaller and Caldwell, 2003; Trimmer and Rhodes, 2004). Na,1.2 has one α-subunit (260 kDa) and one or more β-subunits (33–36 kDa each) (Catterall, 2000b). The α-subunit is comprised of four homologous transmembrane domains (I–IV) that are the pore-forming entity of the channel, as well as intracellular domains that play a vital role in control of channel gating (Cormier et al., 2002; Herzog et al., 2003; Mantegazza et al., 2001). Inactivation or closing of Na,1.2 requires an interaction between two regions of the α-subunit: the intracellular loop that connects domains III and IV and the C-terminal tail (Catterall, 2000a; Cormier et al., 2002; Deschênes et al., 2001; Herzog et al., 2003).

The membrane-proximal half of the C-terminal tail of Na,1.2 has been modeled to contain six α-helices. Patch clamp studies have shown that deletion of the putative sixth α-helix region slows recovery from inactivation by maintaining the channel in a closed, inactivated state (Cormier et al., 2002; Herzog et al., 2003; Mantegazza et al., 2001). A sequence (residues 1901 to 1927, Na1.2IQp) within the putative sixth α-helix also contains a classical calmodulin-binding IQ-motif (IQxxxxBGGxxxB), where X is any amino acid, and B is either Arg or Lys (Figure 4.1a) (Mori et al., 2003; Mori et al., 2000). IQ-motifs found in many proteins such as myosins, neuronal growth proteins, and ion channels have been characterized to bind preferentially to apo (calcium-depleted) calmodulin (CaM) (Bayley et al., 2003; Gerendasy et al., 1995; Houdusse et al., 2006; Liu and Storm, 1990; Mori et al., 2003; Mori et al., 2000; Mori et al., 2004; Shah et al.,
CaM is a small intracellular calcium sensor that is essential to many eukaryotic signal transduction pathways (Klee, 1988; Wang et al., 1980; Yagi et al., 1990; Yap et al., 2000). CaM has two homologous domains (N- and C-domains) that are 4-helix bundles connected by a flexible linker (Figure 4.1b) (Babu et al., 1988; Evans and Shea, 2009; Sorensen and Shea, 1997). Each domain binds two $\text{Ca}^{2+}$ ions cooperatively in neighboring EF-hand motifs, giving rise to a total of 4 bound $\text{Ca}^{2+}$ ions per molecule of CaM (Pedigo and Shea, 1993; Sorensen et al., 2002b). Although the two domains are similar in sequence and structure, the N-domain binds $\text{Ca}^{2+}$ more weakly than the C-domain in the absence of allosteric effectors (Newman and Shea, 2006; Newman, 2008; Vanscyoc and Shea, 2001b). Changes in intracellular $\text{Ca}^{2+}$ levels are linked to many cellular events by the effect of $\text{Ca}^{2+}$ on CaM: binding triggers conformational changes that expose hydrophobic surfaces in both domains of CaM, altering the free energy of association with many target proteins (Crivici and Ikura, 1995; Sorensen and Shea, 1997). Several high resolution structures of (Ca$^{2+}$)$_4$-CaM bound to an IQ-motif show that it adopts a compact ellipsoidal conformation. That conformation has also been observed when calcium-saturated CaM is bound to target regions of most known cyclases, phosphatases, cytoskeletal motors and ion channels (Tjandra et al., 1999). Besides the IQ-motif, the most common CaM-binding sequence is a Basic Amphipathic Alpha-helix (BAA) motif, where the domains of (Ca$^{2+}$)$_4$-CaM adopt an “open” conformation to interact with hydrophobic “anchor” residues located within the BAA motif (Figure 4.1c) (Tjandra et al., 1999). There are only two available high-resolution structures showing the apo C-domain of CaM bound to a peptide (Figure 4.1d, 4.1e): CaM bound to a fragment of myosin V (2IX7) or of the SK channel (1G4Y). Only one of those (2IX7) contains an IQ-motif. However, in both complexes, the apo C-domain of CaM adopted a “semi-open” conformation in which interhelical angles are smaller than those of (Ca$^{2+}$)$_4$-CaM (Houdusse et al., 2006; Schumacher et al., 2001).
All ten human isoforms of the voltage-dependent sodium channel contain a single IQ-motif necessary for regulation by CaM (Yu and Catterall, 2003). Previous studies conducted by the Shea laboratory (Theoharis et al., 2008) showed that both apo and calcium-saturated CaM bind to Na\textsubscript{v}1.2\textsubscript{IQp} with high affinity (i.e., dissociation constants near nanomolar). However, the two homologous domains of CaM have different roles when interacting with Na\textsubscript{v}1.2\textsubscript{IQp} (Theoharis et al., 2008). Circular dichroism and fluorescence spectroscopy showed that Na\textsubscript{v}1.2\textsubscript{IQp} binds preferentially to the C-domain of apo CaM which selectively lowers the calcium-binding affinity of sites III and IV, while having little effect on sites I and II.

To examine the molecular basis of the preferential binding of apo CaM to Na\textsubscript{v}1.2\textsubscript{IQp}, and the roles of each domain of CaM, we used heteronuclear NMR to determine residue-specific responses of CaM to binding the IQ-motif. HSQC spectra showed that, under apo conditions, the C-domain of CaM was necessary and sufficient to bind Na\textsubscript{v}1.2\textsubscript{IQp}, while resonances corresponding to the N-domain of CaM were not affected. NMR studies of \textsuperscript{13}C-\textsuperscript{15}N-labeled CaM were then used to determine a set of high-resolution models of a C-domain fragment of apo CaM (CaM\textsubscript{76-148}) bound to Na\textsubscript{v}1.2\textsubscript{IQp}. This set of structures revealed that apo CaM\textsubscript{76-148} adopts a “semi-open” conformation when bound to Na\textsubscript{v}1.2\textsubscript{IQp} similar to the C-domains of either apo CaM, CaM-like proteins, or essential light chain (ELC) when bound to IQ-motifs in myosin (Houdusse et al., 2006; Swindells and Ikura, 1996; Terrak et al., 2003). It also demonstrated the importance of two Tyr residues in the sequence of Na\textsubscript{v}1.2\textsubscript{IQp}; these are conserved in most IQ-motifs of all isoforms of voltage-dependent sodium channels. Although both Na\textsubscript{v}1.2\textsubscript{IQp} and CaM are very polar, the interface was dominated by hydrophobic interactions. This was consistent with results from NaCl titrations of the apo CaM-IQ complex which remained unperturbed in the presence of elevated levels of NaCl (up to 650 mM).

Although Na\textsubscript{v}1.2\textsubscript{IQp} tightly associates with (Ca\textsuperscript{2+})\textsubscript{4}-CaM, NMR studies showed that participating residues changed dramatically, consistent with an interaction interface
like that found for a peptide bound to an “open” domain of CaM. However, for both apo and calcium-saturated CaM, $^{15}$N-HSQC and fluorescence anisotropy revealed that the N-domain of CaM did not interact with Na$_{v}$1.2$_{IQp}$. This suggests that its preferred binding site lies elsewhere in the sequence Na$_{v}$1.2. Analysis of conserved contact residues in all available high-resolution structures of apo CaM, apo ELC, or apo CaM-like proteins in complex with canonical IQ-motifs suggested that all of the IQ motifs bind with a similar polarity, having the amino-terminus closer to the EF-hand, and the C-terminus closer to the G-H hand of that domain.

**Materials and Methods**

**Overexpression and Purification of Calmodulin**

All isotopes were obtained from Cambridge Isotope Laboratories (Andover, MA). IPTG-induced CaM overexpression was performed using transformed *E. coli* BL21(DE3) cells containing the recombinant pT7-7 vector expressing either the full length or the C-domain of *Paramecium* CaM (a gift from C. Kung, University of Wisconsin, Madison, WI). $^{15}$N-labeled proteins were overexpressed in minimal medium, using 2 g/L unlabeled glucose as a carbon source and 1g/L $^{15}$NH$_4$Cl as the sole nitrogen source. $^{13}$C and $^{15}$N-labeled proteins were produced using 2g/L $^{13}$C-glucose as the sole carbon source and 1 g/L $^{15}$NH$_4$Cl as the sole nitrogen source. CaM was then purified as previously described by Putkey et al (Putkey et al., 1985). The recombinant proteins were 97-99% pure as judged by silver-stained SDS-PAGE gels. Protein concentrations were determined by UV spectroscopy of protein denatured with NaOH (Beaven and Holiday, 1952) or native at pH 7.4 (Crouch and Klee, 1980).

**Prepared Na$_{v}$1.2$_{IQp}$**

A peptide (Nav1.2IQp:KRKQEEVSAIVALIQRAYRRYLLKQKVKK 3.36kDa) representing residues 1901-1927 of the $\alpha$-subunit of Na$_{v}$1.2 was custom-synthesized by
the GenScript Corporation (Scotch Plains, NJ). The peptide was evaluated to be at least 95% pure by HPLC analysis and MALDI-TOF mass spectrometry.

15N-HSQC Monitored Na,1,2IQp and Ca2+ Titration of CaM1-148

15N-HSQC spectroscopy was used to monitor titration of Na,1,2IQp into 15N-labeled apo CaM1-148 (400 µM) in 10 mM D4-imidazole, 100 mM KCl, 50 µM D16-EDTA, 0.01% NaN3, pH 6.5. Upon Na,1,2IQp saturation of apo CaM1-148, CaCl2 was titrated into the sample to a final concentration of 5mM. Na,1,2IQp and Ca2+ saturation of CaM1-148 were confirmed by plateaus in the intensity of peaks corresponding to either the Na,1,2IQp:apo CaM1-148 or Na,1,2IQp:(Ca2+)4-CaM1-148.

Preparation of 13C/15N-CaM76-148:Na,1,2IQp Complex

A 15N-HSQC monitored titration of 12C/14N-Na,1,2IQp into 13C/15N-apo CaM76-148 was performed to ensure saturation of apo CaM76-148 by Na,1,2IQp as determined by a plateau in the intensity of peaks corresponding the apo CaM76-148:Na,1,2IQp complex and the disappearance of peaks corresponding to apo CaM76-148 alone. The sample was then applied to a 200 mL G50 Superdex column. Complex containing fractions were pooled, buffer exchanged into 10 mM D4-imidazole, 100 mM KCl, 50 µM D16-EDTA, 0.01% NaN3, pH 6.5 and concentrated to 1.5 mM. Nitrogen-based experiments were conducted in the previously described buffer conditions in 90% H2O / 10% D2O, while carbon-based experiments were conducted in 100% D2O. All spectra with the exception of amide exchange were conducted with Shigemi (Allison Park, PA) microscale NMR tubes whose magnetic susceptibility was matched to D2O.
**15N-HSQC Monitored Amide Exchange of 15N-CaM76-148:Nav1.2IQp Complex**

A 480 µl sample of 1.5 mM 15N-CaM76-148:Nav1.2IQp complex in 10 mM D4-imidazole, 100 mM KCl, 50 µM D16-EDTA, 0.01% NaN3, pH 6.5 that had been prepared in H2O was lyophilized in a Speed-Vac Model VG-5 (Savant). After lyophilization the complex was then resuspended with 480 µl of 99.96% D2O (Cambridge Isotope Laboratories, Andover, MA) from a freshly broken ampoule. The pH of the sample was not adjusted after addition of D2O, and assumed to be 7.4 as HMQC spectra taken of the sample prior to lyophilization in H2O were identical to that of the D2O spectrum. The resuspended sample was placed immediately in a Bruker 500 MHz Avance II spectrometer and data acquisition began within 5 minutes. 15N-HSQC spectra were acquired at 30 minute intervals for 23 hours. Spectra were processed with NMRPipe (Delaglio et al., 1995b) and individual peak intensities were determined with Sparky (Goddard and Kneller). Peak intensity decays times were fit to a mono-exponential decay model (Equation 4.1) using the Solver function within Microsoft Excel.

\[
I = I_0 e^{-\frac{(t-t_0)}{\tau}} + b
\]  

In this equation, \(I\) represents the intensity at time \(t\), \(I_0\) is the initial intensity at time zero, \(t_0\) is time zero, \(\tau\) is the apparent lifetime, and \(b\) is a constant representing the offset intensity of the baseline.

**NMR Spectroscopy for Structure Determination**

The NMR spectra were collected at 25 °C on a Bruker Avance II 500 or cryo-probe equipped 800 MHz NMR spectrometers. The 1H, 15N, and 13C resonances of the apo CaM76-148 backbone were assigned using triple resonance experiments (HNCA, HN(CO)CA, HNCACB, HN(CO)CACB, HNCO, and HN(CA)CO) (Yamazaki et al., 1994) with the uniformly 15N and 13C-labeled apo CaM76-148 in complex with unlabeled Na,1.2IQp. 1H resonances were assigned from an 15N-edited TOCSY spectrum using a
uniformly $^{15}$N-labeled protein (Clore and Gronenborn, 1994) and from HA(CACO)NH experiment using a uniformly $^{15}$N and $^{13}$C-labeled sample. The side chain signals were assigned from 3D H(CCO)NH-TOCSY, C(CO)NH-TOCSY, HCCH-TOCSY, $^{15}$N-edited TOCSY, and $^{15}$N or $^{13}$C-edited NOESY spectra (Clore and Gronenborn, 1994; Fesik and Zuiderweg, 1988). The unlabeled Na$_v$1.2$_{	ext{iQp}}$ resonances were assigned from several 2D doubly $^{14}$N and $^{12}$C-filtered NOESY and TOCSY spectra acquired with mixing times of 80 to 120 ms for NOESY and 26 to 46 ms for TOCSY (Ikura and Bax, 1992; Vuister et al., 1994). The intermolecular NOEs were assigned from the $^{13}$C-edited and $^{12}$C,$^{14}$N-filtered 3D NOESY spectrum acquired with a mixing time of 140 ms (Vuister et al., 1994). All NMR spectra were processed with the NMRPipe program (Delaglio et al., 1995a) and analyzed using NMRView (Johnson and Blevins, 1994) and Sparky (Goddard and Kneller).

**Apo CaM$_{76-148}$ :Na$_v$1.2$_{	ext{iQp}}$ Structure Calculations**

Structures of the apo CaM$_{76-148}$ :Na$_v$1.2$_{	ext{iQp}}$ complex were generated using a torsion-angle molecular dynamics protocol (Karimi-Nejad et al., 1998; Stein et al., 1997) with the CNS program (Brunger et al., 1998). Structure calculations employed a total of 1812 NMR-derived distance restraints from the analysis of 3D $^{15}$N- and $^{13}$C-resolved NOESY spectra acquired with a mixing time of 120 ms (Fesik and Zuiderweg, 1988). The NOE-derived distance restraints were given upper bounds of 3.0, 4.0, 5.0, and 6.0 Å based upon the measured NOE intensities. From an analysis of the amide exchange rates measured from a series of $^{15}$N/$^1$HN HSQC spectra recorded after the addition of D$_2$O, 47 hydrogen bonds from the $\alpha$-helices were included in the structural calculations. In addition, 44 $\phi$ and $\psi$ angular restraints derived from an analysis of the C, N, C$_{\alpha}$, H$_{\alpha}$, and C$_{\beta}$ chemical shifts using the TALOS program (Cornilescu et al., 1999a; Shen et al., 2009b) were included in the structural calculations. A square-well potential was employed to constrain the NMR-derived distance restraints with F$_{\text{NOE}}$ set to 150 and 50
kcal mol\(^{-1}\) Å\(^{-2}\) during the stages of high temperature and slow-cooling Torsion Angle Dynamics (TAD) and the final stage of conjugate gradient minimization, respectively. Force constants of 100 and 200 kcal mol\(^{-1}\) rad\(^{-2}\) were applied to all torsional restraints during the stage of high temperature TAD and the rest stages of structural calculations, respectively.

Quantification of chemical shifts due to Na\(_{v,1.2}\)IQp or Ca\(^{2+}\) addition

To determine the change in chemical shift upon Na\(_{v,1.2}\)IQp binding to apo CaM\(_{76-148}\), or Ca\(^{2+}\) binding to apo CaM\(_{1-148}\), chemical-shift changes in both the \(^1\)H and \(^{15}\)N dimensions were quantified using the modified Pythagorean theorem shown in Equation 4.2, as described previously (Jaren et al., 2002).

\[
\Delta ppm = \sqrt{(\Delta H ppm)^2 + (0.10134 \cdot \Delta ^{15}N ppm)^2}
\]

In this equation, \(\Delta ppm\) refers to the linear change of a specific resonance peak from its initial starting position in the reference spectrum.

NaCl Titration of apo CaM-Na\(_{v,1.2}\)IQp Complex

Fluorescence anisotropy was used to examine the [NaCl] dependence of Na\(_{v,1.2}\)IQp binding to either apo CaM\(_{76-148}\) or apo CaM\(_{1-148}\). A Fluorolog 3 (Jobin Yvon, Horiba) spectrofluorimeter, equipped with dual auto-assembly Glan-Thompson polarizers was used to monitor the anisotropy change of a fluorescein-labeled Na\(_{v,1.2}\)IQp in 50mM HEPES, 1mM MgCl\(_2\), 5mM NTA, 50µM EGTA, pH 7.4 as it was titrated at 25 °C to a 1:1.2 ratio of Na\(_{v,1.2}\)IQp to either apo CaM\(_{76-148}\) or apo CaM\(_{1-148}\). The complex was then titrated with 50mM HEPES, 5M NaCl, 1mM MgCl\(_2\), 5mM NTA, 50µM EGTA, pH 7.4. The anisotropy of fluorescein-labeled Na\(_{v,1.2}\)IQp was monitored using \(\lambda_{ex}\) 498 nm and \(\lambda_{em}\) 520 nm with 2 nm excitation and 10 nm emission bandpasses. Anisotropy (r) was calculated as shown in Equation 4.3, described previously (Akyol et al., 2004)
where \( I_{VV} \) and \( I_{VH} \) are the intensities of vertically or horizontally emitted light upon vertical excitation respectively, and \( G \) is the instrument correction factor \((G = I_{HV} / I_{HH})\).

\[
r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}}
\]

(4.3)

**Results**

**The C-domain of apo CaM\textsubscript{1-148} is Necessary and Sufficient to Bind Na\textsubscript{v}1.2\textsubscript{IQp}**

Previous studies showed that both CaM\textsubscript{1-148} and a C-domain fragment (CaM\textsubscript{76-148}) exhibit tight, calcium-independent binding to Na\textsubscript{v}1.2\textsubscript{IQp} \((K_d \leq 10 \text{ nM})\), whereas an N-domain fragment of CaM (CaM\textsubscript{1-80}) binds weakly \((K_d \sim 1 \text{ mM})\), regardless of calcium concentration (Theoharis et al., 2008). To understand the structural differences at interfaces responsible for these affinities, \(^{15}\text{N}-\text{HSQC} \) spectra were collected of apo CaM\textsubscript{1-148} ± Nav\textsubscript{v}1.2\textsubscript{IQp} for comparison to spectra of apo CaM\textsubscript{1-80} and apo CaM\textsubscript{76-148}:Na\textsubscript{v}1.2\textsubscript{IQp} complex. When spectra of apo CaM ± Na\textsubscript{v}1.2\textsubscript{IQp} were overlaid (Figure 4.2a), many of the observed peaks for the apo CaM\textsubscript{1-148}:Na\textsubscript{v}1.2\textsubscript{IQp} complex were shifted relative to apo CaM\textsubscript{1-148} alone. Analyzing spectral overlap indicated that the chemical environment of the amide groups of the C-domain were all perturbed upon Na\textsubscript{v}1.2\textsubscript{IQp} addition, while those of the N-domain of apo CaM\textsubscript{1-148} were unperturbed by the peptide.

When spectra of the apo CaM\textsubscript{76-148}:Na\textsubscript{v}1.2\textsubscript{IQp} complex (Figure 4.2b) or apo CaM\textsubscript{1-80} (Figure 4.2c) were compared to the spectrum of apo CaM\textsubscript{1-148} bound to Na\textsubscript{v}1.2\textsubscript{IQp}, it was seen that the spectra were additive. Nearly all of the peaks in the samples of apo CaM\textsubscript{76-148}:Na\textsubscript{v}1.2\textsubscript{IQp} and apo CaM\textsubscript{1-80} alone overlaid upon the full set of those observed for the apo CaM\textsubscript{1-148}:Na\textsubscript{v}1.2\textsubscript{IQp} complex. These results indicated that the interaction of apo CaM\textsubscript{1-148} with Na\textsubscript{v}1.2\textsubscript{IQp} was mediated solely via the C-domain because the spectrum of apo CaM\textsubscript{1-148}:Na\textsubscript{v}1.2\textsubscript{IQp} complex could be reproduced by combining the spectra of apo CaM\textsubscript{1-80} and apo CaM\textsubscript{76-148}:Na\textsubscript{v}1.2\textsubscript{IQp} complex as seen in Figure 4.2d.
Previous studies of Na\textsubscript{v}1.2 IQp binding to CaM showed similar affinities for apo CaM\textsubscript{76-148} or apo CaM\textsubscript{1-148} indicating that the interaction of Na\textsubscript{v}1.2 IQp with apo CaM is mediated solely by the C-domain. These previous data, coupled with the new residue-specific observation that the N-domain of apo CaM\textsubscript{1-148} was unperturbed by Na\textsubscript{v}1.2 IQp binding prompted using NMR to determine the structure of apo CaM\textsubscript{76-148} bound to Na\textsubscript{v}1.2 IQp. Relative to using full-length CaM this complex reduced spectral complexity while still capturing the complete high-affinity binding interface that would exist in the apo CaM\textsubscript{1-148}:Na\textsubscript{v}1.2 IQp complex.

Comparison and quantification of $^{15}$N-HSQC spectra of apo CaM\textsubscript{76-148} $\pm$ Na\textsubscript{v}1.2 IQp indicated significant perturbation (average $\Delta$ppm for all residues was 0.51) upon addition of Na\textsubscript{v}1.2 IQp (Figure 4.3a and 4.3b). It is of interest to point out that Na\textsubscript{v}1.2 IQp addition dramatically increased dispersion of $^{15}$N-HSQC peaks (Figure 4.3a) by breaking the symmetry of similar chemical environments of homologous residues in the paired EF-hands of apo CaM\textsubscript{76-148}. Mapping of chemical shift perturbations upon the structure of apo CaM\textsubscript{76-148} bound to Na\textsubscript{v}1.2 IQp indicated that the highest chemical shift perturbation (average $\Delta$ppm of 1.47) was located in the loop region (residues 108-117) between helices F and G which is in close proximity to the Gln (Q) of the IQ-motif (Figure 4.3c).

**Hydrogen/Deuterium Backbone Amide Exchange**

Analysis of hydrogen/deuterium (H-D) backbone amide exchange of the apo CaM\textsubscript{76-148}:Na\textsubscript{v}1.2 IQp complex was performed to observe the location of persistent hydrogen bonds. These were used as structural restraints, and were analyzed to determine apparent amide exchange rates that correlate with protein packing and flexibility. A total of 30 amide resonances were detected ~30 minutes after addition to D$_2$O to a lyophilized sample of the apo CaM\textsubscript{76-148}:Na\textsubscript{v}1.2 IQp complex. Their identities were included as hydrogen bonding restraints for structure calculations. Apparent amide exchange rates (listed in Table 4.1) were calculated from peak decays such as those...
shown in Figure 4.4a, fit to a mono-exponential decay (Eq. 1, Figure 4.4b) and mapped onto the structure of the apo CaM76-148: Nav1.2IQp complex (Figure 4.4c). The residues with observable exchange rates indicate persistent secondary structure elements. These locations in combination with residue-specific dihedral angles calculated via TALOS (Cornilescu et al., 1999b; Shen et al., 2009a) from backbone chemical shifts were consistent with the pattern of secondary structure elements found in a 4-helix bundle with two anti-parallel β-sheets.

**Determination of apo CaM76-148: Nav1.2IQp Structure**

The structural statistics of the final apo CaM76-148: Nav1.2IQp complex structure are presented in Table 4.2. Favorable linewidths and dispersion exhibited in the NMR spectra greatly facilitated chemical shift assignment and NOE analysis as compared to analysis of CaM alone. Nearly complete $^1$H, $^{13}$C, and $^{15}$N resonance assignments were obtained for apo CaM76-148 when bound to Nav1.2IQp. This structure was determined based on 1974 unambiguous restraints comprised of 457 intra-residue, 298 short (+1), 300 medium (2-4), 331 long (5+), 245 intra-peptide, 188 intermolecular, 46 hydrogen bonds, and 109 dihedral angles. The distribution of restraints was in agreement with the occurrences of residues in a secondary structure element, where a higher number of restraints were observed in α-helical and β-sheet regions than in the loop regions that contain Ca$^{2+}$-binding sites III and IV. Figure 4.5a presents a superposition of the family of 20 lowest energy structures of Nav1.2IQp bound to apo CaM76-148 that best satisfied the experimental restraints. The interhelical angles as calculated by UCSF Chimera (Pettersen et al., 2004) between helices E-F and G-H of these structures were 77.4° ± 1.7 and 78.0° ± 2.3 respectively.

The structure of the apo CaM76-148: Nav1.2IQp complex was well defined, as reflected by the low value of the ensemble RMSD (0.65 ± 0.06 Å) and is of good quality, as indicated by the Ramachandran statistics and energetic terms listed in Table 4.2.
Frayed termini were observed at the N- and C-termini of apo CaM76-148 and Nav1.2IQp in Figure 4.5a. This was attributed to the absence of observable NOEs for these residues, and lack of assignments for peptide residues 1901-1903, 1922-1923, and 1926-1927.

**Interaction Interface of Na\textsubscript{v}1.2IQp with apo CaM\textsubscript{76-148}**

The hydrophobic interface between apo CaM\textsubscript{76-148} and Na\textsubscript{v}1.2IQp was well defined as judged by the positions of interacting residues shown in Figure 4.5b. The change in solvent accessible hydrophobic surface between apo CaM\textsubscript{76-148} and Na\textsubscript{v}1.2IQp was calculated using GETAREA (Fraczkiewicz and Braun, 1998). Upon binding the peptide, 1393 Å\textsuperscript{2} (751 Å\textsuperscript{2} Na\textsubscript{v}1.2IQp, 642 Å\textsuperscript{2} apo CaM\textsubscript{76-148}) of solvent accessible hydrophobic surface area was buried. In apo CaM\textsubscript{76-148}, a subset of hydrophobic residues that consisted of A88, V91, F92, L112 and M145 accounted for 43% of the buried surface, while in Na\textsubscript{v}1.2IQp, hydrophobic residues V1911, I1912, Y1916, Y1919, and L1920 accounted for 53.5% of the buried surface area.

In addition to hydrophobic interactions, favorable electrostatic interactions were observed between apo CaM\textsubscript{76-148} and Na\textsubscript{v}1.2IQp. Shown in Figure 4.5c are the electrostatic surface potentials calculated by APBS (Baker et al., 2001) for coordinates of apo CaM\textsubscript{76-148} and Na\textsubscript{v}1.2IQp corresponding to their conformation in the complex. Examination of the electrostatic potentials of solvent-accessible regions clearly showed that charge complementarity is present between the negatively charged apo CaM\textsubscript{76-148} and the positively charged Na\textsubscript{v}1.2IQp.

All contacts between apo CaM\textsubscript{76-148} and Na\textsubscript{v}1.2IQp that were within 4.5 Å were tabulated using the program *Contacts of Structural Units (CSU)* (Sobolev et al., 1999) and are shown in Figure 4.6a. The interactions of the IQ residues (I1912 and Q1913) with apo CaM\textsubscript{76-148} were of special interest due to their highly conserved nature in IQ-motifs as shown in Figure 4.1a and could be investigated on the basis of numerous NOEs such as those shown in Figure 4.6b and 4.6c. As indicated in the CSU analysis and
displayed in Figure 4.6d, I1912 inserted directly into the shallow hydrophobic pocket of apo CaM_{76-148}. As shown in Figure 4.6e, Q1913 is positioned to form hydrogen bonds with backbone atoms of residues L112 and E114 that are in the loop connecting helices F and G of apo CaM_{76-148}. A comparison of CaM sequences in 102 eukaryotic species (Ataman et al, Supplementary Table 1) showed that residue E114 and its preceding residue, G113, were identical in all species; while L112 was highly conserved (found in 91 of 102 sequences).

**Effect of Ca^{2+} upon apo CaM_{1-148}:Nav_{1.2}IQp Complex**

$^{15}$N-HSQC NMR spectroscopy was used to examine whether apo CaM_{1-148} bound to Nav_{1.2}IQp undergoes a structural transition as a result of Ca^{2+} binding. Shown in Figure 4.7a are spectral overlays of apo CaM_{1-148} (red) and (Ca^{2+})_{4}-CaM_{1-148} (black). Comparison of these indicated a significant change in the chemical environment of apo CaM_{1-148} resonances within the apo CaM_{1-148}:Nav_{1.2}IQp complex upon Ca^{2+} addition. To determine whether the chemical shifts observed upon Ca^{2+} addition to the apo CaM_{1-148}:Nav_{1.2}IQp complex are due to Ca^{2+} induced release of Nav_{1.2}IQp, the spectrum of free (Ca^{2+})_{4}-CaM_{1-148} was compared to the spectrum of the (Ca^{2+})_{4}-CaM_{1-148}:Nav_{1.2}IQp complex (Figure 4.7a). Lack of spectral overlap in Figure 4.7a indicated that both apo CaM_{1-148} and (Ca^{2+})_{4}-CaM_{1-148} were bound toNav_{1.2}IQ, but that their structures were significantly different.

Shown in Figures 4.7b, 4.7c, and 4.7d are plots showing the HSQC signal for selected resonances of the N- and C-domain of apo CaM_{1-148} when bound to Nav_{1.2}IQp at successively higher levels of calcium during a titration. Shown in Figure 4.7b are residues of the N-domain of apo CaM_{1-148} that were uniformly in fast-exchange over the course of the calcium titration. The normalized change in chemical shift plotted against the equivalents of calcium added indicated that most residues within the N-domain titrated fully between 0 and 2 Ca^{2+} equivalents.
Observation of changes in position and intensity of C-domain peaks during the calcium titration (Figure 4.7c) revealed that they were in intermediate or slow exchange. For those, calcium-dependent change in intensities of peaks were used to determine their relative populations over the course of the titration. For apo CaM1-148 bound to Na\textsubscript{v}1.2\textsubscript{IQp}, peaks corresponding to the C-domain of the apo state broadened beyond the limit of detection after addition of 2 Ca\textsuperscript{2+} equivalents (Figure 4.7c). Although amide backbone assignments for the C-domain of (Ca\textsuperscript{2+})\textsubscript{4}-CaM1-148 bound to Na\textsubscript{v}1.2\textsubscript{IQp} are unknown, peaks that appeared at the midpoint of the Ca\textsuperscript{2+} titration are likely to correspond to residues located within the C-domain of (Ca\textsuperscript{2+})\textsubscript{4}-CaM1-148. When the intensities of these peaks were plotted against the ratio of [Ca\textsuperscript{2+}]/[CaM1-148:Na\textsubscript{v}1.2\textsubscript{IQp}], increases in peak intensity were observed at higher calcium stoichiometries than were chemical shift changes. These data indicated that when bound to Na\textsubscript{v}1.2\textsubscript{IQp} the N- and C-domains of CaM1-148 bind 2 Ca\textsuperscript{2+} per domain, where the N-domain has a slightly more favorable Ca\textsuperscript{2+}-binding affinity than the C-domain, consistent with species population simulations conducted previously (Theoharis et al., 2008).

**Effect of Ca\textsuperscript{2+} upon Molecular Size of CaM\textsubscript{1-148:Na\textsubscript{v}1.2\textsubscript{IQp} Complex**

To examine whether Ca\textsuperscript{2+} binding causes the N-domain of the (Ca\textsuperscript{2+})\textsubscript{4}-CaM1-148 to collapse onto Na\textsubscript{v}1.2\textsubscript{IQp} forming a compact ellipsoidal structure, CaCl\textsubscript{2} was titrated into a solution of preformed apo CaM1-148:Na\textsubscript{v}1.2 complex and monitored with fluorescence anisotropy. As shown in Figure 4.8a, apo CaM1-148 bound to the fluoresceinated Na\textsubscript{v}1.2\textsubscript{IQp} stoichiometrically at a 1:1 ratio. Following complex formation, the apo CaM-IQ complex was titrated with 10 mM CaCl\textsubscript{2} in matching buffer (Figure 4.8a). No significant decrease in fluorescence anisotropy was observed, indicating that (Ca\textsuperscript{2+})\textsubscript{4}-CaM1-148 maintains the hydrodynamic behavior of apo CaM1-148 bound to fl-Na\textsubscript{v}1.2\textsubscript{IQp}.
This suggested that $(\text{Ca}^{2+})_4\text{CaM}$ does not adopt a compact ellipsoidal structure when bound to $\text{Na}_v1.2_{\text{IQp}}$ on the basis of raw anisotropy.

**Effect of Na$^+$ upon Na$_v1.2_{\text{IQp}}$ Binding to CaM**

$\text{Na}_v1.2$ and other proteins in the plasma membrane experience a large fluctuation in Na$^+$ ion concentration during $\text{Na}_v1.2$ gating. Thus, it was of interest to determine whether NaCl affected the affinity of $\text{Na}_v1.2_{\text{IQp}}$ for apo or calcium-saturated CaM. Shown in Figure 4.8b are NaCl titrations of complexes of $\text{Na}_v1.2_{\text{IQp}}$ binding apo CaM$_{76-148}$ or apo CaM$_{1-148}$ as monitored by fluorescence anisotropy to examine the NaCl dependence of $\text{Na}_v1.2_{\text{IQp}}$ dissociation. Both apo CaM$_{76-148}$ and apo CaM$_{1-148}$ bound to the fluoresceinated $\text{Na}_v1.2_{\text{IQp}}$ stoichiometrically at a 1:1 ratio. Saturation of $\text{Na}_v1.2_{\text{IQp}}$ was ensured by the addition of a slight excess of either apo CaM$_{76-148}$ or apo CaM$_{1-148}$, then the complex was titrated with NaCl in matching buffer to cover a range from 0 to 650 mM NaCl (Figure 4.8b). This NaCl range was used in attempt to dissociate apo CaM from $\text{Na}_v1.2_{\text{IQp}}$, but proved unsuccessful as the cuvette volume limited the amount of NaCl solution that could be added resulting in 650 mM being the maximum NaCl examined. In NaCl titrations of complexes of $\text{Na}_v1.2_{\text{IQp}}$ bound to apo CaM$_{76-148}$ and apo CaM$_{1-148}$, the anisotropy of $\text{Na}_v1.2_{\text{IQp}}$ was unchanged by NaCl, which indicated a negligible effect on $\text{Na}_v1.2_{\text{IQp}}$ dissociation from CaM at the final concentration of peptide and protein.

**Discussion**

The voltage-dependent sodium channel $\text{Na}_v1.2$ contains a CaM-binding IQ-motif that is required for proper regulation of its physiological function. The high-resolution solution structure of apo CaM$_{76-148}$ bound to $\text{Na}_v1.2_{\text{IQps}}$ presented here demonstrates that apo CaM$_{76-148}$ adopts a “semi-open” conformation when bound to $\text{Na}_v1.2_{\text{IQp}}$, and that it binds such that the F-G loop interacts directly with the glutamine residue of the IQ motif. The structure of this apo CaM$_{76-148}$:$\text{Na}_v1.2_{\text{IQp}}$ complex provides a foundation for studying
other isoforms of voltage-dependent ion channels that interact preferentially with the C-domain of apo CaM.

**A “semi-open” apo C-domain Binds Na,1.2IQp.**

Shown in Figure 4.9a are superpositions of the backbone of (a) apo CaM\textsubscript{76-148} bound to Na,1.2IQp determined in this study (b) the C-domains of two apo CaM\textsubscript{1-148} molecules bound to two neighboring IQ-motifs found in myosin V (2IX7.pdb), (c) apo CaM-like proteins bound to IQ-motifs of MYO2P (1M46.pdb, 1M45.pdb, and 1N2D.pdb) and (d) ELC bound to myosin heavy chain. A feature common to all of these structures is that each set of paired EF-hands of the C-domain adopts a “semi-open” conformation when bound to its respective IQ-motif. Another feature common to all structures shown in Figure 4.9a is that residues located at positions “0” and “1” (defined in Figure 4.1a) of the IQ-motif interact with a similar subset of residues in the apo C-domain. These conserved structural features are also reflected in the sequence conservation of the IQ-motif at these positions Figure 4.1a.

At position “0” we observed that a hydrophobic residue (often a branched chain) is necessary to insert into the exposed hydrophobic core of the “semi-open” C-domain, burying what would otherwise be solvent exposed hydrophobic surface. The absolute conservation of Gln at position “1” of canonical IQ-motifs is reflected by hydrogen bonding interactions made between the carboxamide of Gln at position “1” and the backbone of residues found within the loop connecting helices F and G as shown in Figure 4.6e. Formation of this hydrogen bonding network between Gln at position “1” and the backbone of the loop residues account for the large amide chemical shift at this position observed in the $^{15}$N-HSQC spectrum shown in Figure 4.3a, 4.3b, and 4.3c.
A “Semi-open” apo C-domain is used to Bind a Non IQ-motif Containing Target

There is an example of 1 apo C-domain bound to a peptide that does not have an IQ-motif in the structure of partially Ca\(^{2+}\)-saturated CaM bound to a peptide derived from the small conductance potassium channel (SK-Channel) (Schumacher et al., 2001). When Na\(_v\)1.2IQp bound apo CaM\(_{76-148}\), and the apo C-domain of CaM bound to the SK-channel peptide (SK\(_p\)) were overlaid (Figure 4.9b), both adopt “semi-open” domain conformations. This structural similarity indicates that an IQ-motif is sufficient but not necessary to induce a “semi-open” apo C-domain conformation. Additionally the structural similarity in Figure 4.9b, suggests that a “semi-open” C-domain conformation is not exclusive to IQ-motifs alone but quite possibly all motifs that bind to the C-domain of apo CaM.

Although the SK-Channel does not contain an IQ-motif, it shares key features seen in IQ-motif bound structures of apo CaM, CaM-like proteins, and ELC. Figures 4.9a, and 4.9b, show that a carboxamide-containing side chain of Gln (IQ-motif) or Asn (SK\(_p\)) are required to form a hydrogen bond with the backbone of loop residues located between the F and G helices of CaM. This conserved hydrogen-bonding network between the side chain carboxamide and loop backbone of CaM helps to determine the \(\alpha\)-helical register of the IQ-motif or SK\(_p\) relative to the apo C-domain.

The similarity in \(\alpha\)-helical register between the IQ-motif and SK\(_p\) was also apparent in a conserved hydrophobic interaction between either I1912 (Na\(_v\)1.2IQp) or L428 (SK\(_p\)) and the core of the apo C-domain (Fig 4.9b). Due to the polarity of the N- and C-terminus of their respective motifs, I1912 or L428 differ in primary sequence position relative to the highly conserved carboxamide side chain used to hydrogen bond to the backbone of residues L112 and E114 of CaM (Figure 4.9b). For ease in describing the reversal in polarity between Na\(_v\)1.2IQp and SK\(_p\) relative to the C-domain, we have termed the interactions of Na\(_v\)1.2IQp and SK\(_p\) with apo CaM\(_{76-148}\) as NF-GC and
cF-G_N respectively. These terms result from positioning the 4-helix bundle of the C-domain as depicted in Figure 4.9c, where dependent upon polarity of the peptide N- and C-terminus relative to helices F and G of CaM either a _N_ F-G_C or _c_ F-G_N orientation is adopted.

When an _N_ F-G_C orientation is adopted, as in the interaction of apo CaM with Na_v.1.2IQp, the conserved hydrophobic residue (I1912) that inserts in the core of apo CaM_76-148_ precedes the conserved carboxamide-containing residue (Q1913). However if the peptide polarity is reversed as seen the _c_ F-G_N orientation of SK_p relative to the C-domain of apo-CaM, the residue homologous to I1912 of Na_v.1.2IQp, (L428) is located 2 amino acids away on the C-terminal side of the carboxamide-containing residue (N426).

**Canonical IQ-motifs Bind to apo CaM using Similar Orientations**

Shown in Figure 4.10 are structures of apo and (Ca^{2+})_4-CaM bound to IQ-motif containing peptides derived from various targets, where the directionality of the target interaction with CaM is depicted with right or left arrows respectively. Depicted in Figure 4.9a, are conserved interactions made by all apo CaM or apo CaM-like proteins when bound to IQ-motifs. These conserved features consist of a hydrophobic interaction at IQ-motif position “0” of the peptide contacting the core of CaM as well as the hydrogen bond network between the ultra-conserved Gln residue at position “1” and CaM loop residues L112 and E114 or their CaM-like protein equivalent. If these conserved interactions are maintained throughout all canonical apo CaM binding motifs then all canonical IQ-motifs must bind to the C-domain of apo CaM in a _N_ F-G_C orientation.

Examination of the binding orientation of (Ca^{2+})_4-CaM bound to IQ-motif containing structures is less clear as structures of (Ca^{2+})_4-CaM bound to the IQ-motifs of Ca_v.2.1 and 2.3 differ as to the peptide orientation relative to (Ca^{2+})_4-CaM (Figure 4.10). Ca_v.2.1 and 2.3 peptides of differing length as well as different crystallization conditions
were used to obtain these structures which may account for this discrepancy (Fallon et al., 2009; Fallon et al., 2005; Halling et al., 2009; Houdusse et al., 2006; Kim et al., 2008; Mori et al., 2008; van Petegem et al., 2005). We propose apo and (Ca\(^{2+}\))\(_4\)-CaM bind to Na\(_v\).1.2\(_{IQp}\) and canonical IQ-motifs where a Gln is located at position “1” in a NF-GC orientation. The alternative would require CaM to release from its anti-parallel orientation to the IQ-motif upon Ca\(^{2+}\) influx, and reorient itself into a parallel arrangement for binding to occur.

**Role of the N-domain of CaM\(_{1-148}\) in Na\(_v\).1.2 Regulation**

\(^{15}\)N-HSQC and fluorescence anisotropy monitored Ca\(^{2+}\) titration studies shown in Figures 4.7a and 4.8a, indicated that the N-domain of both apo and (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) do not interact with Na\(_v\).1.2\(_{IQp}\). These observations are consistent with previous studies that indicated that a region consisting of residues 1913-1938 in Na\(_v\).1.2 contains a second CaM-binding BAA-motif (Na\(_v\).1.2\(_{BAA}\)) (Mori et al., 2003; Mori et al., 2000). Based on results presented here, we propose the model presented in Figure 4.9d.

In this model, only the “semi-open” C-domain of apo CaM interacts with the IQ-motif of Na\(_v\).1.2, while under Ca\(^{2+}\)-saturating conditions both CaM N- and C-domains adopt “open” conformations when they bind to the BAA and IQ-motifs of Na\(_v\).1.2 respectively. This model is supported by observations in Figure 4.2 where only the C-domain CaM\(_{1-148}\) is perturbed upon Na\(_v\).1.2\(_{IQp}\) addition, while Figure 4.3 shows that the apo C-domain adopts a “semi-open” conformation when bound to Na\(_v\).1.2\(_{IQp}\). Support for the proposed (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) interactions with Na\(_v\).1.2 are drawn from Figure 4.7 which indicated CaM\(_{1-148}\) binds 4 Ca\(^{2+}\) ions and Figure 4.8a, which showed that the N-domain of (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) does not interact with Na\(_v\).1.2\(_{IQp}\) by collapsing upon it after Ca\(^{2+}\) addition. These observations coupled with studies by Mori et al. (Mori et al., 2003) which showed that (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) bound to both Na\(_v\).1.2\(_{IQp}\) and Na\(_v\).1.2\(_{BAA}\), while only
apo CaM_{1-148} bound to Na_{v}1.2_{IQp} provide strong evidence for the model proposed in Figure 4.9d.

Future studies will focus on uncovering the mechanistic role that CaM plays in Na_{v}1.2 regulation under Ca^{2+}-saturating conditions as well as determination of structures of larger intracellular regions of Na_{v}1.2 in complex with CaM. To accomplish these goals, studies to establish the location of the N-domain binding site within Na_{v}1.2, coupled with structure determination of (Ca^{2+})_{4}-CaM in complex with a peptide that contains both the N-domain binding site and Na_{v}1.2_{IQp} are proposed.
Table 4.1: Apparent amide exchange rates of apo CaM$_{76-148}$ when bound to Na$_{v}$1.2$_{10p}$

<table>
<thead>
<tr>
<th>Residue</th>
<th>Rate*</th>
<th>Error*</th>
</tr>
</thead>
<tbody>
<tr>
<td>86</td>
<td>69.1</td>
<td>0.9</td>
</tr>
<tr>
<td>87</td>
<td>138.9</td>
<td>1.5</td>
</tr>
<tr>
<td>88</td>
<td>162.9</td>
<td>1.7</td>
</tr>
<tr>
<td>89</td>
<td>180.6</td>
<td>2.4</td>
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<tr>
<td>90</td>
<td>122.9</td>
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</tr>
<tr>
<td>91</td>
<td>16.7</td>
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<tr>
<td>92</td>
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<tr>
<td>93</td>
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<td>0.8</td>
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<tr>
<td>99</td>
<td>85.6</td>
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<tr>
<td>100</td>
<td>382.6</td>
<td>5.5</td>
</tr>
<tr>
<td>103</td>
<td>247.4</td>
<td>26.2</td>
</tr>
<tr>
<td>105</td>
<td>3.6</td>
<td>0.5</td>
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<tr>
<td>106</td>
<td>30.9</td>
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<tr>
<td>108</td>
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<td>125.1</td>
<td>2.4</td>
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<tr>
<td>110</td>
<td>17.9</td>
<td>0.4</td>
</tr>
<tr>
<td>111</td>
<td>141.4</td>
<td>7.8</td>
</tr>
<tr>
<td>112</td>
<td>20.2</td>
<td>0.4</td>
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<table>
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<th>Residue</th>
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<th>Error*</th>
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</thead>
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<td>118</td>
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<td>0.4</td>
</tr>
<tr>
<td>121</td>
<td>243.7</td>
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<tr>
<td>146</td>
<td>69.6</td>
<td>1.0</td>
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*Values reported in minutes
Table 4.2: Structural statistics and root-mean-square deviation for 20 structures of apo CaM<sub>76-148</sub>:Nav<sub>1.2</sub>IQ<sub>p</sub> complex

<table>
<thead>
<tr>
<th>Structural statistics&lt;sup&gt;a&lt;/sup&gt;</th>
<th>&lt;SA&gt;</th>
<th>&lt;SA&gt;&lt;sub&gt;r&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rmsd from experimental distance restraints (Å)&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
</tr>
<tr>
<td>All (1865)</td>
<td>0.008 ± 0.001</td>
<td>0.007</td>
</tr>
<tr>
<td>CaM intra-residue (457)</td>
<td>0.007 ± 0.002</td>
<td>0.005</td>
</tr>
<tr>
<td>CaM sequential (298)</td>
<td>0.005 ± 0.004</td>
<td>0.003</td>
</tr>
<tr>
<td>CaM medium range (300)</td>
<td>0.009 ± 0.002</td>
<td>0.008</td>
</tr>
<tr>
<td>CaM long range (331)</td>
<td>0.006 ± 0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>Intra-peptide (245)</td>
<td>0.005 ± 0.002</td>
<td>0.003</td>
</tr>
<tr>
<td>CaM-peptide intermolecular (188)</td>
<td>0.012 ± 0.002</td>
<td>0.011</td>
</tr>
<tr>
<td>hydrogen bond (46)</td>
<td>0.016 ± 0.001</td>
<td>0.021</td>
</tr>
<tr>
<td>Rmsd from experimental torsional angle restraints (deg)&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>φ and ψ angles (109)</td>
<td>0.3 ± 0.03</td>
<td>0.2</td>
</tr>
<tr>
<td>CNS potential energies (kcal mol&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E&lt;sub&gt;tot&lt;/sub&gt;</td>
<td>89 ± 4.8</td>
<td>80</td>
</tr>
<tr>
<td>E&lt;sub&gt;bond&lt;/sub&gt;</td>
<td>5 ± 0.5</td>
<td>1</td>
</tr>
<tr>
<td>E&lt;sub&gt;ang&lt;/sub&gt;</td>
<td>65 ± 2.9</td>
<td>62</td>
</tr>
<tr>
<td>E&lt;sub&gt;imp&lt;/sub&gt;</td>
<td>5 ± 0.6</td>
<td>4</td>
</tr>
<tr>
<td>E&lt;sub&gt;repel&lt;/sub&gt;</td>
<td>6 ± 1.2</td>
<td>6</td>
</tr>
<tr>
<td>E&lt;sub&gt; noe&lt;/sub&gt;</td>
<td>6 ± 1.2</td>
<td>5</td>
</tr>
<tr>
<td>E&lt;sub&gt;cdih&lt;/sub&gt;</td>
<td>1 ± 0.1</td>
<td>1</td>
</tr>
<tr>
<td>Cartesian coordinate rmsd (Å)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;SA&gt; vs. &lt;SA&gt;&lt;sub&gt;r&lt;/sub&gt;</td>
<td>0.31 ± 0.05</td>
<td>0.95 ± 0.11</td>
</tr>
</tbody>
</table>

<sup>a</sup>Where <SA> is the ensemble of 20 NMR-derived solution structures of CaM/peptide; <SA> is the mean atomic structure; <SA><sub>r</sub> is the energy-minimized average structure. The CNS Frepel function was used to simulate van der Waals interactions using a force constant of 4.0 kcal mol<sup>-1</sup> Å<sup>−4</sup> with the atomic radii set to 0.8 times their CHARMM values (Brooks et al., 1983).

<sup>b</sup>Distance restraints were employed with a square-well potential (F<sub> noe </sub> = 50 kcal mol<sup>-1</sup> Å<sup>−2</sup>). Hydrogen bonds were given bounds of 1.8-2.4 Å (H-O) and 2.7-3.3 Å (N-O). No distance restraint was violated by more than 0.3 Å in any of the final structures.

<sup>c</sup>Torsional restraints were applied with values derived from an analysis of the C’, N, C<sub>α</sub>, H<sub>a</sub>, and C<sub>b</sub> chemical shifts using the TALOS program. Force constant of 200 kcal mol<sup>−1</sup> rad<sup>−2</sup> was applied for all torsional restraints.

<sup>d</sup>Rmsd for CaM protein residues 80-128 and 134-146 and peptide residues 1905-1920.
Figure 4.1: CaM and target interaction background
A: Consensus sequence of 208 canonical IQ-motifs derived from 108 proteins of the human genome.
B-C: Structures of CaM (residues 1-75 blue, 76-80 black, 81-148 red) in the absence and presence of peptide targets (green, Ca$^{2+}$ yellow spheres).

/Users/nmr_mike/Thesis/Chapter_IV/Figure4_1.jpg
Figure 4.2: $^{15}$N-HSQC Spectra of apo CaM76-148 and apo CaM1-148 bound to Na$_v$1.2IQp

A: Overlaid $^{15}$N-HSQC spectra of apo CaM1-148 alone (black) and apo CaM1-148 bound to Na$_v$1.2IQp (red).

B: Overlaid $^{15}$N-HSQC spectra of apo CaM76-148 (green) and apo CaM1-148 when bound to Na$_v$1.2IQp. Overlapping resonances are indicated in solid circles, while non-overlapping residues of apo CaM76-148 bound to Na$_v$1.2IQp are indicated by solid hexagons.

C: Overlaid $^{15}$N-HSQC spectra of apo CaM1-80 (blue) and apo CaM1-148 bound to Na$_v$1.2IQp (red). Overlapping resonances are indicated by dashed squares, while non-overlapping apo CaM1-80 resonances are indicated by solid hexagons.

D: Overlaid $^{15}$N-HSQC spectra of apo CaM1-80 (blue), apo CaM76-148 bound to Na$_v$1.2IQp (green), and apo CaM1-148 bound to Na$_v$1.2IQp bound to Na$_v$1.2IQp. Resonances of apo CaM1-80 which overlap with those of apo CaM1-148 bound to Na$_v$1.2IQp are shown in dashed squares, while resonances of apo CaM76-148 bound to Na$_v$1.2IQp that overlay onto apo CaM1-148 bound to Na$_v$1.2IQp are shown in solid circles.

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Figure 4.3: Na\textsubscript{v}1.2\textsuperscript{IQp} binding to apo CaM\textsubscript{76-148} quantified by \textsuperscript{15}N-HSQC spectroscopy

A: Overlaid \textsuperscript{15}N-HSQC spectra of apo CaM\textsubscript{76-148} (blue) and apo CaM\textsubscript{76-148} bound to Na\textsubscript{v}1.2\textsuperscript{IQp} (red).

B: Quantified apo CaM\textsubscript{76-148} amide proton chemical shifts upon Na\textsubscript{v}1.2\textsuperscript{IQp} addition.

C: Magnitude of Na\textsubscript{v}1.2\textsuperscript{IQp} induced chemical shift mapped to the structure of apo CaM\textsubscript{76-148} bound to Na\textsubscript{v}1.2\textsuperscript{IQp} (gray rod) with I and Q residues of the IQ-motif shown in ball and stick.

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Figure 4.4: $^{15}$N-HSQC-monitored amide exchange of apo CaM$_{76-148}$:Nav$_{1.2IQp}$ complex

**A:** $^{15}$N-HSQC monitored amide exchange series of select residues peak intensities as a function of exchange time corresponding to different exchange regimes.

**B:** Fitted exchange curves and rates for residues shown in panel A.

**C:** Magnitude of observed exchange rates mapped to the structure of apo CaM$_{76-148}$ bound to Na$_v$1.2IQp (green rod) with I and Q residues of the IQ-motif shown in ball and stick.

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Figure 4.5: Solution structure of apo CaM_{76-148} bound to Na_v1.2_{IQp}
A: Ensemble of 20 lowest energy structures of apo CaM_{76-148} (black) bound to Na_v1.2_{IQp} (red)  B: Hydrophobic interaction interfaces of 20 lowest energy structures of apo CaM_{76-148} (red) bound to Na_v1.2_{IQp} (green).  C: Electrostatic potentials of apo CaM_{76-148} (left), and Na_v1.2_{IQp} (middle, and right) in 150mM NaCl, pH 6.5.

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Figure 4.6: Binding interfaces of apo CaM_{76-148} and Na_{1.2}IQp  
A: apo CaM_{76-148} residues (red) ≤ 4.5 Å of Na_{1.2}IQp (black)  
B: Select NOE peak between I1912 of Na_{1.2}IQp and apo CaM_{76-148} where individual residue unfiltered and filtered $^{13}$C-NOESY spectral strips are outlined in blue and red respectively.  
C: Select NOE peaks between Q1913 of Na_{1.2}IQp and apo CaM_{76-148}, where individual $^{15}$N-NOESY spectral strips are outlined in black.  
D: Location of apo CaM_{76-148} residues shown in panel B in relation to I1912.  
E: Location of apo CaM_{76-148} residues shown in panel C in relation to Q1913, shown in dashed lines are hydrogen bonds made by the carboxamide of Q1913 to the backbone of residues L112, and E114.

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Figure 4.7: Effect of Ca\(^{2+}\) upon apo CaM\(_{76-148}\) when bound to Na\(_{v}1.2\) IQp

A: \(^{15}\)N-HSQC spectral overlay of apo (red) and (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) (black) bound to Na\(_{v}1.2\) IQp (left). \(^{15}\)N-HSQC spectral overlay of apo CaM\(_{1-148}\) (blue) and (Ca\(^{2+}\))\(_4\)-CaM\(_{1-148}\) bound to Na\(_{v}1.2\) IQp (right).

B: \(^{15}\)N-HSQC Monitored Ca\(^{2+}\) titration of select N-domain residues (upper) with quantified change in chemical shift as Ca\(^{2+}\) was added (lower).

C: \(^{15}\)N-HSQC Monitored Ca\(^{2+}\) titration of select C-domain residues (upper) with quantified change in chemical shift as Ca\(^{2+}\) was added (lower), lines are added to guide the eye.

D: \(^{15}\)N-HSQC Monitored Ca\(^{2+}\) titration of residues from the C-domain of CaM, but whose individual residue identity is unknown (upper) with quantified change in chemical shift as Ca\(^{2+}\) was added (lower).
Figure 4.8: Fluorescence anisotropy monitored titration of apo CaM bound to Na$_{v}$1.2$\text{I}_{op}$

A: Fluorescence anisotropy monitored apo CaM$_{1-148}$ titration of fluoresceinated Na$_{v}$1.2$\text{I}_{op}$ (left) followed by CaCl$_2$ titration of apo CaM$_{76-148}$:Na$_{v}$1.2$\text{I}_{op}$ complex (right).

B: Fluorescence anisotropy monitored of fluoresceinated Na$_{v}$1.2$\text{I}_{op}$ (left) with apo CaM followed by NaCl titration of the apo CaM:Na$_{v}$1.2$\text{I}_{op}$ complex (right).

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Figure 4.9: Superposition of target bound apo CaM and CaM-like protein C-domains
A: Superposition of apo CaM76-148 (red):Nav1.2IQp (green rod) complex onto C-domains of apo CaM and apo CaM-like proteins (gray) bound to canonical IQ-motifs. I and Q residues of all IQ-motifs are shown in sticks, the Cα atom of residue 113 is shown as a sphere as a point of reference. Pdb files used in overlay 2IX7, 1M45, 1M46, 1ND2, and 3JVT. B: Superposition of apo CaM76-148 (red):Nav1.2IQp (green rod) complex and the C-domain of partially Ca2+-saturated CaM1-148 (orange) bound to the SK-Channel peptide (cyan). Residues at positions I and Q of IQ-motif and their SK-channel homologs are shown in sticks, the Cα atom of residue 113 is shown as a sphere as a point of reference. The aligned primary sequences of Nav1.2IQp and SK-channel are shown where residues at IQ-motif defining position are shown in bold, and essential IQ-motif residues or their homolog are boxed. C: Structural depiction of naming scheme used to describe peptide polarity when interacting with the C-domain of CaM. The N- and C-termini of a peptide (blue and magenta respectively) can orient themselves in 2 possible ways with respect to helices F (red) and G (orange) of the C-domain resulting in either a N-F-GC or C-F-GN orientation. D: Proposed model of CaM1-148 interaction with the C-terminal tail of Na1.2 under apo and Ca2+-saturating conditions.
Figure 4.10: Directionality of IQ-motif binding to the C-domain of CaM

The polarity of IQ-motif containing helices is indicated by the color gradient where the N-terminus is shown in blue and C-terminus in purple. CaM helices F and G are colored red and orange, while the I and Q of each IQ-motif are shown as spheres colored cyan and green respectively.

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CHAPTER V
INFLUENCE OF ELECTROSTATIC INTERACTIONS ON
CA\(^{2+}\) AND TARGET BINDING BY CAM

**Introduction**

CaM is a Ca\(^{2+}\) sensor protein that is essential to eukaryotic signal transduction pathways (Pedigo et al., 1992). Changes in intra-cellular Ca\(^{2+}\) levels are linked to cellular events by the effect of Ca\(^{2+}\) on CaM: it triggers conformational changes that expose hydrophobic surfaces in both domains of CaM, altering its binding to many target proteins (VanScyoc and Shea, 2001a; Yagi et al., 1990). In addition to the widely accepted role of hydrophobic interactions in recognition and binding of targets by CaM, electrostatic interactions have an important role in these processes as well (André et al., 2004; Linse et al., 1988; Ogawa and Tanokura, 1984). At physiological conditions (pH 7, 100mM KCl) CaM is a negatively charged protein (pI=4) with net charges of -24 (apo) and -16 (Ca\(^{2+}\)-saturated) while peptide derivatives from the CaMBD’s of its targets typically carry a complementary positive charge (Nav1.2IQp = +8, CaMKIIp = +6). Compared to hydrophobic interactions alone, electrostatic interaction between CaM and its targets allow for recognition of each other at much greater distances, increasing the rate at which they associate (André et al., 2004; Antosiewicz and McCammon, 1995). The strength of electrostatic attraction between CaM and its targets is particularly interesting as it can be influenced in the cell due to spatial and temporal fluctuations in ionic concentrations due to naturally occurring currents through ion channels(Akyol et al., 2004; Cens et al., 2006; Levitan, 1999; Saimi and Kung, 2002).

Hydrophobic and electrostatic interactions are the major forces responsible for CaM-target recognition and binding affinity (Bayley et al., 1996; Tjandra et al., 1999; Yagi et al., 1990). Electrostatic interactions between CaM and its targets guide hydrophobic surfaces located on CaM and its targets into close proximity so that they can interact. Due to their α-helical character, CaMBD’s contain a periodicity within their primary amino acid sequence...
producing a hydrophobic face that is buried from the solvent upon binding to CaM and a basic face that remains solvent exposed. This periodicity has resulted in multiple CaM binding motifs that have been characterized based upon the numerical position of the hydrophobic residues within CaMBD’s that insert into the hydrophobic pockets of the N- and C-domains of CaM (Figure 5.1). This periodicity can also be visualized in helical wheel diagrams of the CaMBDs of proteins regulated by CaM (Figure 5.2). The regions between hydrophobic “anchor” positions are enriched with positively charged residues creating charge complementarity between CaM and its target (Yamniuk and Vogel, 2004).

The extent to which electrostatic interactions contribute to CaM-target recognition and binding affinity may significantly change in the cell due to ion fluxes necessary for the generation and propagation of action potentials (Andre et al., 2006; Suizu et al., 1995). The ions that undergo the largest change in intra and extracellular concentration involved in this process are Na⁺, K⁺, Ca²⁺, and Cl⁻. Shown in Figure 5.3 is a schematic illustrating the ionic strength and concentration gradients present in a resting cell (Hille, 2001).

Large changes in local intracellular ion concentration occur near ion channels as they open to allow their specific ions to pass through the membrane. Given that CaM is an intrinsic subunit of several known Na⁺, K⁺, and Ca²⁺ channels, fluctuations in the strength of electrostatic interaction may alter occupancy of CaM at CaMBDs on these channels (Ehlers et al., 1996; Halling et al., 2005; Pitt, 2005; Saimi and Kung, 1994; Theoharis et al., 2008). To investigate whether these fluctuations significantly alter the affinity of CaM binding to CaMKIIp or Na,1.2IQp, fluorescence anisotropy monitored titrations as a function of varied salt were performed.

**Poisson-Boltzmann Equation**

Electrostatic interactions can be observed between virtually all interacting macromolecules. Creighton has estimated that more than 20% of all amino acids in globular proteins are ionized at physiological pH (Creighton, 1993). Structural methods such as NMR,
and X-ray crystallography are able to provide the spatial arrangement of charged amino acid
groups within a protein or protein-ligand complex, allowing for the calculation of electrostatic
potentials that can be mapped to the macromolecular surface. Solution of Poisson-Boltzmann
equation was first described by Gouy (1910) and Chapman (1913) (Chapman, 1913; Gouy,
1910) allows for the calculation of the electrostatic potential of a macromolecule throughout the
calculated space, as well the distribution the local ions around the macromolecule.

The Poisson-Boltzmann equation (Equation. 5.1)

\[-\nabla \cdot \varepsilon(\chi)\nabla \phi(\chi) + \kappa^{-2}(\chi) \sinh \phi(\chi) = f(\chi)\]

is a second-order nonlinear elliptic partial differential equation that relates the electrostatic
potential ($\phi$) to the dielectric properties of the solute and solvent ($\varepsilon$), the ionic strength of the
solution and the accessibility of ions to the solute interior ($\kappa^{-2}$), and the distribution of solute
atomic partial charges ($f$). To expedite solution of the equation, the nonlinear PBE is often
approximated by the linearized PBE (LPBE) by assuming $\sinh \phi(\chi) \approx \phi(\chi)$. Pioneering work in
this area has been done by many groups including Honig and McCammon. Their studies allow
the calculation of electrostatic potentials of macromolecules such as nucleic acids and proteins
(Allison et al., 1988; Gilson and Honig, 1988; Honig and Nicholls, 1995; Sharp and Honig,
1990). In addition to visualization of electrostatic potentials of a macromolecule in solutions of
varying ionic strength, solution of the linear or non-linear Poisson-Boltzmann equation allows
for calculation of macromolecular solvation energies.

Determination of the solvation energies of the macromolecular complex and the
individual components that comprise them can be used via linkage analysis to calculate the
contribution of electrostatic interactions to overall binding affinity. Multiple ionic strengths can
be used to examine the dependence of the electrostatic binding energy on solution ionic strength.
This dependence can determined by plotting the electrostatic binding energy ($x$-axis) versus the
log [salt] ($y$-axis) and fitting a line to the points. The greater the slope of this plot, the more
dependent the electrostatic component of binding and by default the overall binding energy of
the associating macromolecules are on the ionic strength of the solution.
Materials and Methods

Poisson-Boltzmann calculations

Poisson-Boltzmann calculations were performed using the Adaptive Poisson-Boltzmann Solver (APBS) software package developed by Nathan Baker and coworkers (Baker et al., 2001) to determine the theoretical contribution of electrostatic interactions to the overall binding energy of (Ca\(^{2+}\))\(_4\)-CaM for CaMKII\(_p\). Structural coordinates for the (Ca\(^{2+}\))\(_4\)-CaM:CaMKII\(_p\) complex were obtained from the pdb file 1CM1.pdb, and missing side chains were added using Swiss-PDBViewer (Guex and Peitsch, 1997). From the previously mentioned file, two separate PDB files were created consisting of (Ca\(^{2+}\))\(_4\)-CaM, and CaMKII\(_p\), in the conformation they were observed in the (Ca\(^{2+}\))\(_4\)-CaM:CaMKII\(_p\) complex structure. These files were then converted into PQR format and protonated based on their charge states at pH 7.4 using the PDB2PQR webserver (Sobczak et al., 2002).

Identical calculation grid center and length coordinates were used in calculating electrostatic potentials of each molecule or complex. The dimensions of the coarse grid were \(x=60\ \text{Å},\ y=75\ \text{Å},\ \text{and}\ z=70\ \text{Å}\) with a grid spacing of 0.5 Å, while the fine grid was \(x=41.5\ \text{Å},\ y=52\ \text{Å},\ \text{and}\ z=48\ \text{Å}\) with a grid spacing of 0.25, each was centered at \(x=19.558,\ y=56.112,\ \text{and}\ z=74.867\). The temperature used in the calculation was 295K, while the dielectric constant values used for the protein and solvent were 4.00 and 78.54. The linearized form of the Poisson-Boltzmann equation was solved using single Debye-Huckel boundary conditions with cubic B-spline charge discretization and surface smoothing, while the spline window was set to 0.3Å.

The [NaCl] or [KCl] was varied in 50mM increments over a range between 0 and 2M, while the keeping the [CaCl\(_2\)] and [MgCl\(_2\)] constant to mimic the experimental setup to which these calculations were compared to. The atomic radii of the Ca\(^{2+}\), Mg\(^{2+}\), Cl\(^-\), and (Na\(^+\) or K\(^+\)) used in the calculation were 1.97 Å, 1.60 Å, 1.75 Å, 1.86 Å and 2.27 Å respectively, while the solvent radius was 1.4 Å. The atomic radii of the afore mentioned ions were calculated using APBS by iterative rounds of calculation of the solvation energy of the ion and radius adjustment.
to match experimentally determined solvation values (Burgess, 1988). The electrostatic potentials of the \((\text{Ca}^{2+})_4\text{-CaM:CaMKII}_p\) complex, \((\text{Ca}^{2+})_4\text{-CaM}\), and \(\text{CaMKII}_p\) were then calculated at each [NaCl or KCl]. These electrostatic potentials were then used to determine the electrostatic component of \(\text{CaMKII}_p\) binding to \((\text{Ca}^{2+})_4\text{-CaM}\) at each [NaCl or KCl] by subtracting the electrostatic energy values determined for \((\text{Ca}^{2+})_4\text{-CaM}\) and \(\text{CaMKII}_p\) from the value determined for the \((\text{Ca}^{2+})_4\text{-CaM:CaMKII}_p\) complex as shown in Equation 5.2.

\[
\Delta G_{\text{Electrostatic}} = \text{Complex} - \text{CaM Alone} - Na_{1.2\text{-IQp}} \tag{5.2}
\]

The resulting value from this calculation represents the energy of binding/complex formation.

**Fluorescence Anisotropy Monitored Titrations of CaM**

**Binding to Either CaMKII\(_p\) or Na\(_{1.2\text{-IQp}}\) at Varied [NaCl or KCl]**

Fluorescence anisotropy monitored titrations of CaM binding to either CaMKII\(_p\) or Na\(_{1.2\text{-IQp}}\) at varied [NaCl or KCl] were performed to examine how these salts alter the binding affinity of CaM for these peptides. \((\text{Ca}^{2+})_4\text{-CaM}_{1-148}\) binding to fluorescein-labeled CaMKII\(_p\), or \(\text{CaM}_{76-148}\) or \(\text{CaM}_{1-148}\) binding to fluorescein-labeled Na\(_{1.2\text{-IQp}}\) peptide under apo and Ca\(^{2-}\)-saturating conditions were monitored using a Fluorolog 3 (Jobin Yvon, Horiba) spectrofluorimeter, equipped with dual auto-assembly Glan-Thompson polarizers. The anisotropy of the fluorescein labeled peptides were monitored using \(\lambda_{\text{ex}} 496\) nm and \(\lambda_{\text{em}} 520\) nm with 2nm excitation and 10nm emission bandpasses. Anisotropy \((r)\) was calculated as shown in Equation 5.3 as described previously (Akyol et al., 2004),

\[
r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}} \tag{5.3}
\]

where \(I_{VV}\) and \(I_{VH}\) are the intensities of vertically- or horizontally-emitted light upon vertical excitation, respectively, and \(G\) is the instrument correction factor \((G = I_{HH}/I_{HV})\). Averages of three readings with a 1-sec integration time at each point were recorded. Samples of 100 nM Fl-
CaMKII$_p$ or 1µM Fl-Na$_{v1.2}$$_{IQp}$ in 50 mM HEPES, 100 mM KCl, 50 µM EGTA, 5 mM NTA, 1 mM MgCl$_2$, pH 7.4 in the absence (apo) or presence of 10 mM CaCl$_2$ (Ca$^{2+}$-saturated) at 22 ºC were titrated with concentrated apo or Ca$^{2+}$-saturated CaM. At least three replicate titrations were conducted for each NaCl or KCl concentration examined as well as for apo and (Ca$^{2+}$)$_4$-CaM$_{1-148}$. Titrations involving CaM$_{76-148}$ were less well determined and represent only single trials performed as part of exploratory measurements.

**Analysis of K$_d$ for CaM Binding to Na$_{v1.2}$$_{IQp}$ or CaMKII$_p$**

Affinity estimates of CaM for Na$_{v1.2}$$_{IQp}$ or CaMKII$_p$ were determined by fitting titration data to a one-site binding model using NONLIN (Johnson and Frasier, 1985). Fractional saturation of Na$_{v1.2}$$_{IQp}$ or CaMKII$_p$ was described by Equation 5.4 as described previously (Akyol et al., 2004):

$$
\frac{Y}{1-K_a[X_{free}]} = \frac{K_a[X_{free}]}{1+K_a[X_{free}]}
$$

(5.4)

where $K_a$ represents the association constant for CaM binding to CaMKII or Na$_{v1.2}$$_{IQp}$, and $[X_{free}]$ is the free concentration of CaM in solution, as calculated from the independent variables (total concentration of X, $[X_{total}]$) and (total concentration of M, $[M_{total}]$) according to the quadratic equation described by Equation 5.5 as described previously (Akyol et al., 2004):

$$
[X_{free}] = \frac{\sqrt{b + b^2 - 4K_a[1-X_{total}]} - b}{2K_a}
$$

(5.5)

where $b = (1 + K_a[M_{total}] - K_a[X_{total}])$. Under equilibrium conditions, the concentration of Na$_{v1.2}$$_{IQp}$ or CaMKII ([M$_{total}$]) was low relative to the Kd (dissociation constant, 1/Ka) of CaM binding to the peptide. The free concentration of CaM may be approximated by the total (i.e., $[X_{free}]$ at $[X_{total}]$). This allows for an accurate estimate of the association constant. Under stoichiometric conditions however, the ligand is limiting and $[X_{free}]$ is estimated iteratively in the nonlinear least squares function for saturation as the best solution to the difference between $[X_{total}]$ (calculated on the basis of the total ligand added) and $[X_{bound}]$ (calculated as the product
of \([M_{\text{total}}]\) and \(\bar{Y}\). The value of a binding constant estimated in this way is highly correlated with the precision of the numerical value measured for \([M_{\text{total}}]\); therefore, the dissociation constant of Na\(_v\),1.2\(_{IQp}\) or CaMKII\(_p\) for apo and Ca\(^{2+}\)-saturated CaM\(_{76-148}\) or CaM\(_{1-148}\) in Tables 5.1 and 5.2 is reported as a limiting value. Experimental variations in the observed endpoints of individual titration curves were accounted for by Equation 5.6 described previously (Akyol et al., 2004):

\[
f(X) = Y_{[X]_{\text{low}}} + \bar{Y} \cdot [I(Y_{[X]_{\text{high}}} - Y_{[X]_{\text{low}}}) = \text{Span}
\]

(5.6)

where \(\bar{Y}\) refers to average fractional saturation of Na\(_v\),1.2\(_{IQp}\) or CaMKII and \(Y_{[X]_{\text{low}}}\) corresponds to the intrinsic fluorescence anisotropy of CaMKII or Na\(_v\),1.2\(_{IQp}\) in the absence of CaM. The \(\text{Span}\) describes the magnitude and direction of signal change upon titration, which describes the difference between the high \((Y_{[X]_{\text{high}}}\) and low \((Y_{[X]_{\text{low}}}\) endpoints. The \(\text{Span}\) is positive for an increasing signal and negative for a decreasing signal.

In most titrations, the upper and lower endpoints were well defined experimentally. However, in the equilibrium titrations of Na\(_v\),1.2\(_{IQp}\) with apo and (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\) at 300mM KCl the fluorescence anisotropy of Na\(_v\),1.2\(_{IQp}\) did not reach a plateau at the final CaM concentration tested. To estimate the final anisotropy that might have been reached if Na\(_v\),1.2\(_{IQp}\) had become saturated with apo and (Ca\(^{2+}\))\(_2\)-CaM\(_{76-148}\), the end points of the same titrations done at 100 mM KCl were used. The endpoint \((Y_{[X]_{\text{high}}}\) was fixed at this value in the nonlinear least squares analysis of the affected titrations.

To illustrate the degree of precision which we were able to place on the limiting \(K_d\) values of stoichiometric titrations reported in this chapter binding curves corresponding to \(K_d\) values of 1, 10, 25, and 50 nM were simulated and are shown in Figure 5.5. These curves were simulated assuming a 1\(\mu\)M peptide concentration and plotted against either log \([\text{CaM}]_{\text{total}}\), or \([\text{CaM}]/[\text{Peptide}]\). Plotted upon each graph are identical data points of a (Ca\(^{2+}\))\(_4\)CaM\(_{1-148}\) titration of Na\(_v\),1.2\(_{IQp}\) where the \([\text{Na\(_v\)},1.2\(_{IQp}\}]\) concentration was 1 \(\mu\)M to demonstrate how the representation of the same data points change dependent upon how the x-axis is displayed.
Results

Fluorescence Anisotropy Monitored CaM Titrations of Na,1.2IQp

As shown in Figures 5.4 and 5.6, CaM titrations of Na,1.2IQp monitored by fluorescence anisotropy were used to determine the binding affinities of CaM78-148 and CaM1-148 for Nav1.2IQp under apo and Ca^{2+}-saturating conditions at varied NaCl (CaM1-148 only) and KCl concentrations. Reported in Table 5.2 are estimated binding affinities for CaM76-148 and CaM1-148 for Nav1.2IQp. It should be noted that the value of CaM76-148 titration of Na,1.2IQp is the result of a single trial performed as part of initial exploratory study of salt effects on the affinity of Na,1.2IQp binding to CaM76-148.

A common feature present for all titrations of CaM1-148 was that Nav1.2IQp binding was observed to be in a stoichiometric binding regime regardless of the salt (NaCl, or KCl) used (Figure 5.3). Increasing the KCl concentration shifted Na,1.2IQp binding to both apo and (Ca^{2+})_2-CaM76-148 from a stoichiometric to equilibrium binding regime (Figure 5.2). The stoichiometry of Nav1.2IQp binding to either CaM76-148 or CaM1-148 was determined to be 1:1 under both apo and Ca^{2+}-saturating conditions. A decrease in Na,1.2IQp binding affinity was observed as the concentration of salt was increased in all titrations (Figures 5.1, and 5.2). Comparison of changes in free energy of Nav1.2IQp binding at varied salt concentrations showed that NaCl induced larger changes in Nav1.2IQp binding affinity than KCl (Table 5.2) for both apo and (Ca^{2+})_4-CaM1-148.

Although additional trials of CaM76-148 titrations of Na,1.2IQp are needed to confirm the values of these initial findings, when changes in Na,1.2IQp binding affinities at 100mM and 300mM KCl were compared for CaM76-148 and CaM1-148, larger changes are observed for CaM76-148 than CaM1-148 (Table 5.2).
Fluorescence Anisotropy Monitored CaM Titrations of CaMKII_p

As shown in Figure 5.3 CaM titrations of CaMKII_p monitored by fluorescence anisotropy were used to determine the binding affinity of (Ca^{2+})_4-CaM_{1-148} for CaMKII_p at varied NaCl and KCl concentrations. Reported in Table 5.1 are estimated binding affinities of CaMKII_p for (Ca^{2+})_4-CaM_{1-148}. A common feature present for all titrations was that CaMKII_p binding was observed to be in a stoichiometric binding regime regardless of the salt (NaCl, or KCl) used (Figure 5.3). The stoichiometry of CaMKII_p to either (Ca^{2+})_4-CaM_{1-148} was determined to be 1:1. Though stoichiometric, a decrease in CaMKII_p binding affinity was observed as the concentration of either NaCl or KCl was increased. Comparison of changes in free energy of CaMKII_p binding at varied salt concentrations showed that NaCl induced larger changes in CaMKII_p binding affinity than KCl (Table 5.1) for (Ca^{2+})_4-CaM_{1-148}.

Electrostatic Binding Energy of CaMKII for (Ca^{2+})_4-CaM_{1-148}

Calculated via APBS

To predict the effect of NaCl and KCl on the binding affinity of CaMKII_p for (Ca^{2+})_4-CaM_{1-148}, Poisson-Boltzmann calculations were performed at varied NaCl and KCl concentrations (25 mM–1000 mM). Both NaCl and KCl lowered the binding affinity of CaMKII_p for (Ca^{2+})_4-CaM in a concentration dependent manner shown in Figure 5.4a. Though both NaCl and KCl lowered the affinity of CaMKII_p for (Ca^{2+})_4-CaM, NaCl induced a greater change in affinity at concentrations ranging from 25–400 mM than KCl (Figure 5.4a). Comparison of ΔΔG_{NaCl-KCl} values of CaMKII_p binding at salt concentrations greater than 400 mM indicated a convergence in the degree to which Na^+ or K^+ reduce the binding affinity of CaMKII_p (Figure 5.4a). Shown in Figure 5.4b are structures of (Ca^{2+})_4-CaM_{1-148} and CaMKII_p whose solved exposed surfaces are colored according to their electrostatic potential at varied NaCl or KCl concentrations.
**Discussion**

CaM is a negatively charged protein (pI=4) that binds to CaMBD sequences that are enriched in positive charge. The interaction of charged particles in solution can be lessened by electrostatic shielding by the addition of salt. Results presented in **Chapter V** indicate that increasing the concentration of NaCl or KCl lowered the binding affinity of targets for CaM. Examination of values determined for CaMKII\textsubscript{p} and Na\textsubscript{v},1.2\textsubscript{I\text{Qp}} binding to CaM at the same NaCl or KCl concentration indicated that the Na\textsuperscript{+} ion induced a larger effect on CaMKII\textsubscript{p} or Na\textsubscript{v},1.2\textsubscript{I\text{Qp}} binding affinity than the K\textsuperscript{+} ion.

Poisson-Boltzmann calculations of CaMKII\textsubscript{p} binding to (Ca\textsuperscript{2+})\textsubscript{4}-CaM\textsubscript{1-148} indicated that both NaCl and KCl reduced the binding affinity of CaMKII\textsubscript{p} for (Ca\textsuperscript{2+})\textsubscript{4}-CaM, with NaCl having a larger effect than KCl up to 400 mM. Calculation of NaCl and KCl effects on CaMKII\textsubscript{p} binding to (Ca\textsuperscript{2+})\textsubscript{4}-CaM\textsubscript{1-148} at concentrations greater than 400 mM showed a decrease in Na\textsuperscript{+} or K\textsuperscript{+} ion specific effects indicating a general ion effect applies at salt concentrations above 400 mM. The onset of a general salt effect as NaCl or KCl progress past 400 mM represents a saturation of (Ca\textsuperscript{2+})\textsubscript{4}-CaM\textsubscript{1-148} and CaMKII\textsubscript{p} surfaces with either salt, after which Na\textsuperscript{+} or K\textsuperscript{+} ion specific effects become less pronounced.

Results obtained in this chapter show that electrostatic interactions between CaM and its targets CaMKII\textsubscript{p} and Na\textsubscript{v},1.2\textsubscript{I\text{Qp}} are influenced by changes in solvent ionic strength, influencing their binding affinity for each other. A caveat of this observation is that although the peptide binding affinity of CaM was decreased by increasing the salt concentration, the binding affinity of CaM for CaMKII\textsubscript{p} and Na\textsubscript{v},1.2\textsubscript{I\text{Qp}} remained at levels where they would remain associated with CaM in the cell. Together these observations suggest that CaM and its targets have evolved binding surfaces whose molecular interfaces rely on interactions that are largely independent of changes in intracellular salt concentration.
Salt Dependence of Na,1.2IQp Binding to apo and (Ca\textsuperscript{2+})-CaM

Titrations of Na,1.2IQp binding to CaM at varied NaCl and KCl concentrations showed differential effects upon the Na,1.2IQp binding affinity of CaM. In all cases titrations done in the presence of NaCl showed a greater decrease in the binding affinity of CaMKIIp or Na,1.2IQp for CaM than those done in KCl. This effect can be directly attributed to differences between interactions of the Na\textsuperscript{+} or K\textsuperscript{+} ion and the CaM:target complex. Comparison of the radii of the Na\textsuperscript{+} and K\textsuperscript{+} ion indicate that the Na\textsuperscript{+} ion has a greater charge density than the K\textsuperscript{+} ion (Hille et al. 2001). This characteristic allows for stronger attraction, and greater access to negatively charged cavities found within CaM and either CaMKIIp or Na,1.2IQp than the larger K\textsuperscript{+} ion. These results suggest that as K\textsuperscript{+} ions exchange for Na\textsuperscript{+} ions during an action potential, the affinity of CaM for its targets decreases.

Comparison of salt effects on the binding affinity of Na,1.2IQp for apo and (Ca\textsuperscript{2+})\textsubscript{4}-CaM\textsubscript{1-148} showed that the binding affinity of Na,1.2IQp for apo CaM\textsubscript{1-148} was decreased to a greater extent than for (Ca\textsuperscript{2+})\textsubscript{4}-CaM\textsubscript{1-148} (Table 5.2). This effect is attributed to the electrostatic component of the overall Na,1.2IQp binding affinity for apo CaM\textsubscript{1-148} comprising a greater percentage than (Ca\textsuperscript{2+})\textsubscript{4}-CaM. This difference in contribution of electrostatic interactions to the overall binding affinity is likely due to apo CaM\textsubscript{1-148} have a greater overall net negative charge of -24 compared to -16 of (Ca\textsuperscript{2+})\textsubscript{4}-CaM\textsubscript{1-148} due to Ca\textsuperscript{2+} binding.

Preliminary trials of both apo and (Ca\textsuperscript{2+})\textsubscript{2}-CaM\textsubscript{76-148} at 100 and 300 mM KCl showed a more pronounced change in the affinity of Na,1.2IQp than what was observed for apo and (Ca\textsuperscript{2+})\textsubscript{4}-CaM\textsubscript{1-148}. Additional trials are necessary to confirm the significance of these observed values. Based on the available data for CaM\textsubscript{76-148}, addition of KCl caused a larger decrease in Na,1.2IQp affinity for both apo and (Ca\textsuperscript{2+})\textsubscript{2}-CaM\textsubscript{76-148} than apo and (Ca\textsuperscript{2+})\textsubscript{4}-CaM\textsubscript{1-148} with the largest decrease in Na,1.2IQp binding affinity seen in apo CaM\textsubscript{76-148} (Table 5.2). The greater effect of KCl upon CaM\textsubscript{76-148} than CaM\textsubscript{1-148} may be attributed to KCl induced structural changes specific to CaM\textsubscript{76-148} that are not found in CaM\textsubscript{1-148}. This differential effect is consistent with previous
studies that showed that when separated, the domains of CaM do not behave in an identical manner as when they are covalently linked (Sorensen et al., 2002a; Sorensen and Shea, 1998).

Evaluation of calculated versus experimentally observed changes in the binding affinity of CaMKII$_p$ for (Ca$^{2+}$)$_4$-CaM indicates qualitative effects similar to those described previously for Na$_V$1.2$_{10p}$ binding. In both experimental and calculated measurements, increasing NaCl or KCl concentrations decreased the affinity of CaMKII$_p$ for (Ca$^{2+}$)$_4$-CaM. Quantitative comparison of calculated values determined for CaMKII$_p$ binding to (Ca$^{2+}$)$_4$-CaM showed that the salt dependence of CaMKII$_p$ binding to (Ca$^{2+}$)$_4$-CaM is overestimated. Shown in Figure 5.9 are the salt dependence slopes of experimentally observed and calculated changes in CaMKII$_p$ binding affinity for (Ca$^{2+}$)$_4$-CaM. This difference between observed and predicted results represents a significant overestimate in the calculated salt dependence of CaMKII$_p$ binding to (Ca$^{2+}$)$_4$-CaM.

Comparison of APBS calculated salt dependence slopes of (Ca$^{2+}$)$_4$-CaM binding to CaMKII$_p$ (6.7-6.8) with that calculated for DNA binding to the Lambda repressor DNA binding domain (4.6) indicates a significantly greater salt dependence in the CaM-CaMKII$_p$ system (Sharp et al., 1995). Like CaMKII$_p$ binding to CaM, both DNA and the lambda repressor DNA binding domain are oppositely charged macromolecules indicating a significant contribution of electrostatic forces to the overall binding affinity (Sharp et al., 1995). It is of interest to note that unlike CaM binding CaMKII$_p$, the structure of the DNA binding domain of the lambda repressor does not undergo a large conformational change upon DNA binding (Beamer and Pabo, 1992; Pervushin et al., 1996).

The calculated overestimate in salt dependence of CaMKII$_p$ binding to (Ca$^{2+}$)$_4$-CaM is likely due to multiple factors. APBS calculations do not take into account conformational changes that are known to occur within (Ca$^{2+}$)$_4$-CaM and CaMKII$_p$ upon binding. CaM is a very flexible molecule whose conformation changes it from an extended conformation in the unbound state to a collapsed structure when bound to a target (Figure 1.4). CD data of CaMKII$_p$ peptide in the absence of CaM indicates that it does not form a persistent α-helix in solution (Shifman...
and Mayo, 2002). Another factor that may also contribute the discrepancy between observed and predicted salt dependence values are structural differences that may be present at one salt concentration but not at others.

The discrepancy between predicted salt effects from calculated and observed values suggest that conformational changes occur upon peptide binding or salt-induced structural changes of CaMKIIp to (Ca$^{2+}$)$_4$-CaM, that are not captured by the structures used in these calculations. These results further demonstrate that a folding transition must occur upon CaMKIIp (unstructured) binding to (Ca$^{2+}$)$_4$-CaM (extended) and the final compact ellipsoidal structure of (Ca$^{2+}$)$_4$-CaM bound to CaMKIIp (Figure 1.4) (Tse et al., 2007).
Table 5.1: Calculated effect of salt on CaMKIIp binding to (Ca²⁺)₄-CaM₁-148

| [Salt], mM | $K_d$ (KCl)   | $K_d$ (NaCl)   | Fold Difference  \\
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>10 nM (-10.8*)</td>
<td>12 nM (-10.7*)</td>
<td>0.83</td>
</tr>
<tr>
<td>100</td>
<td>14 nM (-10.6*)</td>
<td>16 nM (-10.5*)</td>
<td>0.88</td>
</tr>
<tr>
<td>200</td>
<td>29 nM (-10.2*)</td>
<td>47 nM (-9.9*)</td>
<td>0.62</td>
</tr>
<tr>
<td>300</td>
<td>55 nM (-9.8*)</td>
<td>85 nM (-9.5*)</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Values reported in kcal/mol
Table 5.2: Effect of salt on Na_{v,1.2}Qp binding to apo and (Ca^{2+})-CaM

<table>
<thead>
<tr>
<th>[Salt], mM</th>
<th>apo</th>
<th>(Ca^{2+})</th>
<th>apo/Ca^{2+}</th>
<th>apo</th>
<th>(Ca^{2+})</th>
<th>apo/Ca^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>≤10 nM (≤-10.8*)</td>
<td>≤10 nM (≤-10.8*)</td>
<td>1</td>
<td>≤1 nM (≤-10.8*)</td>
<td>≤51 nM (≤-9.8*)</td>
<td>0.02</td>
</tr>
<tr>
<td>300</td>
<td>≤85 nM (≤-9.5*)</td>
<td>≤62 nM (≤-9.7*)</td>
<td>1.37</td>
<td>≤122 nM (≤-9.3*)</td>
<td>≤170 nM (≤-9.1*)</td>
<td>0.71</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[Salt], mM</th>
<th>apo</th>
<th>(Ca^{2+})</th>
<th>apo/Ca^{2+}</th>
<th>apo</th>
<th>(Ca^{2+})</th>
<th>apo/Ca^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>≤1 nM (≤-10.8*)</td>
<td>≤1 nM (≤-10.8*)</td>
<td>1</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>300</td>
<td>1 ± 4 µM (-8.1* ±0.2)</td>
<td>2.8 ± 3 µM (-7.5* ±0.3)</td>
<td>0.36</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Values reported in kcal/mol
Table 5.3: Calculated effect of salt on CaMKII$_P$ binding to (Ca$^{2+}$)$_4$-CaM$_{1-148}$

<table>
<thead>
<tr>
<th>[Salt], mM</th>
<th>$\Delta G_{\text{Electrostatic}}$ <em>(KCl)</em></th>
<th>$\Delta G_{\text{Electrostatic}}$ <em>(NaCl)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-11.5</td>
<td>-11.5</td>
</tr>
<tr>
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</tr>
<tr>
<td>50</td>
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</tr>
<tr>
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<td>-6.8</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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</tr>
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</tr>
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</tr>
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<td>-0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>900</td>
<td>0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>1000</td>
<td>0.3</td>
<td>0.9</td>
</tr>
</tbody>
</table>

*Values reported in kcal/mol*
Figure 5.1: CaM-binding domains classified by residues used to interact with the N- and C-domain of CaM.

Highlighted in yellow are the locations hydrophobic residues that insert into the hydrophobic pockets of CaM exposed upon Ca\(^{2+}\) binding. The spacing between these hydrophobic residues dictates the numerically derived name given to each CaMBD.

Figure 5.2: Helical Wheel Diagrams of CaM-Binding Domains
Helical wheel diagrams generated using University of Virginia applet
http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html for various Ca$^{2+}$-dependent and Ca$^{2+}$-independent CaMBDs where nonpolar residues are colored according to the scheme depicted above.
Users/nmr_mike/Thesis/Chapter_V/Figure5_2.jpg
Figure 5.3: Intracellular and extracellular ion concentrations of a resting cell
Intracellular and extracellular ionic strengths of Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\), Mg\(^{2+}\), and Cl\(^{-}\) ions are shown (top), while their concentration gradients across the membrane are qualitatively shown below. Upon membrane depolarization ion channels open to allow for ions to pass into or out of the cell dependent upon their concentration gradients (Hille et al. 2001).

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Figure 5.4: Apo or \((\text{Ca}^{2+})_4\text{-CaM}_{1-148}\) titration of \(\text{Nav}_{1.2\,\text{IQp}}\) at varied [Salt]
Titrations of \(\text{Nav}_{1.2\,\text{IQp}}\) with apo or \((\text{Ca}^{2+})_4\text{-CaM}_{76-48}\) are shown. Titrations were conducted at 5 mM CaCl\(_2\) or 50 \(\mu\text{M}\) EGTA for \(\text{Ca}^{2+}\)-saturating or apo trials respectively. Shown in red are trials performed at 100 mM [Salt], while trials shown in black were performed at 300 mM [Salt]

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Figure 5.5: Simulated fitting curves used to determine Na\textsubscript{v,1.2IQp} binding affinity for CaM. Both plots display identical data plotted in two different formats. Na\textsubscript{v,1.2IQp} binding to (Ca\textsuperscript{2+})\textsubscript{4}-CaM\textsubscript{1-148} (red diamonds) is shown on the left where the log [CaM]\textsubscript{Total} is plotted on the x-axis, while on the x-axis of the titration on the right the stoichiometry of [CaM] / [Na\textsubscript{v,1.2IQp}] is shown. Shown in solid lines are simulated binding curves at varied affinities/binding energies where the [Na\textsubscript{v,1.2IQp}] was set to 1µM. Note that when stoichiometric data is plotted on a log scale x-axis typically used for equilibrium binding curves that the difference between one possible fit or another is significantly reduced.

Users/nmr_mike/Thesis/Chapter_V/Figure5_5.jpg
Figure 5.6: Apo or (Ca$^{2+}$)$_2$-CaM$_{76-148}$ titration of Na$_v$1.2$_{IQp}$ at varied [KCl]
Titrations of Na$_v$1.2$_{IQp}$ with apo or (Ca$^{2+}$)$_4$-CaM$_{76-48}$ are shown. Titrations were conducted at 5 mM CaCl$_2$ or 50 µM EGTA for Ca$^{2+}$-saturating or apo trials respectively. Shown in red are trials performed at 100 mM KCl, while trials shown in black were performed at 300 mM KCl.
Users/nmr_mike/Thesis/Chapter_V/Figure5_6.jpg
Figure 5.7: \((\text{Ca}^{2+})_4\text{CaM}_{1-48}\) titration of CaMKII\textsubscript{p} at varied [KCl] and [NaCl]

Titrations of CaMKII\textsubscript{p} with \((\text{Ca}^{2+})_4\text{CaM}_{1-48}\) are shown. Titrations were conducted at CaCl\textsubscript{2} concentrations of 5mM at 50 mM (blue), 100 mM (red), 200 mM (green), and 300 mM (black) NaCl or KCl.

Users/nmr_mike/Thesis/Chapter_V/Figure5_7.jpg
Figure 5.8: Calculated electrostatic binding energies of CaMKII$_p$ for (Ca$^{2+}$)$_4$-CaM$_{1-148}$

A: Plotted change in CaMKII$_p$ affinity for (Ca$^{2+}$)$_4$-CaM$_{1-148}$ as a function of salt. Plotted on the primary Y-axis are APBS calculated electrostatic binding energies of CaMKII$_p$ for (Ca$^{2+}$)$_4$-CaM$_{1-148}$ as a function of [NaCl] (purple) or [KCl] (orange). Plotted on the secondary Y-axis is the difference between APBS calculated NaCl and KCl binding affinities at similar concentrations (green).

B: Electrostatic surface potentials of (Ca$^{2+}$)$_4$-CaM$_{1-148}$ and CaMKII$_p$ at varied NaCl and KCl concentrations. Positive surface is colored in blue, while negative surface is shown in red.

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Figure 5.9: Comparison of calculated and observed changes in CaMKII$_p$ binding to (Ca$^{2+}$)$_4$-CaM$_{1-148}$ as a function of salt

Comparison of observed (red) and calculated (light blue) changes in the binding affinity of CaMKII$_p$ for (Ca$^{2+}$)$_4$-CaM$_{1-148}$ as a function of either [NaCl] (upper plot) or [KCl] (lower plot).

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CHAPTER VI
SUMMARY AND FUTURE DIRECTIONS

Thesis Summary

The interaction of apo CaM with targets is an emerging area of interest in the CaM research field where historically it was a widely held belief that only (Ca\(^{2+}\))\(_4\)-CaM bound and activated intracellular targets (Cheung, 1980; Klee and Haiech, 1980; Siegel, 1973). Numerous targets such as myosins, ion channels, and growth-associated proteins have been shown to interact with apo CaM via an IQ-motif (Black et al., 2005; Liu and Storm, 1990; Martin and Bayley, 2004; Shah et al., 2006). This thesis investigates how the anti-psychotic drug TFP, and \(\text{Na}_1.2_{\text{IQp}}\) interact with apo CaM at the molecular level to better understand how apo CaM recognizes and binds such diverse targets. A model of how CaM interacts with both of these targets is presented in Figure 6.1.

TFP Binding Induces a Biphasic Response in the Ca\(^{2+}\)-Binding Affinity of CaM

Nearly all targets that bind to CaM either naturally occurring or synthetic “tune” its Ca\(^{2+}\)-binding affinity in some manner (Peersen et al., 1997). Targets such as CaMKII\(_p\), Calcineurin, and the Ryanodine receptor increase the Ca\(^{2+}\)-binding affinity of CaM, while IQ-motif containing proteins such as Na\(_1.2\) lower the Ca\(^{2+}\)-binding affinity of CaM (Evans and Shea, 2009; Newman and Shea, 2006; Quintana et al., 2005; Theoharis et al., 2008). Unlike these previously mentioned protein targets of CaM, the anti-psychotic drug TFP was shown in Chapter II to both increase and decrease the Ca\(^{2+}\)-binding affinity of CaM dependent upon the concentration of TFP examined. To our knowledge this is the first time this behavior has been observed with any allosteric effector of CaM.

The basis of the observed biphasic response can be found in the stoichiometry of TFP binding to CaM. We determined that there are 2 TFP binding sites (one per domain) in apo CaM, while there are 4 TFP binding sites in (Ca\(^{2+}\))\(_4\)-CaM. The TFP induced biphasic response in
Ca$^{2+}$-binding affinity results from each TFP site having a different binding affinity for apo and (Ca$^{2+}$)$_4$-CaM as shown in Chapter II. TFP at ratios of 1:1 and 2:1 have a more favorable binding affinity for apo CaM than (Ca$^{2+}$)$_4$-CaM, and thus via allosteric linkage cause the Ca$^{2+}$-binding affinity of CaM to become less favorable. TFP:CaM ratios of 3:1 and 4:1, are achieved in the Ca$^{2+}$-bound state only, because of this the Ca$^{2+}$-bound state of CaM is selectively stabilized over the apo state resulting in a more favorable Ca$^{2+}$-binding affinity. To further probe the basis of this biphasic response, TFP titrations of (Ca$^{2+}$)$_4$-CaM were performed. We observed biphasic responses in the chemical shift of residues located at the interface between the N- and C-domain of CaM as well as in residues in close (< 4 Å) proximity to multiple TFP binding sites, corroborating the biphasic response seen in Ca$^{2+}$-binding measurements.

**CaM uses Distinct Interfaces to Bind TFP under apo and Ca$^{2+}$-Saturating Conditions**

Ca$^{2+}$-titrations of CaM at multiple ratios of TFP performed in Chapter II, indicated that the Ca$^{2+}$-binding affinity of the C-domain of CaM was affected to a greater extent than that of the N-domain. It was clear in $^{15}$N-HSQC spectra taken of TFP bound to apo and (Ca$^{2+}$)$_4$-CaM that TFP induced non-equivalent chemical shifts which were dependent upon the Ca$^{2+}$-ligation state of CaM. Consistent with Ca$^{2+}$-titration data, quantification of N- and C-domain $^{15}$N-HSQC chemical shifts of apo and (Ca$^{2+}$)$_4$-CaM indicated that in both cases the C-domain of CaM was perturbed to a greater extent than the N-domain. These observations prompted structural studies using the isolated C-domain fragment (CaM$_{76-148}$) performed in Chapter III to gain insight into the differing molecular interfaces used by apo and (Ca$^{2+}$)$_2$-CaM$_{76-148}$ identified in Chapter II.

The crystal structure of TFP bound to (Ca$^{2+}$)$_2$-CaM$_{76-148}$ directly reveals the molecular binding interface and conformation used by (Ca$^{2+}$)$_2$-CaM$_{76-148}$ when binding TFP. In this structure (Ca$^{2+}$)$_2$-CaM$_{76-148}$ adopts an “open” domain conformation observed in all structures of (Ca$^{2+}$)$_4$-CaM alone or when bound to a target. The TFP binding site is located within the hydrophobic pocket of (Ca$^{2+}$)$_2$-CaM$_{76-148}$ and contacts the FLMM tetrad of residues, both
common features seen in all structures of \((\text{Ca}^{2+})_{2}\text{-CaM}_{76-148}\) bound to protein or drug targets (Ataman et al., 2007). A unique feature of this structure was that 2 chains of \((\text{Ca}^{2+})_{2}\text{-CaM}_{76-148}\) were found within the unit cell, and formed a pseudo ellipsoidal structure typically observed when the N- and C-domain of \((\text{Ca}^{2+})_{4}\text{-CaM}_{1-148}\) bind a target. A desirable future set of experiments would be to determine the \(^1\text{H}\), \(^{15}\text{N}\), and \(^{13}\text{C}\) assignments of TFP-bound \((\text{Ca}^{2+})_{2}\text{-CaM}_{76-148}\) to use NMR spectroscopy to observe changes in dynamics upon TFP binding, and compare these changes with areas of chains A and B that differ structurally. This comparison will serve as an orthogonal approach to validate the structural changes observed between chains A and B of \((\text{Ca}^{2+})_{2}\text{-CaM}_{76-148}\) when bound to TFP, as well as allow for \(T_1\) and \(T_2\) experiments to verify its oligomeric state.

NMR studies of residue-specific changes induced upon TFP binding to apo CaM\(_{76-148}\) performed in Chapter III indicated that significant structural changes occurred within apo CaM\(_{76-148}\) as TFP binds. These residue-specific changes were then mapped to a structure of apo CaM\(_{76-148}\) determined in the absences of TFP and clustered onto helices F-H as well as the \(\text{Ca}^{2+}\)-binding loops. Hydrophobic residues dominate TFP binding perturbed resonances within helices F-H, while polar residues are perturbed in the \(\text{Ca}^{2+}\)-binding loops. The chemical shifts induced upon TFP addition to apo CaM\(_{76-148}\) are most likely a result of 2 different phenomena: 1) direct TFP binding or 2) allosteric linkage. Due to the chemical properties of TFP (hydrophobic small molecule) we hypothesize that TFP interacts directly with hydrophobic residues contained within helices F-H, while it allosterically alters the chemical shifts of the \(\text{Ca}^{2+}\)-binding loops. Evidence to support this claim was shown in Chapter II in which TFP was shown to allosterically alter the \(\text{Ca}^{2+}\)-binding affinity of CaM\(_{76-148}\), and that it is unlikely that TFP directly competes with \(\text{Ca}^{2+}\) within the \(\text{Ca}^{2+}\)-binding loops of CaM\(_{76-148}\).

Although TFP significantly perturbed apo CaM\(_{76-148}\) upon binding, the magnitude of change in chemical shifts was much smaller than those observed for TFP binding to C-domain of CaM\(_{1-148}\), suggesting a smaller conformational change occurs under apo conditions. The only conformation observed of the C-domain of apo CaM or CaM-like proteins when bound to a
target has been that of the “semi-open” conformation. We hypothesize that a “semi-open” conformation is used by apo CaM$_{76-148}$ when binding TFP, as this conformation (consistent with magnitude of $^{15}$N-HSQC shifts) requires less structural rearrangement than the “open” conformation. The adoption of a “semi-open” conformation is also evidenced by the slight decrease in T$_2$ relaxation time upon addition of TFP to apo CaM$_{76-148}$, indicative of a slight increase in molecular size. Future studies are proposed to determine changes in T$_2$ relaxation rates upon TFP binding to (Ca$^{2+}$)$_2$-CaM$_{76-148}$. These values would help to confirm that the rate of molecular tumbling due to changes in the hydrodynamic radius of TFP-bound apo CaM$_{76-148}$ is faster that of TFP-bound (Ca$^{2+}$)$_2$-CaM$_{76-148}$. It is anticipated that the average T$_2$ rate of apo CaM > TFP-bound apo CaM$_{76-148}$ > TFP-bound (Ca$^{2+}$)$_2$-CaM$_{76-148}$.

To definitively confirm that CaM uses distinct interfaces to bind TFP under apo and Ca$^{2+}$-saturating conditions high resolution 3 dimensional structures are required. To this end the we have determined the (Ca$^{2+}$)$_2$-CaM$_{76-148}$ adopts an “open” conformation via x-ray crystallography. The future goal of the work described in Chapter III is to determine the solution structure of TFP bound to apo CaM$_{76-148}$. These NMR data have been collected and are awaiting assignment and analysis for calculation the structure of TFP bound to apo CaM$_{76-148}$.

**Conservation of Carboxamide-Containing Side chains in apo CaM Binding Motifs**

Studies presented in Chapter IV indicated the “semi-open” domain conformation is used by the C-domain of CaM and CaM-like proteins when interacting with targets under apo conditions. The structure of the SK-channel bound to a partially Ca$^{2+}$-saturated CaM shows that an IQ-motif is not necessary for binding of the C-domain of apo CaM (Figure 4.9) (Schumacher et al., 2004). The one structural feature observed that was conserved by all apo CaM binding motifs was a Gln or Asn residue whose carboxamide-containing side chain was used to make 2 hydrogen bonds to atoms found in the backbone of residues 112 and 114 located within the loop connecting helices F and G of CaM (Figure 4.9).
To examine the necessity of this hydrogen bonding network for apo C-domain binding additional studies are proposed in which mutations of either Gln→Glu (Na$_v$.1.2$_{IQp}$) or Asn→Asp (SK-Channel) will be made at the Q position of the IQ-motif followed by fluorescence anisotropy monitored binding studies. These mutants would test whether 2 hydrogen bonds are required for apo binding of the C-domain of CaM. The aforementioned study (dependent upon the results) could be repeated except instead of Gln→Glu or Asn→Asp mutations, a Glu or Asn →Ala mutation would be made that completely removes the observed hydrogen bond network between apo CaM and the target peptide.

**Changes in the Electrostatic Environment of CaM Alters its Interaction with Targets**

Studies shown in Chapter V indicate that increasing the concentration of NaCl or KCl in solution lowers the affinity of CaM for its target, due to the screening of electrostatic interactions between CaM and the peptide target. Perturbation of the local electrostatic environment of CaM can also be performed by introducing point mutations that introduce, remove or reverse the intrinsic charge of an amino acid. Structural changes due to this type of perturbation of the electrostatic environment of CaM, will be examined with CaM mutants D95G and H135R. These mutants were identified via a genetic screen of viable Paramecia that exhibited abnormal chemotactic behavior resulting from impaired ion channel function (Kung et al., 1992; Ling et al., 1992). Exploratory studies of D95G and H135R have identified these over-reactive mutants as ideal candidates for structural studies. (Ca$^{2+}$)$_2$-CaM$^{76-148}$ H135R was crystallized and diffracted to 2.2 Å, while the $^{15}$N-HSQC spectrum of D95G (Ca$^{2+}$)$_2$-CaM$^{76-148}$ bound to Na$_v$.1.2$_{IQp}$ showed excellent peak dispersion (Figure 6.2).

Future endeavors to determine to these high-resolution structures will allow for direct observations to be made of the effect of D95G or H135R on CaM alone or when in complex with Na$_v$.1.2$_{IQp}$. Either of these structures would be the first of their kind to have been determined from the genetic screen of viable Paramecium mutants. These structures would lay the
groundwork for structural based conclusions to be made as to the molecular basis of the altered chemotactic behavior observed in *Paramecium* mutants.

**Future Studies**

**Changes in Met 144 Dynamics upon Binding (Ca$^{2+}$)$_2$-CaM$_{76-148}$**

The residue M144 has previously been identified to adopt the most variable conformations when bound to a target of the FLMM tetrad residues, as well as being the least conserved FLMM tetrad residue in 102 CaM sequences from other species. Studies presented in Chapter III indicated that the side chain dynamics of M144 play a key role in selecting the orientation of TFP within the hydrophobic cleft of (Ca$^{2+}$)$_2$-CaM$_{76-148}$. To investigate this observation further, $^{13}$C-methyl relaxation experiments are proposed to examine changes in relaxation rates of (Ca$^{2+}$)$_2$-CaM$_{76-148}$ upon binding TFP (Palmer, 2001; Wand, 2001). This data would help to clarify at a finer level of detail the amount of molecular motion present within the TFP-binding site of (Ca$^{2+}$)$_2$-CaM$_{76-148}$ as x-ray crystallography only provide static images.

This data could then be incorporated into small molecule docking simulations of drugs to CaM in which highly dynamic residues would be allowed to be flexible, instead of being held rigid as is often done for all residues in docking simulations. This treatment would allow for a more realistic docking simulation to be run, yet be less computationally taxing than allowing all-atom flexibility. On a larger scale, incorporation of *in vitro* data into virtual docking simulations of lead compounds against hub-proteins such as CaM, may benefit pharmaceutical discovery in which off target effects are not desired. Virtual screening of a compound library against every known protein structure is considerably more computationally expensive than screening against a select set of hub-proteins who regulate a myriad of downstream targets. *In silico* identification of compounds that alter the function of hub-proteins such as CaM would save in the cost of bringing a compound to clinical trials by identifying a drug or drugs whose interaction with a network of regulatory pathways could result in an undesired off-target effect.
Determination of Binding Site for N-domain of (Ca\(^{2+}\))\(_4\)-CaM on Na\(_{1.2}\)

Studies presented in Chapter IV determined that the N-domain of (Ca\(^{2+}\))\(_4\)-CaM does not bind to Na\(_{1.2}\)IQp, indicating that there is likely to be binding site for the N-domain of (Ca\(^{2+}\))\(_4\)-CaM located in another region of Na\(_{1.2}\). Future studies will build off of the observations made in Chapter IV by using a peptide array. This array will be composed of overlapping 30-residue peptide sequences derived from the cytoplasmic face of Na\(_{1.2}\). Following initial identification of a putative N-domain Na\(_{1.2}\) binding sequence (Na\(_{1.2}\)N) FRET will be used to confirm that donor-acceptor labeled peptides corresponding to Na\(_{1.2}\)IQp, and Na\(_{1.2}\)N are capable of simultaneously binding to the N- and C-domain of (Ca\(^{2+}\))\(_4\)-CaM. Lastly, comparison of patch clamp currents of Na\(_{1.2}\) channel mutants in the area of Na\(_{1.2}\)N, as well as the use of CaM mutants deficient in N-domain Ca\(^{2+}\)-binding will be used to verify functional role binding to the N-domain of (Ca\(^{2+}\))\(_4\)-CaM.

Circular Permutation of Charge Residues in Na\(_{1.2}\)IQp

An IQ-motif peptide derived from another sodium channel variant (Na\(_{1.5}\)IQp) is homologous (78\%) to Na\(_{1.2}\)IQp yet binds with a 16-fold lower affinity to Apo-CaM and 200-fold lower affinity to (Ca\(^{2+}\))\(_4\)-CaM than Na\(_{1.2}\)IQp (Shah et al., 2006). The most notable difference between the two to account for these differences is their charge distribution and overall net charge (Figure 6.3). To investigate the role that charge distribution has on the affinity of apo and Ca\(^{2+}\)-CaM, charged residues of the Na\(_{1.2}\)IQp will be mutated. Initially charged residues of the Na\(_{1.2}\)IQp which are not conserved when compared to Na\(_{1.5}\)IQp will be mutated to their Na\(_{1.5}\)IQp counterpart. The effect of the mutant Na\(_{1.2}\)IQp on its affinity for CaM and effect on Ca\(^{2+}\) binding by CaM will then be measured to clarify how apo- and (Ca\(^{2+}\))-CaM interact. Pinpointing and then mutating key areas of the Na\(_{1.2}\)IQp -CaM interface will explain on a molecular level how the Ca\(^{2+}\)-affinity of the domains of CaM are “tuned” to perform their physiological functions when bound to IQ-motifs of sodium channel variants (Figure 1.8)
**In Vitro Evolution of apo CaM Binding IQ-motifs**

A fascinating aspect of the IQ-motif (Figure 4.1a) is that with the exception of the Q, it is similar in amino acid composition and spacing with a 1-8-14 BAA motif known to only interact with $(\text{Ca}^{2+})_4$-CaM (Yap et al., 2000). Although the IQ-motif as a whole has been characterized to preferentially interact with apo CaM, exceptions to this statement have been observed for IQ motifs found in Na$_\alpha$1.5 and Ca$_\alpha$1.2 ion channels in which more favorable IQ-motif binding is observed for $(\text{Ca}^{2+})_4$- than apo CaM (Shah et al., 2006). To explore the sequence requirements of target binding to CaM, phage display will be used to evolve peptide target sequences or varying affinity to apo and $(\text{Ca}^{2+})_4$-CaM to.

The selection pool will be composed of a phage library in which unconserved positions of the IQ-motif will be fully randomized at positions indicated with an “X” in the following sequence [x(Q/N)xxx(K/R)xxxx(K/R)], while positions in parenthesis will only carry a binary randomization. Bio-panning will first be carried out against immobilized CaM where initial wash steps will include EGTA to insure enrichment of apo CaM binding sequences. In the last biopanning selection round prior to sequencing, the phage library will be split and selected against either apo or $(\text{Ca}^{2+})_4$-CaM using either an EGTA or Ca$^{2+}$ final wash step. After phage sequencing, comparison of these two groups will be made to identify sequences (if any) that only bind to apo CaM, as well as those that are capable of binding both apo and $(\text{Ca}^{2+})_4$-CaM. This process will be repeated again with the exception that initial bio-panning will be done in the presence of Ca$^{2+}$ instead of EGTA, to evolve IQ-motif sequences that preferentially bind $(\text{Ca}^{2+})_4$-CaM (Figure 6.4) The final result will be 4 pools of CaM binding motifs consisting of sequences that: only bind $(\text{Ca}^{2+})$-CaM, bind apo and $(\text{Ca}^{2+})_4$-CaM with preference for $(\text{Ca}^{2+})_4$-CaM, bind apo and $(\text{Ca}^{2+})_4$-CaM with preference for apo CaM, and sequences that only bind apo CaM.

The results from these experiments would help to resolve uncertainties as to what the sequence determinants of IQ-motifs are that allow them to tune their affinity for the multiple
Ca\textsuperscript{2+}-ligation states of CaM. Comparison of \textit{in vitro} evolutionary results with those of phylogenic trees of IQ-motifs evolved \textit{in vivo} will also provide insight into what evolutionary pressures produced naturally occurring IQ-motifs whose evolution are a result of selection pressure of multiple simultaneous factors.

\textbf{Examination of Naturally Occurring Na\textsubscript{x},1.2\textsubscript{IQp} R1902C Mutation on CaM}

The sodium channel variants contain naturally occurring mutations distributed along multiple areas of the channel sequence that are genetically linked to human disorders. One such mutation R1902C is located within the IQ-motif of Na\textsubscript{x},1.2 and is associated with familial autism (Weiss et al., 2003). Future studies are proposed to determine if the R1902C mutation alters previously determined Ca\textsuperscript{2+}-binding affinity values of CaM when bound to Wt-Na\textsubscript{x},1.2, as well as to determine how this mutation alters the binding affinity of Na\textsubscript{x},1.2 to CaM. Structural studies (SAXS, NMR, or X-ray crystallography) are proposed in combination with other biophysical measurements and techniques (CD, analytical ultracentrifugation, and stokes radius) to determine how R1902C may alter the CaM-Na\textsubscript{x},1.2\textsubscript{IQp} complex. It is well known that binding of targets “tune” the Ca\textsuperscript{2+}-binding affinity of CaM, these investigations may provide the 1\textsuperscript{st} example of how alteration of tuned affinities of CaM result in a human disorder. A better understanding of the fundamental molecular basis of this disorder may aid in improved treatment therapies for individuals carrying the R1902C mutation.

\textbf{Structure Determination of Larger Intracellular Fragments of Na\textsubscript{x},1.2 in Complex with CaM}

Structural studies involving CaM bound to peptides derived from target proteins have proven useful in the dissection of the molecular interactions made upon binding. Although valuable, these studies fail to capture the overall structural changes that occur within the entire macromolecular complex that result in their function. This lack of overall mechanistic detail is likely to be most pronounced in IQ-motif containing targets such as Na\textsubscript{x},1.2 where CaM is an
intrinsic subunit under both apo and Ca\(^{2+}\)-saturating conditions. Ideally, to fully understand how CaM regulates Na\(_v\)1.2 gating to allow passage of Na\(^+\) ions across cellular membranes, structures of Na\(_v\)1.2 bound to CaM in all of their physiologically relevant states are required. Given that the determination of the structure of Na\(_v\)1.2 represents a significant challenge that has yet to be overcome by structural biologists, it is unlikely that in the near future structures of apo and (Ca\(^{2+}\))\(_4\)-CaM bound to Na\(_v\)1.2 will be determined. A more tractable path to uncover a deeper mechanistic understanding of how Na\(_v\)1.2 is regulated by CaM beyond that of peptide studies might be found in structural studies involving intracellular domains of Na\(_v\)1.2 identified to bind CaM. The IQ-motif of Na\(_v\)1.2 is found within a larger domain of Na\(_v\)1.2 theorized to be composed of 6 \(\alpha\)-helices, while studies shown in Chapter 4 indicated that a region outside of Na\(_v\)1.2\(_{IQp}\) interacted with the N-domain of CaM. For these reasons, future structural studies are proposed to determine the structure of apo and (Ca\(^{2+}\))\(_4\)-CaM bound to Na\(_v\)1.2\(_{IQp}\) in the context of the entire C-terminal domain of Na\(_v\)1.2. It is also proposed that dependent upon studies to determine the portion of Na\(_v\)1.2 that binds the N-domain of CaM, that this segment be included as well. These proposed studies would help to establish spatial constrains upon where intracellular regions of Na\(_v\)1.2 are located under apo and Ca\(^{2+}\)-saturating conditions.
Figure 6.1: Model of apo and (Ca\textsuperscript{2+})\textsubscript{4}-CaM C-domain conformations used when binding targets. The C-domain of CaM is represented as red cylinders which contain two distinct binding surfaces. The purple surface located near the perimeter of the hydrophobic cleft of CaM is used for target (green triangle) binding under apo conditions, while the light purple surface is used when the C-domain is Ca\textsuperscript{2+}-saturated.

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Figure 6.2: $^{15}$N-HSQC Spectrum and diffraction image of PCaM Mutants
A: $^{15}$N-HSQC spectrum of D95G apo CaM$_{76-148}$ (red) Bound to Na$_{1.2}$10p (green), where the location of the D95G mutation is indicated by a red sphere. B: Diffraction image of H135R (Ca$_{2+}$)$_2$-CaM$_{76-148}$. The location of the H135R mutation is shown as a red sphere on the structure of Wt-CaM$_{78-148}$ (red) where Ca$_{2+}$-ions are colored yellow.
Users/nmr_mike/Thesis/Chapter_VI/Figure6_2.jpg
Figure 6.3: Sequence alignment of Nav1.2 and Nav1.5 IQ-motifs
The IQ-motifs of Nav1.2 and Nav1.5 are shown in green and magenta respectively. Boxed areas represent positions that differ in charge, sequences below represent circular permutations of charged residues of Nav1.2IQp that will be examined via Ca$^{2+}$ titration and fluorescence anisotropy to monitor changes in Ca$^{2+}$ and peptide-binding affinity of CaM.

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Figure 6.4: Schematic of phage display selection procedure
An initial phage library will be generated and enriched to produce IQ-motifs which either selectively bind to apo or (Ca\(^{2+}\))\(_4\)-CaM.
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APPENDIX A

FORTRAN FUNCTION FOR FITTING FLUORESCENCE ANISOTROPY DATA TO A SIMPLE LANGMUIR BINDING ISOTHERM

The following Fortran function was used in nonlinear least squares analysis of the fluorescence anisotropy data presented in Chapter II and Chapter IV. Fits using this function were used to determine the association constant of CaM for a synthetic peptide representing the CaM-binding domain of Na,1.2p or CaMKIIp. This equation fits to a one-site binding model (Equation A.1), using the total concentration of CaM at each point.

\[
\bar{Y} = \frac{K_a[X]}{1 + K_a[X]}
\] (A.1)

Function FX( Ans, X, ydum, ierr, n)
* 
* fxYvsXt1K.f 
* 
* Function for fitting ligand binding to macromolecule 
* when total ligand, rather than free ligand, 
* concentration is known. 
* Model of binding: Monomer binding to equal and 
* independent site(s). 
* Number of Sites: Set by ans parameter ans(3) 
* Original function name: &FNWT1 <831018.1019> 
* 
* The ANS vector has the following form: 
* ANS(1) = macromolecule total concentration 
* ANS(2) = Association binding constant for ligand to 
* macromolecule 
* ANS(3) = N, number of independent sites on 
* macromolecule 
* ANS(4) = Endpoint at Low X 
* ANS(5) = Endpoint at High X 
* 
Real Ans(5), K, MKS 

F = 1.E-7*X ! free ligand is approximated as a fraction of total 
MKS = Ans(1)*Ans(2)*Ans(3) 
K   = Ans(2)
EndLowX = Ans(4)
EndHighX = Ans(5)
Span = EndHighX - EndLowX

Do 10 i = 1, 500
gf = -X + F + MKS*F/(1.+K*F)
gf1 = 1. + MKS/(1.+K*F)/(1.+K*F)
fold = f
F = F -gf/gf1

If(Abs(Fold/F - 1.).lt.1.E-5) Go to 11
10 Continue
11 Fx = EndLowX + Span * F*K/(1.+F*K)

Return
End

SUBROUTINE START(NAME,MAXP,DNAME,MAXD,MAXV)
C
C       THIS ROUTINE IS USED TO SET THE VARIABLE NAMES FOR C    THE
C     PARAMETERS. THESE NAMES MUST CORRESPOND TO
C     THE VARIABLES USED IN THE FX ROUTINE.
CHARACTER*8 NAME(5),NAMES(5)
CHARACTER*8 DNAME(1),DNAMES(1)
DATA NAMES/'[Mtotal]','Ka (M)  ','# sites ','EndLowX','EndHighX'/
DATA DNAMES/'dG@22C'/
C
MAXV=1
C     maxv is the # of independent variables for the fit.
C     This is usually set to 1. However, to fit multiple  C  sets of data it could be set to 2. The
C     second vector C     of X values can then be used to specify which
C     data set is actually being fit. If this is greater
C     than 1 then X in the FX routine must be dimensioned
C     appropriately.
C
MAXP=5
C     maxp is the # of parameters in model being fit by
C     these routines.
DO 10 I=1,MAXP
10 NAME(I)=NAMES(I)
C     NAME is the names of the actual fitting parameters.
MAXD=1
C MAXD specifies the number of derived parameters. A
C derived parameter is simply a quantity that you wish
C evaluated from the fitting answers after the fit is
C finished. By using this feature the program will
C complete the actual error propagation for the derived
C parameters. In the current example, we calculate the
C free energy from the Kd.
c
DO 110 I=1,MAXD
110  DNAME(I)=DNAMES(I)
c DNAME is the names of the derived parameters.

RETURN
END

subroutine derive(old,new)
C This routine is used to map the OLD (fitted)
C parameters into the desired derived (NEW) parameters.
c
real old(3),new(1)
new(1) = -0.58646 * 2.303 * alog10(old(2))  !RT at 22C
return

div
APPENDIX B
FORTRAN FUNCTION FOR ANALYSIS OF
CALCIUM-DEPENDENT CHANGES IN FLUORESCENCE
THAT DESCRIBE
FILLING OF ONLY TWO SITES

The following Fortran function was used for nonlinear least squares analysis to determine the free energies of calcium binding to CaM from calcium-dependent changes in fluorescence during equilibrium calcium titrations. This function uses a model-independent two-site equation (Equation B.1) that describes potentially heterogeneous, cooperative sites.

\[
\bar{Y} = \frac{K_1[X] + 2K_2[X]^2}{2(1 + K_1[X] + K_2[X]^2)}
\]  

FUNCTION FX(ANS,X,Y,INDEX,N)
*********************************************************
* FX2s2K.mpf
* Function file for use with nonlin (M.L.Johnson)
* For analysis of classical 2-site isotherms
* Resolves macroscopic binding energies at 2 sites
* Permits resolution of span and Yzero value
*
* Independent variable: X = [Ligand] free
* Dependent variable: Fractional saturation
* Parameters or ANS vector elements:
* ANS(1) = TEMPERATURE IN CENTIGRADE
* ANS(2) = Macrosopic Delta G for 1 bound
* ANS(3) = Macrosopic Delta G for 2 bound
* ANS(4) = Span
* ANS(5) = Y infinity
*
*********************************************************
*
DIMENSION ANS(5)
REAL X, Span, Yzero, K1X, K2X2, T, RT
REAL Ybar, FX
*
DATA R /.001987/
T = 273.15 + ANS(1)
RT = R*T
K1X = exp(-Ans(2)/RT)*X !k1*X
K2X2 = exp(-Ans(3)/RT)*X*X !k2*X
Ybar = ((K1X + 2.*K2X2)/(1. + K1X + K2X2))/2.
FX = Ybar * Ans(4) + Ans(5)
*
RETURN
END

SUBROUTINE START(NAME, MAXP, DNAME, MAXD, MAXV)
C
C       THIS ROUTINE IS USED TO SET THE VARIABLE NAMES FOR THE
C       PARAMETERS. THESE NAMES MUST CORRESPOND TO THE
C       VARIABLES USED IN THE FX ROUTINE.
C
CHARACTER*8 NAME(5), NAMES(5)
CHARACTER*8 DNAME(3), DNAMES(3)
DATA NAMES/'Temp','dG1 ','dG2 ','Span','Yzero'/
DATA DNAMES/'dG12est', 'EndLowX', 'EndHighX'/
C
MAXV=1
C     maxv is the # of independent variables for the fit.
C     This is usually set to 1. However, to fit multiple
C     set of data it could be set to 2. The second vector
C     of X values can then be used to specify which
C     data set is actually being fit. If this is greater
C     than 1 then X in the
C     FX routine must be dimensioned appropriately.
C
MAXP=5
C     MAXP is the # of parameters in model being fit by
C     these routines.
DO 10 I=1,MAXP
  10 NAME(I)=NAMES(I)
C     NAME is the names of the actual fitting parameters.

MAXD=3
C MAXD specifies the number of derived parameters. A
derived parameter is simply a quantity that you wish
evaluated from the fitting answers after the fit is
finished. By using this feature the program will
complete the actual error propagation for the derived
parameters. In the current
C example, we calculate the area under the exponential C decay.
C
DO 110 I=1,MAXD
110  DNAME(I)=DNAMES(I)
C DNAME is the names of the derived parameters.

RETURN
END

subroutine derive(old,new)
C This routine is used to map the OLD (fitted) C parameters into the C desired derived (NEW) parameters.
C
real old(5),new(1)
DATA R /0.01987/
    T=273.15 + old(1)
    RT = R * T
* Calculate estimate of dG cooperativity for equal * intrinsic affinities
    new(1) = -RT*Aalog(4.) + old(3) - 2. * old(2)
* Calculated endpoint at low [X]
    new(2) = 1. * old(5)
* Calculated endpoint at high [X]
    new(3) = old(4) + old(5)
    return
    end
APPENDIX C

NMR ASSIGNMENTS OF APO PCaM\textsubscript{76-148}

Amide Assignments of Apo PCaM\textsubscript{76-148}

Below are apo PCaM amide assignments determined in 10 mM D\textsubscript{4}-imidazole, 100 mM KCl, 50 µM D\textsubscript{16}-EDTA, 0.01% NaN\textsubscript{3}, pH 6.5, 298.15 K

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### Carbonyl Assignments of apo PCaM<sub>76-148</sub>

Listed below are carbonyl assignments of apo PCaM<sub>76-148</sub> determined in 10 mM D<sub>4</sub>-imidazole, 100 mM KCl, 50 µM D<sub>16</sub>-EDTA, 0.01% NaN<sub>3</sub>, pH 6.5, 298.15 K

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Methine, Methylene, and Methyl Assignments of apo PCaM$_{76-148}$

Listed below are methane, methylene and methyl assignments of apo PCaM$_{76-148}$ determined in 10 mM D$_4$-imidazole, 100 mM KCl, 50 µM D$_{16}$-EDTA, 0.01% NaN$_3$, pH 6.5, 298.15 K

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APPENDIX D

NMR ASSIGNMENTS OF APO PCaM\textsubscript{76-148} WHEN BOUND TO TFP

**Amide Assignments of Apo PCaM\textsubscript{76-148} When Bound to TFP**

Listed below are amide assignments of apo PCaM\textsubscript{76-148} when bound to TFP determined in 10 mM D\textsubscript{4}-imidazole, 100 mM KCl, 50 µM D\textsubscript{16}-EDTA, 0.01% NaN\textsubscript{3}, pH 6.5, 298.15 K

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Carbonyl Assignments of apo PCaM$_{76-148}$ when Bound to TFP

Listed below carbonyl assignments of apo PCaM$_{76-148}$ when bound to TFP determined in 10 mM D$_4$-imidazole, 100 mM KCl, 50 µM D$_{16}$-EDTA, 0.01% NaN$_3$, pH 6.5, 298.15 K

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Methine, Methylene, and Methyl Assignments of apo PCaM$_{76-148}$

When Bound to TFP

Below are listed methine, methylene, and methyl assignments of apo PCaM$_{76-148}$ when bound to TFP determined in 10 mM D$_4$-imidazole, 100 mM KCl, 50 µM D$_{16}$-EDTA, 0.01% NaN$_3$, pH 6.5, 298.15 K

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### APPENDIX E

**NMR ASSIGNMENTS OF APO PCAM76-148 WHEN BOUND TO NA\textsubscript{v}1.2\textsubscript{IQP}**

**Amide Assignments of apo PCaM\textsubscript{76-148} When Bound to Na\textsubscript{v}1.2\textsubscript{IQP}**

Below are amide assignments of apo PCaM\textsubscript{76-148} when bound to Na\textsubscript{v}1.2\textsubscript{IQP} determined in 10 mM D\textsubscript{4}-imidazole, 100 mM KCl, 50 \mu M D\textsubscript{16}-EDTA, 0.01% NaN\textsubscript{3}, pH 6.5, 298.15 K

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Carbonyl assignments of apo CaM\textsubscript{76-148} when bound to Na\textsubscript{v}1.2\textsubscript{IQp} determined in 10 mM D\textsubscript{4}-imidazole, 100 mM KCl, 50 \textmu{}M D\textsubscript{16}-EDTA, 0.01\% NaN\textsubscript{3}, pH 6.5, 298.15 K

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Methine, Methyline, and Methyl Assignments of apo PCaM_{76-148}

When Bound to Na_{1.2}^{+}

Methine, methyline, and methyl assignments of apo PCaM_{76-148} when bound to Na_{1.2}^{+} determined in 10 mM D_{4}-imidazole, 100 mM KCl, 50 μM D_{16}-EDTA, 0.01% NaN_{3}, pH 6.5, 298.15 K

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Intramolecular NOE Assignments of Na$_2$1.2$_{IQp}$

Intramolecular NOE assignments of Na$_2$1.2$_{IQp}$ determined in 10 mM D$_4$-imidazole, 100 mM KCl, 50 µM D$_{16}$-EDTA, 0.01% NaN$_3$, pH 6.5, 298.15 K

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V 146 CA-HA 62.485 4.296 S 147 CB-HB2 64.113 4.019
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APPENDIX F

RESTRAINT FILES USED FOR APO CAM\textsubscript{76-148:NA\textsubscript{V1.2}iQP}

STRUCTURE CALCULATION

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assign ( residue 92 and name HD# ) ( residue 92 and name HE# ) 3.00 1.20 0.30
assign ( residue 92 and name HD# ) ( residue 92 and name HE# ) 4.00 2.20 0.40
assign ( residue 92 and name HE# ) ( residue 92 and name HE# ) 5.00 3.20 0.50
assign ( residue 92 and name HE# ) ( residue 92 and name HE# ) 5.00 3.20 0.50
assign ( residue 98 and name HA# ) ( residue 138 and name HE# ) 5.00 3.20 0.50
assign ( residue 99 and name HA ) ( residue 138 and name HE# ) 5.00 3.20 0.50
assign ( residue 105 and name HA ) ( residue 105 and name HG ) 4.00 2.20 0.40
assign ( residue 105 and name HD# ) ( residue 105 and name HG ) 3.00 1.20 0.30
assign ( residue 105 and name HD# ) ( residue 105 and name HG ) 4.00 2.20 0.40
assign ( residue 105 and name HD# ) ( residue 105 and name HG ) 3.00 1.20 0.30
assign ( residue 106 and name HA ) ( residue 106 and name HG ) 5.00 3.20 0.50
assign ( residue 106 and name HA ) ( residue 106 and name HG ) 4.00 2.20 0.40
assign ( residue 108 and name HA ) ( residue 108 and name HG ) 4.00 2.20 0.40
assign ( residue 108 and name HA ) ( residue 108 and name HG ) 4.00 2.20 0.40
assign ( residue 108 and name HA ) ( residue 108 and name HG ) 3.00 1.20 0.30
assign ( residue 108 and name HA ) ( residue 108 and name HG ) 3.00 1.20 0.30
assign ( residue 111 and name HA ) ( residue 111 and name HG ) 4.00 2.20 0.40
assign ( residue 111 and name HA ) ( residue 111 and name HG ) 4.00 2.20 0.40
assign ( residue 111 and name HA ) ( residue 111 and name HG ) 3.00 1.20 0.30
assign ( residue 111 and name HA ) ( residue 111 and name HG ) 3.00 1.20 0.30
assign ( residue 112 and name HA ) ( residue 112 and name HG ) 4.00 2.20 0.40
assign ( residue 112 and name HA ) ( residue 112 and name HG ) 4.00 2.20 0.40
assign ( residue 112 and name HA ) ( residue 112 and name HG ) 3.00 1.20 0.30
assign ( residue 112 and name HA ) ( residue 112 and name HG ) 3.00 1.20 0.30
assign ( residue 116 and name HA ) ( residue 116 and name HG ) 5.00 3.20 0.50
assign ( residue 116 and name HA ) ( residue 116 and name HG ) 4.00 2.20 0.40
assign ( residue 116 and name HA ) ( residue 116 and name HG ) 3.00 1.20 0.30
assign ( residue 116 and name HA ) ( residue 116 and name HG ) 3.00 1.20 0.30
assign ( residue 123 and name HG# ) ( residue 1920 and name HG ) 5.00 3.20 0.50
assign ( residue 85 and name HG ) ( residue 142 and name HA ) 4.00 2.20 0.40
assign ( residue 85 and name HG ) ( residue 85 and name HG ) 4.00 2.20 0.40
assign ( residue 85 and name HG ) ( residue 85 and name HG ) 4.00 2.20 0.40
assign ( residue 85 and name HD## ) ( residue 85 and name HG ) 3.00 1.20 0.30
assign ( residue 85 and name HD## ) ( residue 85 and name HG ) 3.00 1.20 0.30
assign ( residue 85 and name HG ) ( residue 86 and name HA ) 5.00 3.20 0.50
assign ( residue 85 and name HG ) ( residue 89 and name HD# ) 5.00 3.20 0.50
assign ( residue 85 and name HG ) ( residue 89 and name HE# ) 5.00 3.20 0.50
assign ( residue 99 and name HA ) ( residue 99 and name HG ) 4.00 2.20 0.40
assign ( residue 99 and name HB# ) ( residue 99 and name HG ) 4.00 2.20 0.40
assign ( residue 99 and name HD## ) ( residue 99 and name HG ) 3.00 1.20 0.30
assign ( residue 101 and name HB# ) ( residue 104 and name HG# ) 5.00 3.20 0.50
assign ( residue 104 and name HA ) ( residue 104 and name HG# ) 3.00 1.20 0.30
assign ( residue 104 and name HB# ) ( residue 104 and name HG# ) 3.00 1.20 0.30
assign ( residue 105 and name HA ) ( residue 109 and name HG# ) 5.00 3.20 0.50
assign ( residue 106 and name HA ) ( residue 106 and name HG# ) 4.00 2.20 0.40
assign ( residue 106 and name HG ) ( residue 142 and name HA ) 4.00 2.20 0.40
assign ( residue 106 and name HG ) ( residue 142 and name HA ) 4.00 2.20 0.40
assign ( residue 108 and name HA ) ( residue 108 and name HG ) 4.00 2.20 0.40
assign ( residue 108 and name HA ) ( residue 108 and name HG ) 4.00 2.20 0.40
assign ( residue 108 and name HA ) ( residue 108 and name HG ) 3.00 1.20 0.30
assign ( residue 108 and name HA ) ( residue 108 and name HG ) 3.00 1.20 0.30
assign (residue 106 and name HA) (residue 109 and name HG#) 6.00 4.20 0.60
assign (residue 106 and name HB#) (residue 106 and name HG#) 3.00 1.20 0.30
assign (residue 106 and name HD#) (residue 106 and name HG#) 4.00 2.20 0.40
assign (residue 106 and name HG#) (residue 107 and name HA) 5.00 3.20 0.50
assign (residue 104 and name HG#) (residue 107 and name HB#) 5.00 3.20 0.50
assign (residue 109 and name HA) (residue 109 and name HG#) 5.00 3.20 0.50
assign (residue 109 and name HB#) (residue 109 and name HG#) 3.00 1.20 0.30
assign (residue 109 and name HE#) (residue 1913 and name HG#) 5.00 3.20 0.50
assign (residue 109 and name HE#) (residue 109 and name HG#) 5.00 3.20 0.50
assign (residue 106 and name HG#) (residue 110 and name HB) 5.00 3.20 0.50
assign (residue 111 and name HA) (residue 115 and name HG#) 5.00 3.20 0.50
assign (residue 113 and name HA#) (residue 1913 and name HG#) 5.00 3.20 0.50
assign (residue 114 and name HA) (residue 114 and name HG#) 3.00 1.20 0.30
assign (residue 114 and name HA#) (residue 115 and name HG#) 5.00 3.20 0.50
assign (residue 114 and name HB#) (residue 114 and name HG#) 4.00 2.20 0.40
assign (residue 115 and name HA) (residue 115 and name HG#) 4.00 2.20 0.40
assign (residue 115 and name HB#) (residue 115 and name HG#) 3.00 1.20 0.30
assign (residue 115 and name HD#) (residue 115 and name HG#) 4.00 2.20 0.40
assign (residue 115 and name HE#) (residue 115 and name HG#) 4.00 2.20 0.40
assign (residue 116 and name HA) (residue 1913 and name HG#) 5.00 3.20 0.50
assign (residue 116 and name HB#) (residue 116 and name HG#) 5.00 3.20 0.50
assign (residue 116 and name HD1#) (residue 1913 and name HG#) 4.00 2.20 0.40
assign (residue 116 and name HD2#) (residue 1913 and name HG#) 5.00 3.20 0.50
assign (residue 109 and name HG#) (residue 116 and name HD2#) 5.00 3.20 0.50
assign (residue 116 and name HG) (residue 1913 and name HG#) 5.00 3.20 0.50
assign (residue 120 and name HB#) (residue 120 and name HG#) 4.00 2.20 0.40
assign (residue 123 and name HA) (residue 123 and name HG#) 4.00 2.20 0.40
assign (residue 123 and name HB#) (residue 123 and name HG#) 3.00 1.20 0.30
assign (residue 124 and name HA) (residue 124 and name HG#) 3.00 1.20 0.30
assign (residue 124 and name HB#) (residue 124 and name HG#) 4.00 2.20 0.40
assign (residue 126 and name HA) (residue 126 and name HG#) 4.00 2.20 0.40
assign (residue 126 and name HB#) (residue 126 and name HG#) 3.00 1.20 0.30
assign (residue 126 and name HD#) (residue 126 and name HG#) 5.00 3.20 0.50
assign (residue 127 and name HA) (residue 127 and name HG#) 5.00 3.20 0.50
assign (residue 127 and name HB#) (residue 127 and name HG#) 4.00 2.20 0.40
assign (residue 90 and name HG#) (residue 138 and name HE#) 5.00 3.20 0.50
assign (residue 139 and name HA) (residue 139 and name HG#) 4.00 2.20 0.40
assign (residue 139 and name HB#) (residue 139 and name HG#) 3.00 1.20 0.30
assign (residue 127 and name HG#) (residue 141 and name HA) 5.00 3.20 0.50
assign (residue 141 and name HA) (residue 144 and name HG#) 5.00 3.20 0.50
assign (residue 124 and name HG#) (residue 141 and name HD#) 6.00 4.20 0.50
assign (residue 127 and name HG#) (residue 141 and name HD#) 5.00 3.20 0.50
assign (residue 124 and name HG#) (residue 141 and name HE#) 5.00 3.20 0.50
assign (residue 142 and name HA) (residue 144 and name HG#) 5.00 3.20 0.50
assign (residue 142 and name HA) (residue 145 and name HG#) 5.00 3.20 0.50
assign (residue 139 and name HG#) (residue 142 and name HG##) 4.00 2.20 0.40
| Residue 143 | Name HD# | Residue 143 | Name HG# | 4.00 | 2.20 | 0.40 |
| Residue 144 | Name HA | Residue 144 | Name HG# | 4.00 | 2.20 | 0.40 |
| Residue 144 | Name HB# | Residue 144 | Name HG# | 4.00 | 2.20 | 0.40 |
| Residue 144 | Name HE# | Residue 145 | Name HG# | 5.00 | 3.20 | 0.50 |
| Residue 145 | Name HA | Residue 145 | Name HG# | 5.00 | 3.20 | 0.50 |
| Residue 145 | Name HB# | Residue 145 | Name HG# | 5.00 | 3.20 | 0.50 |
| Residue 85 | Name HB# | Residue 146 | Name HG## | 6.00 | 4.20 | 0.60 |
| Residue 144 | Name HG# | Residue 146 | Name HG## | 5.00 | 3.20 | 0.50 |
| Residue 77 | Name HA | Residue 77 | Name HG# | 4.00 | 2.20 | 0.40 |
| Residue 77 | Name HB# | Residue 77 | Name HG# | 4.00 | 2.20 | 0.30 |
| Residue 77 | Name HD# | Residue 77 | Name HG# | 4.00 | 2.20 | 0.30 |
| Residue 77 | Name HE# | Residue 78 | Name HG# | 4.00 | 2.20 | 0.40 |
| Residue 78 | Name HA | Residue 78 | Name HG# | 4.00 | 2.20 | 0.40 |
| Residue 78 | Name HB# | Residue 78 | Name HG# | 3.00 | 1.20 | 0.30 |
| Residue 79 | Name HA | Residue 79 | Name HG# | 4.00 | 2.20 | 0.30 |
| Residue 79 | Name HB# | Residue 79 | Name HG# | 4.00 | 2.20 | 0.30 |
| Residue 80 | Name HB# | Residue 1914 | Name HG# | 6.00 | 4.20 | 0.60 |
| Residue 83 | Name HA | Residue 83 | Name HG# | 3.00 | 1.20 | 0.30 |
| Residue 83 | Name HB# | Residue 83 | Name HG# | 3.00 | 1.20 | 0.30 |
| Residue 84 | Name HA | Residue 84 | Name HG# | 3.00 | 1.20 | 0.30 |
| Residue 84 | Name HB# | Residue 84 | Name HG# | 3.00 | 1.20 | 0.30 |
| Residue 90 | Name HA | Residue 90 | Name HG# | 3.00 | 1.20 | 0.30 |
| Residue 90 | Name HB# | Residue 90 | Name HG# | 3.00 | 1.20 | 0.30 |
| Residue 90 | Name HE# | Residue 90 | Name HG# | 4.00 | 2.20 | 0.40 |
| Residue 91 | Name HA | Residue 91 | Name HG## | 6.00 | 4.20 | 0.50 |
| Residue 91 | Name HB | Residue 91 | Name HG# | 5.00 | 3.20 | 0.50 |
| Residue 91 | Name HG## | Residue 1905 | Name HG# | 3.00 | 1.20 | 0.30 |
| Residue 92 | Name HD# | Residue 1905 | Name HG# | 5.00 | 3.20 | 0.50 |
| Residue 92 | Name HG# | Residue 93 | Name HB# | 5.00 | 3.20 | 0.50 |
| Residue 94 | Name HA | Residue 94 | Name HG# | 4.00 | 2.20 | 0.40 |
| Residue 94 | Name HD# | Residue 94 | Name HG# | 3.00 | 1.20 | 0.30 |
| Residue 100 | Name HA | Residue 100 | Name HG1# | 4.00 | 2.20 | 0.40 |
| Residue 100 | Name HB | Residue 100 | Name HG1# | 3.00 | 1.20 | 0.30 |
| Residue 100 | Name HD# | Residue 100 | Name HG1# | 4.00 | 2.20 | 0.40 |
| Residue 109 | Name HE# | Residue 109 | Name HE# | 3.00 | 1.20 | 0.30 |
| Residue 117 | Name HA | Residue 121 | Name HG## | 5.00 | 3.20 | 0.50 |
| Residue 118 | Name HA | Residue 121 | Name HG# | 3.00 | 1.20 | 0.30 |
| Residue 118 | Name HB# | Residue 121 | Name HG## | 5.00 | 3.20 | 0.50 |
| Residue 121 | Name HG## | Residue 122 | Name HA | 5.00 | 3.20 | 0.50 |
| Residue 122 | Name HA | Residue 125 | Name HG1# | 5.00 | 3.20 | 0.50 |
| Residue 121 | Name HG## | Residue 122 | Name HB# | 5.00 | 3.20 | 0.50 |
| Residue 125 | Name HA | Residue 125 | Name HG1# | 4.00 | 2.20 | 0.40 |
| Residue 125 | Name HB | Residue 125 | Name HG1# | 3.00 | 1.20 | 0.30 |
assign (residue 125 and name HD#) (residue 125 and name HG1#) 3.00 1.20 0.30
assign (residue 129 and name HA) (residue 136 and name HG1#) 5.00 3.20 0.50
assign (residue 129 and name HB#) (residue 136 and name HG1#) 5.00 3.20 0.50
assign (residue 130 and name HA) (residue 130 and name HG1#) 5.00 3.20 0.50
assign (residue 130 and name HB) (residue 130 and name HG1#) 5.00 3.20 0.50
assign (residue 130 and name HD#) (residue 136 and name HG1#) 4.00 2.20 0.40
assign (residue 134 and name HA#) (residue 136 and name HG1#) 4.00 2.20 0.40
assign (residue 135 and name HA) (residue 136 and name HG1#) 5.00 3.20 0.50
assign (residue 135 and name HB#) (residue 136 and name HG1#) 5.00 3.20 0.50
assign (residue 136 and name HA) (residue 136 and name HG1#) 4.00 2.20 0.40
assign (residue 136 and name HB) (residue 136 and name HG1#) 5.00 3.20 0.50
assign (residue 136 and name HD#) (residue 136 and name HG1#) 5.00 3.20 0.50
assign (residue 86 and name HG1#) (residue 138 and name HB#) 5.00 3.20 0.50
assign (residue 86 and name HG1#) (residue 139 and name HA) 6.00 4.20 0.60
assign (residue 86 and name HG1#) (residue 139 and name HB) 5.00 3.20 0.50
assign (residue 86 and name HG1#) (residue 142 and name HG##) 6.00 4.20 0.60
assign (residue 86 and name HG1#) (residue 1911 and name HG##) 4.00 2.20 0.40
assign (residue 84 and name HA) (residue 1911 and name HG##) 5.00 3.20 0.50
assign (residue 84 and name HB) (residue 1911 and name HG##) 4.00 2.20 0.40
assign (residue 84 and name HG) (residue 1911 and name HG##) 4.00 2.20 0.40
assign (residue 87 and name HA) (residue 91 and name HG##) 6.00 4.20 0.60
assign (residue 88 and name HB) (residue 911 and name HG##) 3.00 1.20 0.30
assign (residue 88 and name HG) (residue 91 and name HG##) 4.00 2.20 0.40
assign (residue 88 and name HD) (residue 91 and name HG##) 4.00 2.20 0.40
assign (residue 89 and name HA) (residue 91 and name HG##) 5.00 3.20 0.50
assign (residue 89 and name HB) (residue 142 and name HG##) 5.00 3.20 0.50
assign (residue 89 and name HD) (residue 142 and name HG##) 4.00 2.20 0.40
assign (residue 92 and name HA) (residue 100 and name HG1#) 5.00 3.20 0.50
assign (residue 92 and name HA) (residue 108 and name HG##) 5.00 3.20 0.50
assign (residue 92 and name HB) (residue 100 and name HG1#) 5.00 3.20 0.50
assign (residue 93 and name HA) (residue 100 and name HG1#) 5.00 3.20 0.50
assign (residue 100 and name HA) (residue 100 and name HG2#) 3.00 1.20 0.30
assign (residue 100 and name HA) (residue 136 and name HG2#) 6.00 4.20 0.60
assign ( residue 100 and name HB  ) ( residue 100 and name HG2# )   3.00  1.20  0.30
assign ( residue 100 and name HB  ) ( residue 136 and name HG2# )   5.00  3.20  0.50
assign ( residue 100 and name HD1# ) ( residue 100 and name HG2# )   4.00  2.20  0.40
assign ( residue 100 and name HD1# ) ( residue 136 and name HG2# )   5.00  3.20  0.50
assign ( residue 100 and name HG1# ) ( residue 100 and name HG2# )   3.00  1.20  0.30
assign ( residue 100 and name HG1# ) ( residue 136 and name HG2# )   5.00  3.20  0.50
assign ( residue 100 and name HG2# ) ( residue 136 and name HG2# )   3.00  1.20  0.30
assign ( residue 100 and name HG2# ) ( residue 102 and name HA   )   5.00  3.20  0.50
assign ( residue 102 and name HA   ) ( residue 125 and name HG2# )   5.00  3.20  0.50
assign ( residue 102 and name HB#  ) ( residue 125 and name HG2# )   4.00  2.20  0.40
assign ( residue 104 and name HA   ) ( residue 108 and name HG## )   6.00  4.20  0.60
assign ( residue 105 and name HA   ) ( residue 108 and name HG## )   4.00  2.20  0.40
assign ( residue 105 and name HB#  ) ( residue 108 and name HG2# )   5.00  3.20  0.50
assign ( residue 105 and name HD1# ) ( residue 108 and name HG2# )   3.00  1.20  0.30
assign ( residue 105 and name HD2# ) ( residue 108 and name HG2# )   5.00  3.20  0.50
assign ( residue 105 and name HG#  ) ( residue 121 and name HG## )   5.00  3.20  0.50
assign ( residue 106 and name HA   ) ( residue 110 and name HG2# )   5.00  3.20  0.50
assign ( residue 106 and name HA   ) ( residue 121 and name HG## )   3.00  1.20  0.30
assign ( residue 106 and name HB#  ) ( residue 110 and name HG2# )   5.00  3.20  0.50
assign ( residue 106 and name HD#  ) ( residue 110 and name HG2# )   5.00  3.20  0.50
assign ( residue 106 and name HG#  ) ( residue 121 and name HG## )   5.00  3.20  0.50
assign ( residue 107 and name HB#  ) ( residue 108 and name HG2# )   5.00  3.20  0.50
assign ( residue 108 and name HA   ) ( residue 108 and name HG## )   3.00  1.20  0.30
assign ( residue 109 and name HA   ) ( residue 109 and name HG#  )   5.00  3.20  0.50
assign ( residue 109 and name HB#  ) ( residue 109 and name HG#  )   5.00  3.20  0.50
assign ( residue 109 and name HG#  ) ( residue 109 and name HE#  )   3.00  1.20  0.30
assign ( residue 109 and name HE#  ) ( residue 121 and name HG## )   5.00  3.20  0.50
assign ( residue 108 and name HG## ) ( residue 109 and name HG#  )   5.00  3.20  0.50
assign ( residue 110 and name HB ) ( residue 110 and name HG2# ) 3.00 1.20 0.30
assign ( residue 110 and name HB ) ( residue 121 and name HG## ) 5.00 3.20 0.50
assign ( residue 108 and name HG## ) ( residue 111 and name HB# ) 5.00 3.20 0.50
assign ( residue 108 and name HG## ) ( residue 112 and name HB# ) 5.00 3.20 0.50
assign ( residue 108 and name HG## ) ( residue 112 and name HD## ) 5.00 3.20 0.50
assign ( residue 110 and name HG2# ) ( residue 115 and name HA ) 5.00 3.20 0.50
assign ( residue 110 and name HG2# ) ( residue 115 and name HG ) 5.00 3.20 0.50
assign ( residue 110 and name HG2# ) ( residue 116 and name HB# ) 5.00 3.20 0.50
assign ( residue 110 and name HG2# ) ( residue 116 and name HB# ) 5.00 3.20 0.50
assign ( residue 110 and name HG2# ) ( residue 116 and name HA ) 5.00 3.20 0.50
assign ( residue 117 and name HA ) ( residue 117 and name HG2# ) 3.00 1.20 0.30
assign ( residue 110 and name HG2# ) ( residue 117 and name HB ) 5.00 3.20 0.50
assign ( residue 117 and name HB ) ( residue 117 and name HG## ) 3.00 1.20 0.30
assign ( residue 110 and name HG2# ) ( residue 118 and name HA ) 4.00 2.20 0.40
assign ( residue 117 and name HG2# ) ( residue 118 and name HA ) 4.00 2.20 0.40
assign ( residue 110 and name HG2# ) ( residue 120 and name HB# ) 5.00 3.20 0.50
assign ( residue 110 and name HG2# ) ( residue 121 and name HA ) 5.00 3.20 0.50
assign ( residue 121 and name HA ) ( residue 121 and name HG## ) 3.00 1.20 0.30
assign ( residue 110 and name HG2# ) ( residue 121 and name HB ) 4.00 2.20 0.40
assign ( residue 121 and name HB ) ( residue 121 and name HG## ) 3.00 1.20 0.30
assign ( residue 110 and name HG2# ) ( residue 121 and name HG## ) 4.00 2.20 0.40
assign ( residue 122 and name HA ) ( residue 125 and name HG2# ) 5.00 3.20 0.50
assign ( residue 121 and name HG## ) ( residue 124 and name HB# ) 5.00 3.20 0.50
assign ( residue 121 and name HG## ) ( residue 124 and name HE# ) 4.00 2.20 0.40
assign ( residue 121 and name HG## ) ( residue 124 and name HG# ) 5.00 3.20 0.50
assign ( residue 125 and name HA ) ( residue 125 and name HG2# ) 3.00 1.20 0.30
assign ( residue 125 and name HA ) ( residue 136 and name HG2# ) 5.00 3.20 0.50
assign ( residue 125 and name HB ) ( residue 125 and name HG2# ) 3.00 1.20 0.30
assign ( residue 125 and name HB ) ( residue 136 and name HG2# ) 5.00 3.20 0.50
assign ( residue 125 and name HD# ) ( residue 125 and name HG2# ) 3.00 1.20 0.30
assign ( residue 125 and name HG1# ) ( residue 125 and name HG2# ) 3.00 1.20 0.30
assign ( residue 125 and name HG1# ) ( residue 136 and name HG2# ) 3.00 1.20 0.30
assign ( residue 130 and name HA ) ( residue 130 and name HG2# ) 3.00 1.20 0.30
assign ( residue 130 and name HA ) ( residue 130 and name HG2# ) 4.00 2.20 0.40
assign ( residue 130 and name HB ) ( residue 130 and name HG2# ) 3.00 1.20 0.30
assign ( residue 130 and name HB ) ( residue 136 and name HG2# ) 5.00 3.20 0.50
assign ( residue 130 and name HG1# ) ( residue 130 and name HG2# ) 5.00 3.20 0.50
assign ( residue 130 and name HG1# ) ( residue 136 and name HG2# ) 5.00 3.20 0.50
assign ( residue 130 and name HG2# ) ( residue 134 and name HA# ) 5.00 3.20 0.50
assign ( residue 136 and name HA ) ( residue 136 and name HG2# ) 3.00 1.20 0.30
assign ( residue 100 and name HG2# ) ( residue 136 and name HB ) 5.00 3.20 0.50
assign ( residue 136 and name HB ) ( residue 136 and name HG2# ) 4.00 2.20 0.40
assign ( residue 136 and name HD# ) ( residue 136 and name HG2# ) 3.00 1.20 0.30
assign ( residue 136 and name HG1# ) ( residue 136 and name HG2# ) 3.00 1.20 0.30
assign ( residue 136 and name HG2# ) ( residue 137 and name HA  )  5.00  3.20  0.50
assign ( residue 136 and name HG2# ) ( residue 137 and name HB#  )  5.00  3.20  0.50
assign ( residue 136 and name HG2# ) ( residue 138 and name HA  )  4.00  2.20  0.40
assign ( residue 86 and name HG2# ) ( residue 138 and name HB#  )  4.00  2.20  0.40
assign ( residue 100 and name HG2# ) ( residue 138 and name HB#  )  5.00  3.20  0.50
assign ( residue 86 and name HG2# ) ( residue 138 and name HE#  )  5.00  3.20  0.50
assign ( residue 100 and name HG2# ) ( residue 138 and name HE#  )  5.00  3.20  0.50
assign ( residue 86 and name HG2# ) ( residue 138 and name HB#  )  4.00  2.20  0.40
assign ( residue 100 and name HG2# ) ( residue 138 and name HD#  )  5.00  3.20  0.50
assign ( residue 100 and name HG2# ) ( residue 138 and name HD#  )  4.00  2.20  0.40
assign ( residue 86 and name HG2# ) ( residue 138 and name HE#  )  3.00  1.20  0.30
assign ( residue 100 and name HG2# ) ( residue 138 and name HD#  )  5.00  3.20  0.50
assign ( residue 100 and name HG2# ) ( residue 138 and name HE#  )  5.00  3.20  0.50
assign ( residue 86 and name HG2# ) ( residue 138 and name HD#  )  3.00  1.20  0.30
assign ( residue 100 and name HG2# ) ( residue 138 and name HD#  )  5.00  3.20  0.50
assign ( residue 100 and name HG2# ) ( residue 138 and name HE#  )  5.00  3.20  0.50
assign ( residue 86 and name HG2# ) ( residue 138 and name HE#  )  3.00  1.20  0.30
assign ( residue 100 and name HG2# ) ( residue 138 and name HD#  )  5.00  3.20  0.50
assign ( residue 100 and name HG2# ) ( residue 138 and name HE#  )  5.00  3.20  0.50
assign ( residue 86 and name HG2# ) ( residue 138 and name HE#  )  3.00  1.20  0.30
assign ( residue 100 and name HG2# ) ( residue 138 and name HD#  )  5.00  3.20  0.50
assign ( residue 100 and name HG2# ) ( residue 138 and name HE#  )  5.00  3.20  0.50
assign ( residue 86 and name HG2# ) ( residue 138 and name HE#  )  3.00  1.20  0.30
assign ( residue 100 and name HG2# ) ( residue 138 and name HD#  )  5.00  3.20  0.50
assign ( residue 100 and name HG2# ) ( residue 138 and name HE#  )  5.00  3.20  0.50
assign ( residue 86 and name HG2# ) ( residue 138 and name HE#  )  3.00  1.20  0.30
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<th>Name HA</th>
<th>Residue 142</th>
<th>Name HN</th>
<th>Distance (Å)</th>
<th>Bond Angle (°)</th>
<th>Torsion Angle (°)</th>
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assign (residue 86 and name HG) (residue 86 and name HN) 4.00 2.20 0.40
assign (residue 86 and name HD) (residue 86 and name HN) 4.00 2.20 0.40
assign (residue 86 and name HB) (residue 86 and name HN) 3.00 1.20 0.30
assign (residue 84 and name HB) (residue 84 and name HN) 3.00 1.20 0.30
assign (residue 84 and name HG) (residue 84 and name HN) 3.00 1.20 0.30
assign (residue 83 and name HA) (residue 83 and name HN) 5.00 3.20 0.50
assign (residue 83 and name HG) (residue 83 and name HN) 4.00 2.20 0.40
assign (residue 83 and name HA) (residue 83 and name HN) 3.00 1.20 0.30
assign (residue 83 and name HB) (residue 83 and name HN) 3.00 1.20 0.30
assign (residue 82 and name HN) (residue 142 and name HG) 5.00 3.20 0.50
assign (residue 80 and name HB) (residue 82 and name HN) 5.00 3.20 0.50
assign (residue 81 and name HB) (residue 82 and name HN) 5.00 3.20 0.50
assign (residue 82 and name HN) (residue 146 and name HB) 5.00 3.20 0.50
assign (residue 82 and name HN) (residue 84 and name HN) 5.00 3.20 0.50
assign (residue 82 and name HN) (residue 83 and name HN) 4.00 2.20 0.40
assign (residue 82 and name HA) (residue 82 and name HN) 3.00 1.20 0.30
assign (residue 82 and name HB) (residue 83 and name HB) 5.00 3.20 0.50
assign (residue 81 and name HA) (residue 81 and name HN) 4.00 2.20 0.40
Hydrogen Bonding Restraint File

Hydrogen bonding restraint file derived from amide exchange data
assign (residue 114 and name N) (residue 1913 and name OE1) 2.90 0.20 0.40
assign (residue 114 and name HN) (residue 1913 and name OE1) 1.80 0.00 0.60
assign (residue 83 and name HN) (residue 79 and name O) 1.80 0.00 0.60
assign (residue 86 and name HN) (residue 82 and name O) 1.80 0.00 0.60
assign (residue 87 and name HN) (residue 83 and name O) 1.80 0.00 0.60
assign (residue 88 and name HN) (residue 84 and name O) 1.80 0.00 0.60
assign (residue 89 and name HN) (residue 85 and name O) 1.80 0.00 0.60
assign (residue 90 and name HN) (residue 86 and name O) 1.80 0.00 0.60
assign (residue 91 and name HN) (residue 87 and name O) 1.80 0.00 0.60
assign (residue 105 and name HN) (residue 101 and name O) 1.80 0.00 0.60
assign (residue 106 and name HN) (residue 102 and name O) 1.80 0.00 0.60
assign (residue 108 and name HN) (residue 104 and name O) 1.80 0.00 0.60
assign (residue 109 and name HN) (residue 105 and name O) 1.80 0.00 0.60
assign (residue 110 and name HN) (residue 106 and name O) 1.80 0.00 0.60
assign (residue 111 and name HN) (residue 107 and name O) 1.80 0.00 0.60
assign (residue 112 and name HN) (residue 108 and name O) 1.80 0.00 0.60
assign (residue 116 and name HN) (residue 112 and name O) 1.80 0.00 0.60
assign (residue 121 and name HN) (residue 117 and name O) 1.80 0.00 0.60
assign (residue 122 and name HN) (residue 118 and name O) 1.80 0.00 0.60
assign (residue 123 and name HN) (residue 119 and name O) 1.80 0.00 0.60
assign (residue 124 and name HN) (residue 120 and name O) 1.80 0.00 0.60
assign (residue 125 and name HN) (residue 121 and name O) 1.80 0.00 0.60
assign (residue 126 and name HN) (residue 122 and name O) 1.80 0.00 0.60
assign (residue 128 and name HN) (residue 124 and name O) 1.80 0.00 0.60
assign (residue 138 and name HN) (residue 98 and name O) 1.80 0.00 0.60
assign (residue 142 and name HN) (residue 138 and name O) 1.80 0.00 0.60
assign (residue 143 and name HN) (residue 139 and name O) 1.80 0.00 0.60
assign (residue 144 and name HN) (residue 140 and name O) 1.80 0.00 0.60
assign (residue 145 and name HN) (residue 141 and name O) 1.80 0.00 0.60
assign (residue 146 and name HN) (residue 142 and name O) 1.80 0.00 0.60
assign (residue 100 and name HN) (residue 136 and name O) 1.80 0.00 0.60
assign (residue 136 and name HN) (residue 100 and name O) 1.80 0.00 0.60
assign (residue 1913 and name HE2#) (residue 112 and name O) 1.80 0.00 0.60
assign (residue 114 and name HN) (residue 1913 and name OE1) 1.80 0.00 0.60
assign (residue 83 and name N) (residue 1913 and name OE1) 2.90 0.20 0.40
assign (residue 86 and name N) (residue 82 and name O) 2.90 0.20 0.40
assign (residue 87 and name N) (residue 83 and name O) 2.90 0.20 0.40
assign (residue 88 and name N) (residue 84 and name O) 2.90 0.20 0.40
assign (residue 89 and name N) (residue 85 and name O) 2.90 0.20 0.40
assign (residue 90 and name N) (residue 86 and name O) 2.90 0.20 0.40
assign (residue 91 and name N) (residue 87 and name O) 2.90 0.20 0.40
assign (residue 105 and name N) (residue 101 and name O) 2.90 0.20 0.40
assign (residue 106 and name N) (residue 102 and name O) 2.90 0.20 0.40
assign (residue 108 and name N) (residue 104 and name O) 2.90 0.20 0.40
assign (residue 109 and name N) (residue 105 and name O) 2.90 0.20 0.40
assign (residue 110 and name N) (residue 106 and name O) 2.90 0.20 0.40
assign (residue 111 and name N) (residue 107 and name O) 2.90 0.20 0.40
assign (residue 112 and name N) (residue 108 and name O) 2.90 0.20 0.40
assign (residue 116 and name N) (residue 112 and name O) 2.90 0.20 0.60
assign (residue 121 and name N) (residue 117 and name O) 2.90 0.20 0.40
assign (residue 122 and name N) (residue 118 and name O) 2.90 0.20 0.40
assign (residue 123 and name N) (residue 119 and name O) 2.90 0.20 0.40
assign (residue 124 and name N) (residue 120 and name O) 2.90 0.20 0.40
assign (residue 125 and name N) (residue 121 and name O) 2.90 0.20 0.40
assign (residue 126 and name N) (residue 122 and name O) 2.90 0.20 0.40
assign (residue 128 and name N) (residue 124 and name O) 2.90 0.20 0.40
assign (residue 142 and name N) (residue 138 and name O) 2.90 0.20 0.40
assign (residue 143 and name N) (residue 139 and name O) 2.90 0.20 0.40
assign (residue 144 and name N) (residue 140 and name O) 2.90 0.20 0.40
assign (residue 145 and name N) (residue 141 and name O) 2.90 0.20 0.40
assign (residue 146 and name N) (residue 142 and name O) 2.90 0.20 0.40
assign (residue 100 and name N) (residue 136 and name O) 2.90 0.20 0.40
assign (residue 136 and name N) (residue 100 and name O) 2.90 0.20 0.40
assign (residue 1913 and name NE2) (residue 112 and name O) 2.90 0.20 0.40
assign (residue 114 and name N) (residue 1913 and name OE1) 2.90 0.20 0.40
assign (residue 1908 and name HN) (residue 1904 and name O) 1.80 0.00 0.60
assign (residue 1909 and name HN) (residue 1905 and name O) 1.80 0.00 0.60
assign (residue 1910 and name HN) (residue 1906 and name O) 1.80 0.00 0.60
assign (residue 1911 and name HN) (residue 1907 and name O) 1.80 0.00 0.60
assign (residue 1912 and name HN) (residue 1908 and name O) 1.80 0.00 0.60
assign (residue 1913 and name HN) (residue 1909 and name O) 1.80 0.00 0.60
assign (residue 1914 and name HN) (residue 1910 and name O) 1.80 0.00 0.60
assign (residue 1915 and name HN) (residue 1911 and name O) 1.80 0.00 0.60
assign (residue 1916 and name HN) (residue 1912 and name O) 1.80 0.00 0.60
assign (residue 1917 and name HN) (residue 1913 and name O) 1.80 0.00 0.60
assign (residue 1918 and name HN) (residue 1914 and name O) 1.80 0.00 0.60
assign (residue 1919 and name HN) (residue 1915 and name O) 1.80 0.00 0.60
assign (residue 1920 and name HN) (residue 1916 and name O) 1.80 0.00 0.60
assign (residue 1921 and name HN) (residue 1917 and name O) 1.80 0.00 0.60
assign (residue 1904 and name HA) (residue 1905 and name HA) 5.00 3.20 0.50
assign (residue 1904 and name HA) (residue 1907 and name HA) 4.00 2.20 0.40
assign (residue 1904 and name HE2) (residue 1904 and name HB) 5.00 3.20 0.50
assign (residue 1904 and name HG) (residue 1904 and name HA) 5.00 3.20 0.50
assign (residue 1905 and name HA) (residue 1906 and name HA) 5.00 3.20 0.50
assign (residue 1905 and name HB) (residue 1905 and name HA) 4.00 2.20 0.40
assign (residue 1905 and name HG) (residue 1905 and name HB) 5.00 3.20 0.50
assign (residue 1905 and name HG) (residue 1905 and name HB) 3.00 1.20 0.30
assign (residue 1904 and name HN) (residue 1905 and name HN) 5.00 3.20 0.50

\( Na_{1.2 \text{op}} \) Intramolecular Restraint File

assign (residue 1904 and name HA) (residue 1904 and name HN) 5.00 3.20 0.50
assign (residue 1904 and name HA) (residue 1905 and name HN) 5.00 3.20 0.50
assign (residue 1904 and name HA) (residue 1907 and name HN) 4.00 2.20 0.40
assign (residue 1904 and name HB) (residue 1904 and name HA) 4.00 2.20 0.40
assign (residue 1904 and name HG) (residue 1904 and name HB) 4.00 2.20 0.40
assign (residue 1904 and name HG) (residue 1904 and name HE2) 5.00 3.20 0.50
assign (residue 1904 and name HG) (residue 1905 and name HB) 5.00 3.20 0.50
assign (residue 1904 and name HG) (residue 1905 and name HA) 4.00 2.20 0.40
assign (residue 1905 and name HA) (residue 1908 and name HA) 5.00 3.20 0.50
assign (residue 1905 and name HB) (residue 1905 and name HA) 4.00 2.20 0.40
assign (residue 1905 and name HG) (residue 1905 and name HB) 5.00 3.20 0.50
assign (residue 1905 and name HG) (residue 1905 and name HB) 3.00 1.20 0.30
assign (residue 1904 and name HN) (residue 1905 and name HN) 5.00 3.20 0.50
assign (residue 1905 and name HN) (residue 1905 and name HA)  5.00 3.20 0.50
assign (residue 1905 and name HN) (residue 1905 and name HB#)  4.00 2.20 0.40
assign (residue 1905 and name HN) (residue 1905 and name HG#)  5.00 3.20 0.50
assign (residue 1906 and name HB#) (residue 1906 and name HA)  5.00 3.20 0.50
assign (residue 1906 and name HB#) (residue 1906 and name HB#)  3.00 1.20 0.30
assign (residue 1906 and name HB#) (residue 1907 and name HN)  4.00 2.20 0.40
assign (residue 1906 and name HB#) (residue 1906 and name HG#)  5.00 3.20 0.50
assign (residue 1906 and name HB#) (residue 1909 and name HB#)  5.00 3.20 0.50
assign (residue 1907 and name HB) (residue 1907 and name HA)  5.00 3.20 0.50
assign (residue 1907 and name HB) (residue 1908 and name HN)  4.00 2.20 0.40
assign (residue 1907 and name HB) (residue 1908 and name HG##)  5.00 3.20 0.50
assign (residue 1908 and name HB#) (residue 1908 and name HA)  5.00 3.20 0.50
assign (residue 1908 and name HB#) (residue 1909 and name HB#)  5.00 3.20 0.50
assign (residue 1908 and name HB#) (residue 1909 and name HB#)  5.00 3.20 0.50
assign (residue 1909 and name HB#) (residue 1910 and name HB#)  5.00 3.20 0.50
assign (residue 1909 and name HB#) (residue 1910 and name HN)  5.00 3.20 0.50
assign (residue 1910 and name HA) (residue 1910 and name HN)  5.00 3.20 0.50
assign (residue 1910 and name HA) (residue 1910 and name HG1#)  4.00 2.20 0.40
assign (residue 1910 and name HG1#) (residue 1910 and name HB)  3.00 1.20 0.30
assign (residue 1910 and name HG1#) (residue 1910 and name HN)  4.00 2.20 0.40
assign (residue 1910 and name HN) (residue 1910 and name HN)  5.00 3.20 0.50
assign (residue 1910 and name HN) (residue 1910 and name HB)  5.00 3.20 0.50

assign (residue 1911 and name HA) (residue 1912 and name HN) 5.00 3.20 0.50
assign (residue 1911 and name HB) (residue 1911 and name HA) 5.00 3.20 0.50
assign (residue 1911 and name HB) (residue 1912 and name HN) 4.00 2.20 0.40
assign (residue 1908 and name HA) (residue 1911 and name HG##) 5.00 3.20 0.50
assign (residue 1911 and name HG##) (residue 1911 and name HA) 3.00 1.20 0.30
assign (residue 1911 and name HG##) (residue 1911 and name HB) 3.00 1.20 0.30
assign (residue 1911 and name HG##) (residue 1913 and name HN) 5.00 3.20 0.50
assign (residue 1910 and name HN) (residue 1911 and name HN) 3.00 1.20 0.30
assign (residue 1911 and name HN) (residue 1911 and name HA) 4.00 2.20 0.40
assign (residue 1911 and name HN) (residue 1911 and name HB) 4.00 2.20 0.40
assign (residue 1911 and name HN) (residue 1911 and name HG##) 4.00 2.20 0.40
assign (residue 1912 and name HA) (residue 1912 and name HB) 4.00 2.20 0.40
assign (residue 1912 and name HA) (residue 1912 and name HD#) 5.00 3.20 0.50
assign (residue 1912 and name HA) (residue 1916 and name HN) 5.00 3.20 0.50
assign (residue 1912 and name HB) (residue 1912 and name HD#) 4.00 2.20 0.40
assign (residue 1909 and name HA) (residue 1912 and name HD1#) 5.00 3.20 0.50
assign (residue 1909 and name HB#) (residue 1912 and name HD1#) 5.00 3.20 0.50
assign (residue 1912 and name HD1#) (residue 1912 and name HA) 5.00 3.20 0.50
assign (residue 1912 and name HD1#) (residue 1912 and name HB) 4.00 2.20 0.40
assign (residue 1912 and name HD1#) (residue 1912 and name HN) 5.00 3.20 0.50
assign (residue 1911 and name HN) (residue 1912 and name HN) 4.00 2.20 0.40
assign (residue 1912 and name HN) (residue 1912 and name HA) 5.00 3.20 0.50
assign (residue 1912 and name HN) (residue 1912 and name HB) 4.00 2.20 0.40
assign (residue 1912 and name HN) (residue 1912 and name HD#) 5.00 3.20 0.50
assign (residue 1913 and name HA) (residue 1914 and name HN) 5.00 3.20 0.50
assign (residue 1913 and name HB#) (residue 1913 and name HA) 5.00 3.20 0.50
assign (residue 1913 and name HB#) (residue 1913 and name HB) 5.00 3.20 0.50
assign (residue 1913 and name HB#) (residue 1913 and name HG#) 5.00 3.20 0.50
assign (residue 1913 and name HG#) (residue 1913 and name HA) 5.00 3.20 0.50
assign (residue 1913 and name HG#) (residue 1913 and name HB#) 5.00 3.20 0.50
assign (residue 1913 and name HG#) (residue 1913 and name HG2#) 5.00 3.20 0.50
assign (residue 1912 and name HA) (residue 1913 and name HN) 5.00 3.20 0.50
assign (residue 1912 and name HB) (residue 1913 and name HN) 5.00 3.20 0.50
assign (residue 1912 and name HN) (residue 1913 and name HN) 5.00 3.20 0.50
assign (residue 1913 and name HN) (residue 1913 and name HA) 5.00 3.20 0.50
assign (residue 1913 and name HN) (residue 1913 and name HB#) 5.00 3.20 0.50
assign (residue 1911 and name HG##) (residue 1914 and name HA) 5.00 3.20 0.50
assign (residue 1914 and name HA) (residue 1914 and name HD#) 5.00 3.20 0.50
assign (residue 1913 and name HN) (residue 1914 and name HB#) 5.00 3.20 0.50
assign (residue 1914 and name HG#) (residue 1914 and name HA) 4.00 2.20 0.40
assign (residue 1914 and name HG#) (residue 1914 and name HB#) 5.00 3.20 0.50
assign (residue 1914 and name HG#) (residue 1914 and name HD#) 5.00 3.20 0.50
assign (residue 1913 and name HB#) (residue 1914 and name HN) 5.00 3.20 0.50
assign (residue 1913 and name HN) (residue 1914 and name HN) 5.00 3.20 0.50
assign (residue 1914 and name HN) (residue 1914 and name HA) 5.00 3.20 0.50
assign (residue 1914 and name HN) (residue 1914 and name HB#) 5.00 3.20 0.50
assign (residue 1914 and name HN) (residue 1914 and name HD#) 5.00 3.20 0.50
assign (residue 1914 and name HN) (residue 1914 and name HG#) 5.00 3.20 0.50
assign (residue 1915 and name HA) (residue 1916 and name HN) 5.00 3.20 0.50
assign (residue 1915 and name HB#) (residue 1915 and name HA) 4.00 2.20 0.40
assign (residue 1915 and name HB#) (residue 1916 and name HN) 4.00 2.20 0.40
assign (residue 1915 and name HB#) (residue 1916 and name HB#) 5.00 3.20 0.50
assign (residue 1915 and name HB#) (residue 1916 and name HD#) 5.00 3.20 0.50
assign (residue 1915 and name HB#) (residue 1916 and name HE#) 3.00 1.20 0.30
assign (residue 1916 and name HA) (residue 1917 and name HD#) 5.00 3.20 0.50
assign (residue 1916 and name HA) (residue 1917 and name HB#) 4.00 2.20 0.40
assign (residue 1916 and name HA) (residue 1917 and name HE#) 5.00 3.20 0.50
assign (residue 1916 and name HA) (residue 1918 and name HD#) 5.00 3.20 0.50
assign (residue 1916 and name HE#) (residue 1918 and name HN) 5.00 3.20 0.50
assign (residue 1916 and name HA) (residue 1918 and name HB#) 4.00 2.20 0.40
assign (residue 1916 and name HA) (residue 1918 and name HD#) 5.00 3.20 0.50
assign (residue 1916 and name HA) (residue 1918 and name HE#) 3.00 1.20 0.30
assign (residue 1917 and name HA) (residue 1918 and name HD#) 5.00 3.20 0.50
assign (residue 1917 and name HA) (residue 1918 and name HE#) 5.00 3.20 0.50
assign (residue 1917 and name HB#) (residue 1918 and name HN) 5.00 3.20 0.50
assign (residue 1917 and name HB#) (residue 1918 and name HD#) 5.00 3.20 0.50
assign (residue 1917 and name HB#) (residue 1918 and name HE#) 4.00 2.20 0.40
assign (residue 1917 and name HB#) (residue 1918 and name HD#) 5.00 3.20 0.50
assign (residue 1918 and name HG#) (residue 1918 and name HN) 5.00 3.20 0.50
assign (residue 1918 and name HG#) (residue 1918 and name HD#) 5.00 3.20 0.50
assign (residue 1918 and name HG#) (residue 1918 and name HB#) 5.00 3.20 0.50
assign (residue 1918 and name HG#) (residue 1918 and name HD#) 5.00 3.20 0.50
assign (residue 1918 and name HG#) (residue 1918 and name HB#) 5.00 3.20 0.50
assign (residue 1918 and name HG#) (residue 1918 and name HD#) 5.00 3.20 0.50
assign (residue 1918 and name HG#) (residue 1918 and name HB#) 5.00 3.20 0.50
assign (residue 1918 and name HN) (residue 1918 and name HD#) 5.00 3.20 0.50
assign (residue 1918 and name HN) (residue 1918 and name HG#) 5.00 3.20 0.50
assign (residue 1919 and name HA) (residue 1920 and name HN) 5.00 3.20 0.50
assign (residue 1916 and name HN) (residue 1919 and name HB#) 4.00 2.20 0.40
assign (residue 1919 and name HB#) (residue 1919 and name HA) 5.00 3.20 0.50
assign (residue 1919 and name HD#) (residue 1919 and name HA) 5.00 3.20 0.50
assign (residue 1919 and name HD#) (residue 1919 and name HB#) 4.00 2.20 0.40
assign (residue 1919 and name HD#) (residue 1919 and name HN) 5.00 3.20 0.50
assign (residue 1919 and name HE#) (residue 1919 and name HB#) 5.00 3.20 0.50
assign (residue 1919 and name HE#) (residue 1919 and name HD#) 3.00 1.20 0.30
assign (residue 1919 and name HA) (residue 1919 and name HN) 5.00 3.20 0.50
assign (residue 1919 and name HN) (residue 1919 and name HB#) 4.00 2.20 0.40
assign (residue 1919 and name HN) (residue 1919 and name HD##) 5.00 3.20 0.50
assign (residue 1919 and name HD#) (residue 1920 and name HA) 5.00 3.20 0.50
assign (residue 1920 and name HA) (residue 1921 and name HN) 5.00 3.20 0.50
assign (residue 1919 and name HB#) (residue 1920 and name HN) 4.00 2.20 0.40
assign (residue 1919 and name HE#) (residue 1920 and name HB#) 5.00 3.20 0.50
assign (residue 1920 and name HB#) (residue 1920 and name HA) 4.00 2.20 0.40
assign (residue 1917 and name HA) (residue 1920 and name HG) 6.00 4.20 0.50
assign (residue 1920 and name HG) (residue 1920 and name HN) 5.00 3.20 0.50
assign (residue 1920 and name HG) (residue 1920 and name HB#) 3.00 1.20 0.30
assign (residue 1920 and name HG) (residue 1920 and name HD##) 4.00 2.20 0.40
assign (residue 1919 and name HB#) (residue 1920 and name HN) 5.00 3.20 0.50
assign (residue 1919 and name HN) (residue 1920 and name HB#) 4.00 2.20 0.40
assign (residue 1920 and name HN) (residue 1920 and name HD##) 5.00 3.20 0.50
assign (residue 1920 and name HN) (residue 1920 and name HA) 4.00 2.20 0.40
assign (residue 1920 and name HN) (residue 1920 and name HB#) 4.00 2.20 0.40
assign (residue 1920 and name HN) (residue 1920 and name HA) 3.00 1.20 0.30
assign (residue 1921 and name HA) (residue 1920 and name HG) 5.00 3.20 0.50
assign (residue 1920 and name HA) (residue 1921 and name HN) 5.00 3.20 0.50
assign (residue 1921 and name HA) (residue 1921 and name HG) 5.00 3.20 0.50
assign (residue 1921 and name HB#) (residue 1921 and name HA) 4.00 2.20 0.40
assign (residue 1921 and name HB#) (residue 1921 and name HD##) 4.00 2.20 0.40
assign (residue 1921 and name HB#) (residue 1921 and name HA) 4.00 2.20 0.40
assign (residue 1921 and name HB#) (residue 1921 and name HD##) 3.00 1.20 0.30
assign (residue 1921 and name HB#) (residue 1921 and name HA) 3.00 1.20 0.30
assign (residue 1921 and name HD##) (residue 1921 and name HB#) 5.00 3.20 0.50
assign (residue 1921 and name HD##) (residue 1921 and name HA) 5.00 3.20 0.50
assign (residue 1925 and name HB) (residue 1925 and name HA) 4.00 2.20 0.40
assign (residue 1925 and name HG##) (residue 1925 and name HA) 4.00 2.20 0.40
assign (residue 1925 and name HG##) (residue 1925 and name HB) 3.00 1.20 0.30
assign (residue 1907 and name HA) (residue 1910 and name HB) 4.00 2.20 0.00
assign (residue 1908 and name HA) (residue 1911 and name HB) 5.00 3.20 0.00
assign (residue 1909 and name HA) (residue 1912 and name HB) 4.00 2.20 0.00
assign (residue 1912 and name HA) (residue 1915 and name HB##) 4.00 2.20 0.00
assign (residue 1914 and name HA) (residue 1917 and name HB#) 4.00 2.20 0.00
assign (residue 1915 and name HA) (residue 1918 and name HB#) 4.00 2.20 0.00
assign (residue 1916 and name HA) (residue 1919 and name HB#) 4.00 2.20 0.00
assign (residue 1917 and name HA) (residue 1920 and name HB#) 4.00 2.20 0.00
assign (residue 1910 and name HA) (residue 1913 and name HB#) 4.00 2.20 0.00
assign (residue 1911 and name HA) (residue 1914 and name HB#) 4.00 2.20 0.00
assign (residue 1913 and name HA) (residue 1916 and name HB#) 4.00 2.20 0.00
assign (residue 1918 and name HA) (residue 1921 and name HB#) 4.00 2.20 0.00
assign (residue 1908 and name HA) (residue 1911 and name HN) 4.00 2.20 0.00
assign (residue 1909 and name HA) (residue 1912 and name HN) 4.00 2.20 0.00
assign (residue 1911 and name HA) (residue 1914 and name HN) 4.00 2.20 0.00
assign (residue 1912 and name HA) (residue 1915 and name HN) 4.00 2.20 0.00
assign (residue 1913 and name HA) (residue 1916 and name HN) 4.00 2.20 0.00
assign (residue 1916 and name HA) (residue 1919 and name HN) 4.00 2.20 0.00
assign (residue 1917 and name HA) (residue 1920 and name HN) 4.00 2.20 0.00
assign (residue 1910 and name HA) (residue 1913 and name HN) 4.00 2.20 0.00
assign (residue 1911 and name HA) (residue 1914 and name HN) 4.00 2.20 0.00
assign (residue 1913 and name HA) (residue 1916 and name HN) 4.00 2.20 0.00
assign (residue 1916 and name HA) (residue 1919 and name HN) 4.00 2.20 0.00
assign (residue 1917 and name HA) (residue 1920 and name HN) 4.00 2.20 0.00
assign (residue 1912 and name HD1#) (residue 1912 and name HG2#) 4.00 2.20 0.40
assign (residue 1912 and name HD1#) (residue 1912 and name HG2#) 5.00 3.20 0.50
assign (residue 1912 and name HD1#) (residue 1912 and name HG1#) 4.00 2.20 0.40
assign (residue 1912 and name HA) (residue 1912 and name HN) 5.00 3.20 0.50
assign (residue 1911 and name HG##) (residue 1912 and name HA) 5.00 3.20 0.50
assign (residue 1912 and name HG##) (residue 1915 and name HA) 5.00 3.20 0.50
assign (residue 1912 and name HG2#) (residue 1912 and name HA) 5.00 3.20 0.50
assign (residue 1912 and name HG2#) (residue 1912 and name HB) 3.00 1.20 0.30
assign (residue 1912 and name HG2#) (residue 1912 and name HD#) 3.00 1.20 0.30
assign (residue 1912 and name HG2#) (residue 1916 and name HN) 5.00 3.20 0.50
assign (residue 1912 and name HG2#) (residue 1913 and name HA) 5.00 3.20 0.50
assign (residue 1912 and name HG2#) (residue 1913 and name HN) 5.00 3.20 0.50
assign (residue 1912 and name HG1#) (residue 1912 and name HA) 5.00 3.20 0.50
assign (residue 1912 and name HG1#) (residue 1912 and name HB) 5.00 3.20 0.50
assign (residue 1912 and name HG1#) (residue 1912 and name HD#) 3.00 1.20 0.30
assign (residue 1912 and name HG1#) (residue 1912 and name HG2#) 4.00 2.20 0.40
TALOS Dihedral Angle Restraint File

Values derived using chemical shift index

assign (resid 81 and name C )
(resid 82  and name N )
(resid 82  and name CA )
(resid 82  and name C )
 1.0  -59.000  14  2

assign (resid 82 and name N )
(resid 82  and name CA )
(resid 82  and name C )
(resid 83  and name N )
 1.0  -39.000  18  2

assign (resid 82 and name C )
(resid 83  and name N )
(resid 83  and name CA )
(resid 83  and name C )
 1.0  -61.000  10  2

assign (resid 83 and name N )
(resid 83  and name CA )
(resid 83  and name C )
(resid 84  and name N )
 1.0  -42.000  12  2

assign (resid 83 and name C )
(resid 84  and name N )
(resid 84  and name CA )
(resid 84  and name C )
 1.0  -64.000  12  2

assign (resid 84 and name N )
(resid 84  and name CA )
(resid 84  and name C )
(resid 85  and name N )
 1.0  -42.000  8  2

assign (resid 84 and name C )
(resid 85  and name N )
(resid 85  and name CA )
(resid 85  and name C )
 1.0  -63.000  12  2

assign (resid 85 and name N )
(resid 85 and name CA )
(resid 85 and name C )
(resid 86 and name N )
1.0  -45.000 12 2

assign (resid 85 and name C )
(resid 86 and name N )
(resid 86 and name CA )
(resid 86 and name C )
1.0  -61.000 8 2

assign (resid 86 and name N )
(resid 86 and name CA )
(resid 86 and name C )
(resid 87 and name N )
1.0  -44.000 14 2

assign (resid 86 and name C )
(resid 87 and name N )
(resid 87 and name CA )
(resid 87 and name C )
1.0  -61.000 12 2

assign (resid 87 and name N )
(resid 87 and name CA )
(resid 87 and name C )
(resid 88 and name N )
1.0  -43.000 12 2

assign (resid 87 and name C )
(resid 88 and name N )
(resid 88 and name CA )
(resid 88 and name C )
1.0  -67.000 6 2

assign (resid 88 and name N )
(resid 88 and name CA )
(resid 88 and name C )
(resid 89 and name N )
1.0  -41.000 18 2

assign (resid 88 and name C )
(resid 89 and name N )
(resid 89 and name CA )
(resid 89 and name C )
1.0  -65.000 8 2
assign (resid 89 and name N )
  (resid 89 and name CA )
  (resid 89 and name C )
  (resid 90 and name N )
  1.0  -43.000 6 2

assign (resid 89 and name C )
  (resid 90 and name N )
  (resid 90 and name CA )
  (resid 90 and name C )
  1.0  -64.000 14 2

assign (resid 90 and name N )
  (resid 90 and name CA )
  (resid 90 and name C )
  (resid 91 and name N )
  1.0  -39.000 16 2

assign (resid 90 and name C )
  (resid 91 and name N )
  (resid 91 and name CA )
  (resid 91 and name C )
  1.0  -70.000 18 2

assign (resid 91 and name N )
  (resid 91 and name CA )
  (resid 91 and name C )
  (resid 92 and name N )
  1.0  -32.000 22 2

assign (resid 91 and name C )
  (resid 92 and name N )
  (resid 92 and name CA )
  (resid 92 and name C )
  1.0  -103.000 24 2

assign (resid 92 and name N )
  (resid 92 and name CA )
  (resid 92 and name C )
  (resid 93 and name N )
  1.0  5.000 24 2

assign (resid 98 and name C )
  (resid 99 and name N )
  (resid 99 and name CA )
(resid 99 and name C )
1.0 -127.000 34 2

assign (resid 99 and name N )
(resid 99 and name CA )
(resid 99 and name C )
(resid 100 and name N )
1.0 153.000 20 2

assign (resid 99 and name C )
(resid 100 and name N )
(resid 100 and name CA )
(resid 100 and name C )
1.0 -132.000 22 2

assign (resid 100 and name N )
(resid 100 and name CA )
(resid 100 and name C )
(resid 101 and name N )
1.0 142.000 30 2

assign (resid 100 and name C )
(resid 101 and name N )
(resid 101 and name CA )
(resid 101 and name C )
1.0 -95.000 60 2

assign (resid 101 and name N )
(resid 101 and name CA )
(resid 101 and name C )
(resid 102 and name N )
1.0 123.000 44 2

assign (resid 101 and name C )
(resid 102 and name N )
(resid 102 and name CA )
(resid 102 and name C )
1.0 -59.000 12 2

assign (resid 102 and name N )
(resid 102 and name CA )
(resid 102 and name C )
(resid 103 and name N )
1.0 -40.000 24 2

assign (resid 102 and name C )

(resid 103 and name N )
(resid 103 and name CA )
(resid 103 and name C )
1.0 -64.000 12 2

assign (resid 103 and name N )
(resid 103 and name CA )
(resid 103 and name C )
(resid 104 and name N )
1.0 -43.000 10 2

assign (resid 103 and name C )
(resid 104 and name N )
(resid 104 and name CA )
(resid 104 and name C )
1.0 -66.000 12 2

assign (resid 104 and name N )
(resid 104 and name CA )
(resid 104 and name C )
(resid 105 and name N )
1.0 -43.000 12 2

assign (resid 104 and name C )
(resid 105 and name N )
(resid 105 and name CA )
(resid 105 and name C )
1.0 -65.000 12 2

assign (resid 105 and name N )
(resid 105 and name CA )
(resid 105 and name C )
(resid 106 and name N )
1.0 -40.000 16 2

assign (resid 105 and name C )
(resid 106 and name N )
(resid 106 and name CA )
(resid 106 and name C )
1.0 -64.000 12 2

assign (resid 106 and name N )
(resid 106 and name CA )
(resid 106 and name C )
(resid 107 and name N )
1.0 -41.000 10 2
assign (resid 106 and name C )
  (resid 107 and name N )
  (resid 107 and name CA )
  (resid 107 and name C )
  1.0 -63.000 6 2

assign (resid 107 and name N )
  (resid 107 and name CA )
  (resid 107 and name C )
  (resid 108 and name N )
  1.0 -47.000 10 2

assign (resid 107 and name C )
  (resid 108 and name N )
  (resid 108 and name CA )
  (resid 108 and name C )
  1.0 -64.000 22 2

assign (resid 108 and name N )
  (resid 108 and name CA )
  (resid 108 and name C )
  (resid 109 and name N )
  1.0 -40.000 28 2

assign (resid 108 and name C )
  (resid 109 and name N )
  (resid 109 and name CA )
  (resid 109 and name C )
  1.0 -83.000 22 2

assign (resid 109 and name N )
  (resid 109 and name CA )
  (resid 109 and name C )
  (resid 110 and name N )
  1.0 -30.000 18 2

assign (resid 117 and name C )
  (resid 118 and name N )
  (resid 118 and name CA )
  (resid 118 and name C )
  1.0 -59.000 10 2

assign (resid 118 and name N )
  (resid 118 and name CA )
  (resid 118 and name C )
(resid 119 and name N )
1.0 -39.000 14 2

assign (resid 118 and name C )
(resid 119 and name N )
(resid 119 and name CA )
(resid 119 and name C )
1.0 -65.000 10 2

assign (resid 119 and name N )
(resid 119 and name CA )
(resid 119 and name C )
(resid 120 and name N )
1.0 -41.000 8 2

assign (resid 119 and name C )
(resid 120 and name N )
(resid 120 and name CA )
(resid 120 and name C )
1.0 -68.000 8 2

assign (resid 120 and name N )
(resid 120 and name CA )
(resid 120 and name C )
(resid 121 and name N )
1.0 -41.000 14 2

assign (resid 120 and name C )
(resid 121 and name N )
(resid 121 and name CA )
(resid 121 and name C )
1.0 -64.000 20 2

assign (resid 121 and name N )
(resid 121 and name CA )
(resid 121 and name C )
(resid 122 and name N )
1.0 -41.000 8 2

assign (resid 121 and name C )
(resid 122 and name N )
(resid 122 and name CA )
(resid 122 and name C )
1.0 -62.000 10 2

assign (resid 122 and name N )
(resid 122 and name CA )
(resid 122 and name C )
(resid 123 and name N )
1.0 -40.000 18 2

assign (resid 122 and name C )
(resid 123 and name N )
(resid 123 and name CA )
(resid 123 and name C )
1.0 -69.000 14 2

assign (resid 123 and name N )
(resid 123 and name CA )
(resid 123 and name C )
(resid 124 and name N )
1.0 -38.000 14 2

assign (resid 123 and name C )
(resid 124 and name N )
(resid 124 and name CA )
(resid 124 and name C )
1.0 -63.000 12 2

assign (resid 124 and name N )
(resid 124 and name CA )
(resid 124 and name C )
(resid 125 and name N )
1.0 -44.000 10 2

assign (resid 124 and name C )
(resid 125 and name N )
(resid 125 and name CA )
(resid 125 and name C )
1.0 -64.000 10 2

assign (resid 125 and name N )
(resid 125 and name CA )
(resid 125 and name C )
(resid 126 and name N )
1.0 -44.000 16 2

assign (resid 125 and name C )
(resid 126 and name N )
(resid 126 and name CA )
(resid 126 and name C )
1.0 -61.000 14 2
assign (resid 126 and name N )
(resid 126 and name CA )
(resid 126 and name C )
(resid 127 and name N )
1.0 -42.000 14 2

assign (resid 126 and name C )
(resid 127 and name N )
(resid 127 and name CA )
(resid 127 and name C )
1.0 -75.000 24 2

assign (resid 127 and name N )
(resid 127 and name CA )
(resid 127 and name C )
(resid 128 and name N )
1.0 -29.000 24 2

assign (resid 127 and name C )
(resid 128 and name N )
(resid 128 and name CA )
(resid 128 and name C )
1.0 -84.000 28 2

assign (resid 128 and name N )
(resid 128 and name CA )
(resid 128 and name C )
(resid 129 and name N )
1.0 -16.000 34 2

assign (resid 134 and name C )
(resid 135 and name N )
(resid 135 and name CA )
(resid 135 and name C )
1.0 -117.000 54 2

assign (resid 135 and name N )
(resid 135 and name CA )
(resid 135 and name C )
(resid 136 and name N )
1.0 136.000 54 2

assign (resid 135 and name C )
(resid 136 and name N )
(resid 136 and name CA )
assign (resid 136 and name N )
(resid 136 and name CA )
(resid 136 and name C )
(resid 137 and name N )
1.0 -110.000 64 2

assign (resid 136 and name C )
(resid 137 and name N )
(resid 137 and name CA )
(resid 137 and name C )
1.0 -86.000 28 2

assign (resid 137 and name N )
(resid 137 and name CA )
(resid 137 and name C )
(resid 138 and name N )
1.0 110.000 36 2

assign (resid 138 and name C )
(resid 139 and name N )
(resid 139 and name CA )
(resid 139 and name C )
1.0 -67.000 22 2

assign (resid 139 and name N )
(resid 139 and name CA )
(resid 139 and name C )
(resid 140 and name N )
1.0 -33.000 24 2

assign (resid 139 and name C )
(resid 140 and name N )
(resid 140 and name CA )
(resid 140 and name C )
1.0 -60.000 16 2

assign (resid 140 and name N )
(resid 140 and name CA )
(resid 140 and name C )
(resid 141 and name N )
1.0 -39.000 16 2

assign (resid 140 and name C )
(resid 141 and name N )
(resid 141 and name CA )
(resid 141 and name C )
1.0  -60.000 16 2

assign (resid 141 and name N )
(resid 141 and name CA )
(resid 141 and name C )
1.0  -39.000 16 2

assign (resid 141 and name C )
(resid 142 and name N )
(resid 142 and name CA )
(resid 142 and name C )
1.0  -62.000 6 2

assign (resid 142 and name N )
(resid 142 and name CA )
(resid 142 and name C )
(resid 143 and name N )
1.0  -44.000 14 2

assign (resid 142 and name C )
(resid 143 and name N )
(resid 143 and name CA )
(resid 143 and name C )
1.0  -62.000 12 2

assign (resid 143 and name N )
(resid 143 and name CA )
(resid 143 and name C )
(resid 144 and name N )
1.0  -41.000 12 2

assign (resid 143 and name C )
(resid 144 and name N )
(resid 144 and name CA )
(resid 144 and name C )
1.0  -63.000 8 2

assign (resid 144 and name N )
(resid 144 and name CA )
(resid 144 and name C )
(resid 145 and name N )
1.0  -40.000 8 2
assign (resid 144 and name C )
    (resid 145 and name N )
    (resid 145 and name CA )
    (resid 145 and name C )
    1.0   -71.000 22 2

assign (resid 145 and name N )
    (resid 145 and name CA )
    (resid 145 and name C )
    (resid 146 and name N )
    1.0   -37.000 24 2

assign (resid 145 and name C )
    (resid 146 and name N )
    (resid 146 and name CA )
    (resid 146 and name C )
    1.0   -89.000 50 2

assign (resid 146 and name N )
    (resid 146 and name CA )
    (resid 146 and name C )
    (resid 147 and name N )
    1.0   -17.000 38 2

assign
    (residue 1903 and name C )
    (residue 1904 and name N )
    (residue 1904 and name CA )
    (residue 1904 and name C )
    1.00   -65.00 20.00  2

assign
    (residue 1904 and name C )
    (residue 1905 and name N )
    (residue 1905 and name CA )
    (residue 1905 and name C )
    1.00   -65.00 20.00  2

assign
    (residue 1905 and name C )
    (residue 1906 and name N )
    (residue 1906 and name CA )
    (residue 1906 and name C )
    1.00   -65.00 20.00  2
assign (residue 1906 and name C )
(residue 1907 and name N )
(residue 1907 and name CA )
(residue 1907 and name C )
  1.00  -65.00  20.00  2

assign (residue 1907 and name C )
(residue 1908 and name N )
(residue 1908 and name CA )
(residue 1908 and name C )
  1.00  -65.00  20.00  2

assign (residue 1908 and name C )
(residue 1909 and name N )
(residue 1909 and name CA )
(residue 1909 and name C )
  1.00  -65.00  20.00  2

assign (residue 1909 and name C )
(residue 1910 and name N )
(residue 1910 and name CA )
(residue 1910 and name C )
  1.00  -65.00  20.00  2

assign (residue 1910 and name C )
(residue 1911 and name N )
(residue 1911 and name CA )
(residue 1911 and name C )
  1.00  -65.00  20.00  2

assign (residue 1911 and name C )
(residue 1912 and name N )
(residue 1912 and name CA )
(residue 1912 and name C )
  1.00  -65.00  20.00  2

assign (residue 1912 and name C )
(residue 1913 and name N )
(residue 1913 and name CA )
assign
(residue 1913 and name C )
(residue 1914 and name N )
(residue 1914 and name CA )
(residue 1914 and name C )
  1.00  -65.00   20.00  2
assign
(residue 1914 and name C )
(residue 1915 and name N )
(residue 1915 and name CA )
(residue 1915 and name C )
  1.00  -65.00   20.00  2
assign
(residue 1915 and name C )
(residue 1916 and name N )
(residue 1916 and name CA )
(residue 1916 and name C )
  1.00  -65.00   20.00  2
assign
(residue 1916 and name C )
(residue 1917 and name N )
(residue 1917 and name CA )
(residue 1917 and name C )
  1.00  -65.00   20.00  2
assign
(residue 1917 and name C )
(residue 1918 and name N )
(residue 1918 and name CA )
(residue 1918 and name C )
  1.00  -65.00   20.00  2
assign
(residue 1918 and name C )
(residue 1919 and name N )
(residue 1919 and name CA )
(residue 1919 and name C )
  1.00  -65.00   20.00  2
assign
(residue 1919 and name C )
(residue 1920 and name N )
(residue 1920 and name CA )
(residue 1920 and name C )
 1.00  -65.00  20.00  2

assign
(residue 1920 and name C )
(residue 1921 and name N )
(residue 1921 and name CA )
(residue 1921 and name C )
 1.00  -65.00  20.00  2
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