Abstract

Ryanodine Receptors (RyR) are large ion channels that are responsible for the release of Ca\(^{2+}\) from the sarco/endoplasmic reticulum. The channel consists of a large cytosolic cap which functions as a giant allosteric protein, capable of being modulated by an assortment of binding partners and small molecules. To understand its function and mechanisms one needs to dissect the channel to its smallest parts. Using a combination of isothermal titration calorimetry and x-ray crystallography, two areas have been analyzed: binding by calmodulin (CaM) and the structure of a RyR domain, SPRY2.

Calmodulin (CaM) is a Ca\(^{2+}\) binding protein that can regulate RyR under conditions of both high and low Ca\(^{2+}\) by tuning their Ca\(^{2+}\) sensitivity to channel opening and closing in an isoform-specific manner. I analyze the binding of CaM and its individual domains to three different RyR CaM binding regions using isothermal titration calorimetry. I compared binding to skeletal muscle (RyR1) and cardiac (RyR2) isoforms, under both Ca\(^{2+}\)-loaded and Ca\(^{2+}\) free conditions. I find that CaM is able to bind all three regions, but with different binding modes, between the isoforms. Disease mutations target one of the three sites and affect CaM binding and energetics.

The SPRY2 domain is one of three repeats of the same fold that are present within the RyR. It has been suggested as a key protein interaction site with dihydropyridine receptors to mediate excitation-contraction coupling in skeletal muscle tissue. RyR1 and RyR2 SPRY2 domains were crystallized and reveal differences with several other known SPRY domain structures. Docking of the RyR1 SPRY2 structure places it in between the central rim and the clamp region. The structure of a disease mutant causing cardiomyopathy is also determined and shows local misfolding. Finally, RyR1 SPRY2 binding to the DHPR II-III loops is undetectable by isothermal titration calorimetry.
Preface


Chapter 2 is adapted from a published article Lau, K., Chan, M.M.Y, Van Petegem, F. Lobe-Specific Calmodulin Binding to Different Ryanodine Isoforms. Biochemistry. (2014) 53(5) 932-946. Mandy Chan performed preliminary cloning, expression, purification and ITC experiments on CaMBD2 and CaMBD1 peptides. I cloned, expressed, purified and ITC experiments on CaMBD1-3 peptides and their mutants. I prepared all the figures and jointly wrote the manuscript. My supervisor designed, supervised and jointly wrote the manuscript.

Chapter 3 is adapted from work that has accepted for publication in ‘Nature Communications’ as Lau, K., Van Petegem, F. Crystal structures of wild type and disease mutant forms of the Ryanodine Receptor SPRY2 domain. I cloned, expressed, purified, performed mutagenesis, crystallized both RyR1 and RyR2 constructs and related mutants, collected their diffraction data and solved the structures, analyzed the structure and performed thermal melting experiments. I prepared all the figures, except Figure 3.5 which was done by my supervisor, and jointly wrote the manuscript. My supervisor designed and supervised the project.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADP+Pi</td>
<td>Adenosine diphosphate + inorganic phosphate</td>
</tr>
<tr>
<td>ApoCaM</td>
<td>Apocalmodulin</td>
</tr>
<tr>
<td>ARVD2</td>
<td>Arrhythmogenic Right Ventricular Dysplasia Type 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>Ca$^{2+}$/CaM</td>
<td>Calcium-bound calmodulin</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMBD</td>
<td>Calmodulin Binding Domain</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca$^{2+}$/CaM dependent kinase II</td>
</tr>
<tr>
<td>Ca$_v$</td>
<td>Voltage-gated calcium channel</td>
</tr>
<tr>
<td>CCD</td>
<td>Central core disease</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium-induced calcium release</td>
</tr>
<tr>
<td>CPVT</td>
<td>Catecholaminergic Polymorphic Ventricular Tachycardia</td>
</tr>
<tr>
<td>Cryo-EM</td>
<td>Cryogenic-electron microscopy</td>
</tr>
<tr>
<td>CV</td>
<td>Column volume</td>
</tr>
<tr>
<td>E-C</td>
<td>Excitation contraction</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMDB</td>
<td>Electron microscopy databank</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DHPR</td>
<td>Dihydropyridine receptor</td>
</tr>
<tr>
<td>DR</td>
<td>Divergent region</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK-506 binding protein</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster Resonance Energy Transfer</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>II-III loop</td>
<td>Dihydropyridine receptor loop between domains 2 and 3</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>IP3R</td>
<td>Inositol 1,4,5-triphosphate receptor</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>K$^+$</td>
<td>Potassium ions</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption ionization-time-of-flight</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose binding protein</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>Magnesium ion</td>
</tr>
<tr>
<td>MH</td>
<td>Malignant hyperthermia</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>Na$_v$</td>
<td>Voltage-gated sodium channel</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium-calcium exchanger</td>
</tr>
<tr>
<td>OD$_{600}$</td>
<td>Optical density at 600 nm</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptors</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
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## List of Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>$K_d$</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>$\Delta G$</td>
<td>Gibbs free energy</td>
</tr>
<tr>
<td>$\Delta H$</td>
<td>Change in enthalphy</td>
</tr>
<tr>
<td>$\Delta S$</td>
<td>Change in entropy</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to thank my supervisor Dr. Filip Van Petegem for the wonderful opportunity to be part of his lab. His mentorship and guidance will be forever valued and I am extremely grateful. I would also like to thank the members of my supervisory committee Dr. Lawrence McIntosh and Dr. Masa Numata for their roles in guiding me towards this point.

Second, I would like to thank all the members of my lab as well as colleagues in other labs within the Life Sciences Centre that have made my graduate career an extremely fun and rewarding experience. Special thanks goes especially to Jett Tung, Lynn Kimlicka, Dr. Michael Yuchi, Paolo Lobo, Dr. Sarah Chow and Dr. Maen Sarhan for their invaluable assistance and for endless hours of discussion.

Finally I would like to thank my parents and my family for their unconditional support throughout my graduate career. I thank them for their boundless love and persistent encouragement to pursue my dreams and interests.
Dedication

This dissertation is dedicated to my grandmother who passed away shortly before the completion of this document. You raised me ever since I was a child and I know you are proud of me.
Chapter 1 - Introduction
1.1 Calcium as a Biological Signal

The formation of membranes that separated external and internal environments from the cytosol allowed for the formation of large concentration gradients between compartments. The change in concentrations of molecules in time and space allows for distinct messages or signals to be transmitted. Calcium ions (Ca$^{2+}$) have been exploited during evolution as one of several secondary messengers that are critical for signalling within a cell. Initially, Ca$^{2+}$, did not take on this role as early cells only maintained strict control over cytosolic Ca$^{2+}$ levels as an excess is toxic due to the formation of phosphate precipitates, aggregation of nucleic acids, and disruption of the cell membrane. The cell thus expends tremendous resources to maintain a Ca$^{2+}$ homeostatic system that differs by up to 20,000 times between the intracellular (~10-100nM) and extracellular environments (mM). Ca$^{2+}$ is actively transported out of the cell by pumps and transporters or into intracellular stores. Early cells eventually exploited this large concentration difference by evolving proteins that can sense Ca$^{2+}$ fluctuations over a concentration range of (nM to mM). These proteins functioned to buffer Ca$^{2+}$ levels but also serve as triggers for cellular processes such as cell division, motility, immunity, muscle contraction and apoptosis.

1.2 Calcium and Muscles

Skeletal and cardiac muscles are made up of muscle fibres that compose a bundle known as a fascicle. Each fibre’s entire length contains thread-like sub-structures known as myofilaments composed of actin which alternates with bundles of myosin filaments. It was known as early as the 1960s that Ca$^{2+}$ released from the sarcoplasmic reticulum was the trigger that allowed for the contraction of muscles. Seemingly, upon membrane depolarization, an electrical signal could be transmitted into a cell that subsequently released
Another mystery was how a signal could diffuse through the diameter of a muscle fibre (50-100 µm) in only 1-2 msec to the centre of a fibre to trigger a contraction.²⁶ Invaginations of the plasma membrane or sarcolemma into the muscle fibres provided an answer. By folding inwards, the interior of the fibre is never more than 1 µm away from a membrane, similar to the cristae found in mitochondria.⁸ These depressions known as T-tubules are continuous with the surface and allow for signals to penetrate deep within muscle fibres.⁸-¹⁰

In the 1970s, the T-tubules were discovered to be rich in voltage-gated calcium channels (Caᵥ), also known as dihydropyridine receptors (DHPR), that open when depolarized and allow for the rapid influx of extracellular Ca²⁺ upon the arrival of an action potential.⁶ The presence of these channels led to the hypothesis that they were the voltage sensors that detect membrane depolarization.¹¹-¹³ However, a component was still missing because the DHPRs are located on T-tubules and thus could not directly mediate SR Ca²⁺ release.¹⁴ The missing link was a calcium release channel that was first observed in electron micrographs as a large electron dense foot located on the SR.¹⁵-¹⁷ The molecular identity of these gateways that release SR Ca²⁺ was not even known until one was cloned later in 1989.¹⁸,¹⁹ These large channels, now called Ryanodine Receptors (RyR) are the gateways in which the calcium stores of the SR are released into the cytosol when they are activated. Thus, the electrical stimulus via DHPR acts as a signal to open the RyR that is then triggered to release Ca²⁺ from intracellular stores.

The actual contracture is described by the sliding filament theory where the rapid change in Ca²⁺ is first sensed by troponin C.⁵,²⁰ Upon binding, troponin and tropomyosin, located on actin-containing filaments of myofibrils, undergo a conformational change revealing myosin binding sites on actin. Myosin forms bridges with actin and repeated cycles
of release of ADP+Pi and hydrolysis of ATP allow for myosin head movement along an actin filament producing force and motion ultimately leading to contracture. As available Ca\(^{2+}\) is reduced due to active pumping by SERCA, troponin returns back to its resting state resulting in the loss of myosin binding sites on actin and relaxation.\(^5\)

The entire process from the propagation of the action potential down the sarcolemma to the muscle movement is called excitation-contraction coupling (E-C coupling).\(^6\) Different types of muscle tissue undergo E-C coupling in distinct ways. In cardiac muscle, Ca\(^{2+}\) influx from DHPR is sensed by RyRs that then act as a signal amplifier to increase amounts of cytosolic Ca\(^{2+}\) levels in a process called Ca\(^{2+}\)-induced-Ca\(^{2+}\)-release (CICR). (Figure 1.1)
Figure 1.1 An overview of processes involved in RyR activation in cardiac myocytes.

Particular voltage-gated calcium channels known as dihydropyridine receptors (DHPR) allow an influx of Ca\(^{2+}\) upon the arrival of an action potential. Through CICR, the RyR releases Ca\(^{2+}\) from the sarcoplasmic reticulum, triggering muscle contraction. Also shown is a PKA-dependent phosphorylation pathway that activates RyR upon β-adrenergic stimulation. The Sodium-Calcium Exchanger pumps remove one Ca\(^{2+}\) ion for 3 Na\(^{+}\) in return. The Sarco/endoplasmic Reticulum Ca\(^{2+}\)-ATPase (SERCA) allows for reuptake of Ca\(^{2+}\) from the cytosol. It is regulated by phospholamban (PLB) that is also a substrate target for PKA.
Skeletal muscle contraction does not require CICR, but can proceed through another mechanism. It is thought that skeletal muscle DHPR and RyR1 are directly coupled at the T-tubule and that the electrical signal that opens DHPR also induces a conformational change within its II-III loop. The II-III loop provides a critical link with the RyR allowing it to activate RyRs even in the absence of Ca\(^{2+}\) influx. Many see this coupling as a way for the DHPR to act as a voltage sensor for the RyR that becomes the pore of a multi-transmembrane complex.

1.3 Ryanodine Receptors: Discovery and Early Insights

RyRs play a critical role in muscle contraction and their discovery and naming have only been achieved since the 1980s. The channel was observed in junctional SR vesicles isolated from cardiac and skeletal muscle tissue as electron dense “feet” that resembled objects first seen in intact tissues. The name is derived from the initial observation that Ca\(^{2+}\) efflux from SR vesicles could be enhanced or inhibited depending on the amount applied of an alkaloid insecticide, Ryanodine. Further work with SR vesicles showed that Ca\(^{2+}\) release could be modulated by various small molecules like ATP, Mg\(^{2+}\), and Ca\(^{2+}\). Early biochemical evidence first showed that the channel exists as a homotetramer, with each monomer having a mass of \(~550\) kDa. This makes RyRs the largest known ion channels at \(~2.2\) MDa. Recordings made in planar lipid bilayers showed that RyRs are permeable only to small inorganic and organic cations and are completely impermeable to anions.

Mammalian organisms contain three known RyR isoforms. In 1989, Takeshima et al. reported the first primary structure and cloning of cDNA of RyR derived from rabbit skeletal muscle (RyR1). The following year, the group of MacLennan published the cloning
of the human skeletal muscle RyR and rabbit cardiac muscle isoform (RyR2). A third isoform (RyR3), distinct from both the cardiac and skeletal muscle isoforms, was originally cloned from brain tissue but is expressed in a variety of other tissues as well. The genes of all the isoforms are located on different chromosomes in humans. RyR1 is found on chromosome 19q13.2 spanning 104 exons. The gene encoding RyR2 is on chromosome 1q43 (102 exons) and RyR3 is encoded on 15q13.3-14 (103 exons). In addition to mammalian organisms, RyRs have also been identified in non-mammalian vertebrates with two isoforms, RyRα (homologous to RyR1) and RyRβ (similar to RyR3). Moreover, RyR genes have also been identified in invertebrates including Caenorhabditis elegans and Drosophila melanogaster.

The importance of RyRs is evident from several knockout (KO) studies. RyR1 double KO mice die immediately after birth, whereas an RyR2 KO is embryonically lethal. RyR3 KO mice survive, but have impaired learning abilities.

1.4 Disease Mutations

Since Ca²⁺ is a potent second messenger, mutations in proteins involved in Ca²⁺ release can lead to genetic disorders. As such, numerous disease-causing mutations have been identified in RyR1 and RyR2. RyR1 mutations commonly lead to either malignant hyperthermia or congenital myopathies and RyR2 mutations have been linked to catecholaminergic polymorphic ventricular tachycardia and cardiac hypertrophy.

1.4.1 Malignant Hyperthermia

Malignant hyperthermia (MH) is a disorder that requires both the presence of a trigger and a genetic mutation to cause disease. The triggers are volatile anaesthetics such
as halothane, sevoflurane and desflurane, and the muscle relaxant, succinylcholine.\textsuperscript{41} An MH episode is characterized by a sudden rise in core body temperature, muscle rigidity, and acidosis.\textsuperscript{42} This is caused by myocytes being in a hypermetabolic state to continuously remove Ca\textsuperscript{2+} leaked through the RyR from the cytosol.\textsuperscript{43} Incidence in the population could be as high as 1:2000; however, a true figure is hard to determine as less than 2\% of the population will ever be in a situation that requires any of the pharmacological triggers.\textsuperscript{44} Although fatal historically, most surgery rooms keep a stock supply of dantrolene, a RyR1 blocker that can rapidly reverse the symptoms. Fatalities are greater in young men with the cause unknown.\textsuperscript{45} It is thought that this drug affects RyR by inhibiting Ca\textsuperscript{2+} release; however, evidence for direct binding is controversial.\textsuperscript{46,47}

1.4.2 CCD

Mutations in RyR1 also lead to myopathies with central core disease (CCD) comprising the largest group. CCD is typified by extensive muscle weakness, a decrease in muscle tone, skeletal deformities and scoliosis. Diagnosis is usually confirmed by the presence of lesions or “cores” within the centre of myocytes that are devoid of metabolic activity and lack mitochondria.\textsuperscript{48-50} Multi-mini core disease (MmD) is similar to CCD but patients exhibit multiple cores instead of just one. In addition, CCD is autosomal dominant while MmD is mostly inherited recessively.\textsuperscript{49}

1.4.3 CPVT

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is the main disease resulting from mutations in RyR2. It is typically diagnosed in young patients that have family members with known CPVT, and by the presentation of symptoms such as syncope and
sudden death. Like RyR1, it is thought that CPVT is triggered by exercise or even emotional stress. These activities release catecholamines and trigger a β-adrenergic response leading to direct phosphorylation of RyR2 increasing its activity or through phosphorylation of proteins that modulate RyR2 or other components involved in Ca\(^{2+}\) handling like phospholamban. In addition to RyR2, mutations in proteins that interact with RyR2 like calsequestrin, triadin and CaM have also been shown produce CPVT phenotypes.

In both isoforms, it is thought that the presence of mutations cause gain-of-function “leaky” channels allowing for excess Ca\(^{2+}\) release leading to prolonged or premature calcium signals within the cell. Mutations that lead to CCD can also be due to loss-of-function. In cardiac myocytes, the excess Ca\(^{2+}\) resulting from CPVT mutations, may activate the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) on the cytoplasmic membrane that exchanges one Ca\(^{2+}\) out for every three Na\(^+\) ions. The new influx of positive charges generates inwardly depolarizing currents causing delayed-after-depolarizations (DAD) leading to cardiac arrhythmias. In skeletal muscle myocytes, the massive release of Ca\(^{2+}\) stimulates activity of Ca\(^{2+}\)-ATPases, ultimately depleting the ATP reserves and leading the cells into a hypermetabolic state.

Disease mutations in RyR1 and RyR2 seem to cluster in three different regions of the receptor genes (hot spots). These three locations in both receptors match, suggesting important functional roles for those portions. They are located near the N-terminus, in a central domain region, and a C-terminal region covering the transmembrane segments.

1.5 Structure

RyRs are the largest known ion channels, with molecular weights ~2.2 MDa. They form homotetrameric arrangements. The three isoforms found in mammals (RyR1, RyR2 and
RyR3) are highly similar with amino acid sequence identities ~70%. Of note are several divergent regions (DR) with the greatest dissimilarity between isoforms, DR1, DR2 and DR3. DR1 encompasses a large part of the C-terminus and has the greatest dissimilarity. Interestingly, RyRs have significant sequence and structural homology to the inositol-1,4,5-triphosphate (IP₃) receptor, another intracellular Ca²⁺ release channel. Their N-terminal domains are structurally homologous and it has been suggested that both channels evolved from a single ancestor.

Early work utilized negative-stain electron microscopy (EM) to visualize sub-cellular localization of RyRs along with other proteins that compose the EC machinery. Images of the junction region between the SR and T-tubules of muscle tissues revealed large “foot” structures. Improvements to resolution revealed that these feet were four-fold symmetric and provided evidence for large-scale organization of RyRs due to their ability to form two-dimensional arrays in micrographs. In addition, tetrads of skeletal muscle DHPR co-localized with RyRs, further suggesting a physical connection between these two membrane proteins. Cryo-EM has been utilized to provide a higher-resolution structure of the channel, showing that RyRs are mushroom-shaped and composed of multiple globular domains. Studies have imaged open and closed states in the presence of certain ligands. However, since RyRs can adopt multiple conformational states that likely become averaged during the imaging, the resolution of the cryo-EM has thus far been limited to ~12Å.

Due to their large size and membrane nature RyRs have posed a challenge for high-resolution structural studies. Most methods in structural biology require large amounts of protein at high purity. Due to RyRs inherent size and the presence of hydrophobic transmembrane regions, proper folding requires chaperones and post-translational modifications that may not be present in heterologous expression systems. Only since 2006
have atomic resolution structures of portions of RyR been determined by NMR and X-ray crystallography.

1.5.1 Cryo-EM

The cryo-EM structures revealed what typifies RyRs, a large mushroom-cap cytosolic region that consists of 90% of the protein that is connected to a stem that leads to the transmembrane pore. The cap resembles a small prism (290x290x130Å) sitting upon the pore-forming transmembrane region (120x120x70Å).67 Performed on a closed state RyR1, the highest resolution 3D reconstruction to 12Å showed that the cap is a compact assembly of at least 10 globular domains that arranged themselves in a web-like scaffold.74(Figure 1.2) The domains are organized into regions that were described in lower resolution maps: the clamp, handle and central rim.75 Large solvent channels in the assembly account for over 50% of the enclosed volume and this maximizes the total surface area and allows for a variety of ligands and proteins to bind.74,76 Reconstructions of RyR2 and RyR3 show remarkably similar architecture to RyR1 that is expected due to their high amino acid sequence similarity to RyR1.77,78 In addition to the closed state architecture, cryo-EM studies have revealed the structure of RyR1 in the open state at 10.2Å.69 Upon opening, the channel undergoes large conformational changes in the cytosolic region, along with motions in the pore-forming part of the transmembrane region. The RyR is therefore a bona fide allosteric protein, whereby motions in the pore are coupled to regulatory domains in the cytosolic portion.
Figure 1.2 A 12Å cryo-EM map of RyR1.

The map shows the channel viewed from the top (A) cytosolic side and from the ‘side’, parallel to the membrane (B). The colouring and numbering represents individual regions of density that form globular regions that may be composed of one or more protein domains. Dotted outlines indicate common names of regions use in describing RyR. Reproduced with permission from (Serysheva et al. 2008)\textsuperscript{74}
Binding sites of accessory proteins such as calmodulin (CaM), FKBP12/12.6 and the
toxin imperatoxin A have been visualized by examining the difference densities and in the
presence and absence of these proteins.\textsuperscript{70,79-81} Owing to the large size of RyR, attempts have
been made to correlate the sequence of the primary structure within an intact tetramer.
Recombinant RyRs with green fluorescent protein (GFP) or glutathione S-transferase (GST)
insertions at various points have allowed for the visualization of additional density in cryo-
EM reconstructions.\textsuperscript{78,82-86} Antibodies have also been targeted to specific sequences and the
extra density owing to the antibody is interpreted as the region where the targeted sequence
might be present.\textsuperscript{87,88}

1.5.2 Domains of the Ryanodine Receptor

Through primary structure analysis, structural homology to other channels and
proteins and secondary structure prediction, RyRs appear to be built up by multiple
individual domains.\textsuperscript{89} (Figure 1.3) Prior to the work described in this thesis, X-ray
crystallography and NMR structures have been described for two different regions (N-
terminal region and phosphorylation domain) within the RyR sequence.\textsuperscript{90-95} These structures
represent only \(\sim12\%\) of the total channel.
Figure 1.3 The primary structure of RyR1 and its domains.

This primary structure map shows the approximate locations of domains. Each coloured region represents a predicted or currently known domain. The names of the domains are listed at the top. Dark blue lines represent the location of known disease causing mutations in RyR1. Dotted red boxes represent three regions of sequence divergence between RyR1 and RyR2 or divergent regions (DR1-3).

1.5.2.1 The N-terminal Domains

Domains within the N-terminal region were the first to be described. Initially, single domains of the very N-terminus of both RyR1\textsuperscript{91} and RyR2\textsuperscript{90} were described. Later, structures encompassing the first three domains in RyR1\textsuperscript{92} and RyR2\textsuperscript{96} were determined. For simplicity, we refer to these three domains as domains A, B and C. The ABC domains comprises the largest fragment that has been solved. It is approximately 550 amino acids in length in both RyR1 and RyR2 or about 10% of the full-length RyR. The region is composed of 3 domains, two β-trefoil domains comprising 12 β-strands each (domains A 1-204, and B 205-394; RyR1 rabbit) and an α-helical bundle resembling armadillo repeat (domain C 395-532).\textsuperscript{92,96}(Figure 1.4) The three domains together form a compact triangular arrangement with significant domain-domain interactions that are mainly hydrophilic in nature.\textsuperscript{92} In RyR2, an ion pair network, responsible for domain-domain interactions, has been replaced by a central
The N-terminal region represents a part of the channel that is a target for 80 mutations, and it has therefore been suggested as a disease 'hot spot'. Several structures of the disease mutants have been determined and show that disruption of salt bridges through mutations causes relative domain reorientations.

Figure 1.4 The N-terminal domains and their location within the Ryanodine Receptor.

A. Domains A, B and C (PDB 2XOA) are arranged as a compact triangle mediated by hydrophilic interactions at the interface between domains. B. ABC docks within the 10.3Å map (EMDB 5014) in the central rim region of the RyR. It forms a 4-fold symmetric vestibule on the cytosolic face, far away from the pore-forming region.

Docking of ABC into cryo-EM maps reveal that the domain forms a four-fold symmetric ring at the cytosolic surface of the channel forming a central vestibule. In addition to the interfaces formed between the three domains, there are 5 additional interfaces with the rest of the receptor. The pseudo-atomic model reveals that, although it is far from the transmembrane region, the N-terminal domains are connected to the pore through electron-dense columns. Docking of the N-terminal domains into different cryo-
EM maps and FRET measurements show that the domains are mobile during opening and closing of the channel and that they are allosterically coupled to the pore region. Some disease-causing mutations are buried within individual domains, but the bulk are found at domain-domain interfaces. This suggests that proper domain-domain interactions are required for a normal functioning RyR.

Domains ABC can be further subdivided and purified as either domain A or domains BC. Domain A was the first RyR domain structure to be solved by X-ray crystallography and by NMR. Even in such a short segment, domain A contains 32 mutations between RyR1 and RyR2. An unusual deletion mutant of domain A also exists where the entirety of exon3 (Δexon3) is missing. Individuals carrying this mutation were found to suffer from a severe form of CPVT. Surprisingly the structure could be determined and revealed wide-ranging changes. Instead of missing the β-strand structural element that exon 3 provided, a flexible alpha helix encoded by exon 4 replaces the missing strand, rescuing the fold of the protein. (Figure 1.5) This novel structural rescue mechanism suggests that alternative splicing may be used as a mechanism to fine-tune channel activity.
Figure 1.5 Domain A Δexon3 mutant is rescued by exon4.
A. Domain A (PDB 2MC3) is coloured in blue, exon3 is in orange and exon 4 is in red. B. The Δexon3 is a mutation that removes all of exon3 that encodes a crucial β-strand. Surprisingly in the mutant, the β-strand is replaced by exon4, which undergoes a helix-to-strand conformational change.

The structure of ABC also revealed its similarities with RyR’s sister channel, the IP3 receptor (IP3R). The structures of the first three domains of IP3R1 (isoform 1) have been determined and show remarkable structural homology to domains ABC. The first domain of IP3R1 is the “suppressor” domain which is analogous to domain A while the IP3-binding core represents domains BC. In IP3Rs, ligand binding occurs within the IP3-binding core which then activates the channel. The suppressor domain inhibits this process by forming interactions with the IP3-binding core preventing it from undergoing conformational changes that allow it to bind IP3. Structural and functional parallels to RyRs suggest that mutations or ligand binding that weaken domain interfaces may promote channel opening as well. Supporting this is cryo-EM docking of the N-terminal domains of
IP3R1 that also locate them to the corresponding positions in the receptor. Interestingly, chimeras whereby IP3R domain A is replaced by the corresponding domain from RyR2 still produce functional channels. Since IP3 binding can stimulate channel opening, a similar ligand may be modulating RyR activity. However, no ligands have been described so far that can bind to the RyR N-terminal region.

Figure 1.6 The N-terminus of the IP3 receptor superimposed on Domains ABC
A. Superposition of the N-terminus of the IP3 receptor (PDB 3UJ4) yields a striking similarity to domains ABC with an RMSD of 1.2Å. The largest difference is the presence of longer helices within the IP3R domain A. Domains ABC are coloured as before and the IP3 receptor is in beige.

1.5.2.2 Phosphorylation Domain
The RyR amino acid sequence contains a short region that is repeated 4 times. These repeats are found in tandem pairs in two areas (Figure 1.3). The second such tandem pair, encoded by residues 2743-2940 (rabbit RyR1), 2699-2904 (mouse RyR2) and 2597-2800 (human RyR3) contains multiple phosphorylation sites (Ser/Thr/Tyr), and is often referred
to as the phosphorylation domain. This domain has two-fold symmetry with each half adopting a helix-strand-helix structure.\textsuperscript{93,106} It has been a region of intense interest and controversy due to the presence of multiple phosphorylation sites that have been implicated in heart failure.\textsuperscript{107-114}

The phosphorylation domain was crystallized in all three isoforms and revealed phosphorylation sites that are thought to be crucial to channel regulation. In RyR2 several sites are located on a flexible loop that were not well resolved suggesting a highly dynamic nature. (Figure 1.7) Disease mutations in RyR1 cluster around the phosphorylation sites, suggesting that the mutations and phosphorylation may promote similar structural changes.\textsuperscript{93}

\textbf{Figure 1.7 The phosphorylation domain of RyR2 and its docked location in a cryo-EM map}

A. The phosphorylation domain consists of two pseudo-symmetric halves connected by a flexible loop. The latter contains several phosphorylation sites that have been linked to CPVT. Sites capable of being phosphorylated \textit{in vitro} are in red (PKA) and blue (CaMKII). All of these sites, together with additional ones (no dots), have also been identified by mass spectrometry of native tissue. B. The docking of the phosphorylation domain (black circle) places it right at the tip of the clamp region of the channel. Adapted from Yuchi, Lau and Van Petegem, 2012.
Docking of the phosphorylation domain into cryo-EM maps localized it to the tip of the clamp domain. (Figure 1.7) As that is a region that undergoes significant movement as observed between closed/open state maps, phosphorylation may play a role in the transition between the two states. However, no neighbouring domain has been identified.

Interestingly, no other eukaryotic protein is known to contain these tandem repeats, but they have been identified in prokaryotes and viruses, and that is where they are thought to have originated.105 The other tandem repeats, located between the first two SPRY domains, currently has an unknown function. (Figure 1.3)

1.5.2.3 SPRY Domains

SPRY domains were first identified as a repeat domain found multiple times within a splA kinase from Dictyostelium discoideum and within RyRs.104,115 SPRY domains have now been identified in over 177 mammalian proteins and are usually modules mediating specific protein-protein interactions that play a role in immunity.116 They are composed of a conserved twisted β-barrel core of 12 strands connected by highly variable loops.116 SPRY domains segregate into two families based upon their sequence phylogeny: ‘SPRY-only” and “PRYSPRY/B30.2” domains.117 Historically, the SPRY core was predicted in both families, with an additional Pre-SPRY (PRY) motif in the B30.2 family. Structures have revealed that the SPRY-only family also contains elements that are structurally homologous to the PRY motif, but differ significantly in sequence. These structural features are the addition of two to three helices and three β-strands that are N-terminal to the SPRY core. (Figure 1.8)
Figure 1.8 SPRY domains of different classes

Three representative SPRY domains are shown here in cartoon representation with their PDB accession codes below. A. The SPRY domain of Ash2L does not contain the N-terminal helices present in other SPRYs. B & C. Although bioinformatically the ‘SPRY-only’ and B30.2 class are recognized as distinct and separate families, other than the β-barrel core, structural features such as their N-terminal helices are conserved.

Although named partly after RyRs, no SPRY structure from the channel had been solved prior to the work described in this thesis. Within the RyR, there are three repeats (SPRY1 659-797, SPRY2 1084-1207, SPRY3 1430-1570; RyR1 human) (Figure 1.3) that show low sequence identity to known SPRY domains (~20%). Of the three only SPRY2 has been implicated in a RyR function. SU120 SPRY2 is contained within the region that has been implicated as the interaction site for the II-III loop of Ca,1.1 mediating excitation-contraction coupling. Cryo-EM studies utilizing antibodies against peptides and segments of SPRY2 have visualized extra density near the clamp region.
1.5.2.4 Calcium Binding Domains/CaM-like Domains

The ryanodine receptor not only conducts Ca\(^{2+}\) but is also activated and regulated by Ca\(^{2+}\) through CICR. CaM is able to fine-tune the channel’s Ca\(^{2+}\) sensitivity by binding to a CaM binding domain (CaMBD; 3614-3643; RyR1 rabbit). Depending on the free cytosolic Ca\(^{2+}\) concentration CaM is able to activate or inhibit the channel.\(^{121}\) CaM contains EF-hand motifs which allow it to act as Ca\(^{2+}\)-sensors.\(^{122}\) Upon binding Ca\(^{2+}\), the EF-hands undergo conformational changes allowing CaM to bind differently to RyR.\(^{94}\)

Interestingly, the RyR itself contains several EF-hand motifs. Through sequence alignments with known EF-hand proteins, Xiong \textit{et al.}, have identified several regions within RyRs that have a surprising homology to CaM.\(^{123,124}\) In RyR1, the 4064-4210 region could be purified and approximates the size of CaM. Initial biochemical data using equilibrium dialysis suggests that not all the EF-hands are functional, as only two Ca\(^{2+}\) were able to bind.\(^{123}\) These EF-hands may function as the RyR’s intrinsic Ca\(^{2+}\) sensors and be involved in CICR.

1.6 Modulation and Regulation of Ryanodine Receptors

Since cytosolic Ca\(^{2+}\) needs to be strictly regulated, RyRs have evolved to become sensitive signal integrators, receiving input from many proteins and small molecules. These mostly bind to the cytosolic cap and provide positive or negative input. In addition proteins in the ER lumen are also able to bind and regulate channel function. The sites of action for most of these interactions are still to be discovered. Figure 1.9 shows the multitude of positive and negative regulators of RyR. A few of the regulators are discussed below.
Figure 1.9 The Ryanodine Receptor is modulated by diverse proteins and small molecules.

Regulators in the cytosol and within the SR modulate RyRs. Proteins and compounds within the blue circle are activators and those within the red circle inhibit the channel. Some are able to both activate and inhibit the channel.

1.6.1 FKBP

FK506-binding proteins were initially discovered as they are tightly bound to RyR and are copurified. They are immunophilins that exhibit cis-trans-peptidyl-prolyl isomerase activity but can also stabilize the closed state of RyR. A skeletal muscle isoform of 12 kDa and a cardiac muscle isoform of 12.6 kDa isoform exists (FKBP12 and FKBP12.6) and thus the former typically binds RyR1 and the latter binds RyR2. The exact mechanism of action of FKBP12.6 on RyR2 is still unknown but is of intense interest as loss of the protein has been implicated in heart failure and arrhythmias. In experiments where FKBP12 was absent on RyR1, the channels were observed to enter multiple sub-conductance states, to have higher open probabilities, resulting in increased Ca\(^{2+}\) leak and reduced EC coupling. One group has reported that FKBP12 promotes coupling between neighbouring RyR allowing for activation of one channel to affect its neighbours.
EM studies have shown difference density in the 16Å RyR1-FKBP12 and 33Å RyR2-FKBP12.6 maps between the handle and clamp regions and show that 4 FKBP8s bind to one RyR.\textsuperscript{79,131} Through yeast-two hybrid experiments, it has been proposed that RyR1 residues 2361-2496 are involved in binding but further experiments are still required to confirm.\textsuperscript{107}

1.6.2 Phosphorylation

RyRs are regulated by various kinases (PKA, PKG and CaMKII) and phosphatases that are able to modulate channel activity and connect RyRs to stress signalling.\textsuperscript{132-134} Protein kinase A (PKA) has been found to phosphorylate multiple residues on RyR (S2843 in human RyR1; S2030 and S2808 in mouse RyR2).\textsuperscript{114,133,135} Calmodulin kinase II (CaMKII) is also able to phosphorylate S2808 in RyR2 but also S2814.\textsuperscript{136,137} Mass spectrometry has shown that an even greater number of sites could possibly be phosphorylated \textit{in vivo}.\textsuperscript{138} Several reports have suggested that hyperphosphorylation of S2808 leads to FKBP12.6 loss that results in heart failure.\textsuperscript{107,135,139} However, conflicting studies have shown either both increased or unchanged activity in single channel recordings of RyR in the presence of PKA or phosphomimetic mutant S2808D.\textsuperscript{137,140} Similarly, in studies with S2808A and S2808D mice, PKA phosphorylation affected Ca\textsuperscript{2+} spark frequencies and amplitudes, but this could not be repeated by others.\textsuperscript{112,135} No study has been able to attribute changes to a particular phosphorylation site because a modification may simultaneously affect other sites. One cannot eliminate the possibility of cross-talk between phosphorylation sites. In addition, several groups have been unable to reproduce the FKBP12.6 dissociation under conditions of heart failure.\textsuperscript{111,112,141} The effect of phosphorylation on RyR is one of the most discussed topics in the field of E-C coupling.\textsuperscript{53,110,142}
1.6.3  EF-Hand Proteins

A primary ligand for triggering RyR opening is cytosolic Ca\(^{2+}\). However, as Ca\(^{2+}\) levels in the cytosol increase, RyRs close, suggesting that there are multiple Ca\(^{2+}\) binding sites with different affinities and kinetics.\(^{23,143}\) A plot of channel activity (as observed through its Ca\(^{2+}\) efflux rate) versus cytosolic Ca\(^{2+}\) concentration shows a bell-shaped curve.\(^{144,145}\) (Figure 1.10)

![Figure 1.10](image)

**Figure 1.10 The Ca\(^{2+}\) efflux rate constant from Ryanodine Receptors is dependent on external Ca\(^{2+}\) levels.**

This bell-shaped curve shows the dependence of the Ca\(^{2+}\) efflux rate constant on Ca\(^{2+}\) concentrations. Low levels of Ca\(^{2+}\) promote channel activity with maximal rate constant of 1.5s\(^{-1}\) at 5µM. At high concentrations of external Ca\(^{2+}\), efflux is inhibited. This suggests the presence of high-affinity activating and low-infinity inhibitory Ca\(^{2+}\) binding sites. Adapted with permission from Meissner, Darling, Eveleth, 1986.\(^{145}\)

Multiple calcium sensors can also bind RyR and modulate its Ca\(^{2+}\) sensitivity. These proteins contain EF-hand motifs that consist of a helix-loop-helix structure. The loop includes key acidic residues and backbone oxygens that mediate Ca\(^{2+}\) binding with pentagonal bipyramidal coordination.\(^{122,146,147}\) (Figure 1.11)
An EF-hand of CaM (PDB 1EXR) consists of a helix-loop-helix that coordinates Ca\(^{2+}\) (green). The ion is bound by a combination of acidic residues, backbone oxygens and waters in a pentagonal bipyramidal geometry.

In addition, pseudo and non-canonical EF-hand proteins also exist whereby their sequences vary significantly from the typical ‘EF-handome’. Usually present as a pair, EF-hands form structurally stable four-helix arrangements. EF-hands are one of the most common structural motifs found in proteins with up to 1000 known and their most common function is to bind protein substrates in a Ca\(^{2+}\)-dependent manner.

The first EF-hand crystal structure was described for parvalbulmin in the 1970s. Over the years further structures determined by X-ray crystallography and NMR have provided details on the dynamic nature of Ca\(^{2+}\) binding in these proteins and nuances that allow them to specifically target a protein sequence. The following section will provide an overview of two EF-hand proteins before a discussion on EF-hand modulation of RyR.
1.6.3.1 Calmodulin

Calmodulin (CaM) is the best known EF-hand containing protein. It is a eukaryotic protein that mediates many Ca\(^{2+}\)-dependent processes of target enzymes such as myosin light chain kinase (MLCK), CaM-dependent kinases and phosphatase, nitric oxide synthase and Ca\(^{2+}\)-ATPases.\(^ {159-161}\)

CaM is a small protein of 17 kDa and is composed of 4 EF-hands arranged as two pairs or lobes that can bind up to 4 Ca\(^{2+}\).\(^ {162}\) CaM resembles a dumb-bell with its two lobes, commonly termed the N-lobe or C-lobe (N-terminus and C-terminus), connected by a highly flexible linker. The two lobes share 46% sequence identity and 75% sequence homology and their C\(_\alpha\) backbone superimposes very well with an RMSD of 0.7Å.\(^ {163,164}\) The differences are reflected in their calcium affinities where the C-lobe has a higher calcium affinity (\(K_d \sim 10^{-6}\text{M}\)) than N-lobe (\(K_d \sim 10^{-5}\text{M}\)).\(^ {163,164}\) There is positive co-operativity between the lobes upon binding the first Ca\(^{2+}\) allowing for CaM to be responsive over a large range of cellular Ca\(^{2+}\) levels.\(^ {122,165,166}\) When bound to many target proteins, the affinity of Ca\(^{2+}\) for CaM increases due to a significant decrease in the dissociation rate constant \(k_{\text{off}}\).\(^ {167,169}\)

CaM is essential as knockouts are lethal in model organisms such as yeast and \textit{Drosophila}.\(^ {170,171}\) Mutations in CaM are also deleterious causing recurrent cardiac arrest in infants and CPVT in patients.\(^ {57,172}\) CaM is abundant near membranes (50%) and the nucleus with a low free concentration in the cytosol (50-75nM).\(^ {173-175}\) CaM can be tethered to some of its target proteins allowing it to function as a dedicated Ca\(^{2+}\)-sensor.\(^ {176,177}\) This allows for spatially-segregated, fast CaM-mediated processes as soon as cytosolic Ca\(^{2+}\) levels rise. Its highly conserved amino acid sequence (100%) between all vertebrates underlies its importance for normal cellular processes.\(^ {178}\)
The most important feature of CaM is its conformational plasticity and ability to bind to an abundant assortment of protein targets.\textsuperscript{163,179} In the absence of Ca\textsuperscript{2+}, CaM exists in a compact conformation with the lobes closed and packed in towards the central linker.\textsuperscript{180} This limits its ability to bind and apoCaM indeed only associates with a select number of targets.\textsuperscript{181} Upon binding Ca\textsuperscript{2+}, the lobes undergo a conformational change whereby they open and expose hydrophobic residues that form a concave pocket that is able to accommodate a wide array of substrates.\textsuperscript{147,166,182,183} (Figure 1.12) The binding of Ca\textsuperscript{2+} into the EF-hands causes significant alterations in the relative orientations of the helices in each lobe.\textsuperscript{184} The lobes rotate away from the central linker allowing CaM to adopt an open, extended state which permits a wide diversity of binding modes with high affinities (K\textsubscript{d} = 10\textsuperscript{-7} – 10\textsuperscript{-11}M).\textsuperscript{185}

Figure 1.12 Apocalmodulin and Ca\textsuperscript{2+}/Calmodulin

The two forms of CaM exist in very distinct conformations. A. Apocalmodulin (PDB 1CFD) with no Ca\textsuperscript{2+} bound is compact and closed. Upon binding Ca\textsuperscript{2+} (green spheres) calmodulin undergoes a conformational change to become Ca\textsuperscript{2+}/calmodulin (B; PDB 1EXR). Hydrophobic residues including numerous methionine residues (orange) are exposed upon this change allowing for target recognition and binding. It should be noted that NMR structures do not show the central linker as a continuous linker.\textsuperscript{186}
The high number of methionine residues that form the hydrophobic pocket is uncommon in proteins and accounts for CaM’s promiscuity in target binding.\textsuperscript{179,187} (Figure 1.12) Sulfur is highly polarizable, more so than carbon, as it contains a larger electron cloud that allows it to interact with a variety of hydrophobic residues through van der Waals interactions but still being compatible with aqueous environments.\textsuperscript{187} In addition, the sidechain’s flexibility allows the hydrophobic pocket to be conformationally pliable. Charged residues lining the binding pocket stabilize and orient the target via electrostatic interactions.

1.6.3.1.1 CaM Target Motifs

Classically, CaM interacts with short peptide segments between 16-30 amino acids in length that form amphipathic helices.\textsuperscript{163,185,188} Bulky hydrophobic residues (Trp, Tyr, Phe, Val, Ille) that provide anchor points for CaM’s hydrophobic clefts are interspersed with basic residues along a helix. It is thought that many of these helices are initially present as intrinsically disordered regions in the host proteins and fold upon binding to CaM.\textsuperscript{189} As more CaM binding domains (CaMBD) have been identified there have been great efforts to develop consensus sequences that could be predicted to bind CaM. These motifs can be assigned based upon their spacing of the hydrophobic residues ie. 1-10, 1-14, 1-16, which indicates the position of the hydrophobic anchors.\textsuperscript{163,188} Further subgroups such as 1-5-10 and 1-5-8-14 have been established as intervening hydrophobic residues have been identified to play a role in certain sequences. Table 1.1 provides examples of these motifs.
Table 1.1 Representative examples of CaM target motifs based upon their anchor spacing.

<table>
<thead>
<tr>
<th>Target Peptide</th>
<th>Motif Spacing</th>
<th>PDB</th>
</tr>
</thead>
<tbody>
<tr>
<td>smMLCK</td>
<td>1-14 (1-8-14)</td>
<td>1CDL</td>
</tr>
<tr>
<td>skMLCK</td>
<td>1-14 (1-5-8-14)</td>
<td>2BBM</td>
</tr>
<tr>
<td>CaMKII</td>
<td>1-10 (1-5-10)</td>
<td>1CDM</td>
</tr>
<tr>
<td>CaMKK</td>
<td>1-16</td>
<td>1CKK</td>
</tr>
<tr>
<td>RyR1</td>
<td>1-17</td>
<td>2BCX</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}-ATPase</td>
<td>1-18</td>
<td>2KNE</td>
</tr>
</tbody>
</table>

Overall motif class with parentheses indicating the subgroup if applicable.

The so-called “IQ” motif has been found to bind apoCaM. It corresponds to a (ILV)Qxxx(RK)Gxxx(RK) consensus sequence where x represents any amino acid. IQ motifs share similar features with Ca\textsuperscript{2+}/CaM binding domains as they still contain all the common structural elements: a basic, amphipathic helix that contains several hydrophobic anchor points. Many IQ motifs are able to bind both apoCaM and Ca\textsuperscript{2+}/CaM and some preferentially bind apoCaM. For example, the apoCaM binding to Na\textsubscript{1.2}’s IQ domain reduces the C-lobe’s affinity for Ca\textsuperscript{2+} due to constraints imposed to maintain the apoCaM complex.

1.6.3.1.2 CaM Binding Modes

Characterization through fluorescence and small-angle X-ray scattering experiments of Ca\textsuperscript{2+}/CaM and peptides indicated that these ternary complexes have a very compact structure. This suggested early on that the central linker was required to bend and kink to allow for the lobes to come into closer proximity to form a closed state. The first structures of CaM bound to peptides were of skeletal muscle and smooth muscle myosin light chain kinase (skMLCK and smMLCK) and of CaM-dependent kinase II\textalpha (CaMKII\textalpha). In the Ca\textsuperscript{2+}/CaM/CaMKII\textalpha structure, the linker is unwound allowing the
lobes to rotate by 120° and bend by 100° relative to Ca\textsuperscript{2+}/CaM. The peptide is an \(\alpha\) helix with its bulky hydrophobic residues largely buried inside both Ca\textsuperscript{2+}-bound lobes. This example typified most Ca\textsuperscript{2+}/CaM structures but is no longer the case. (Figure 1.13) Structures of Ca\textsuperscript{2+}/CaM complexes have now shown a multitude of binding modes. CaM has been observed to be able to bridge two peptide segments\textsuperscript{197,198} as well as bind peptides in an extended open conformation\textsuperscript{155,156}. A single target region is also capable binding multiple CaM molecules.\textsuperscript{161} CaM is also able of binding deep within a target protein and not an exposed segment.\textsuperscript{152} Finally, there are instances where binding with both Ca\textsuperscript{2+} and apo lobes occur simultaneously.\textsuperscript{158} The diversity of binding modes of CaM with its targets allows for a variety of “custom-tailored” regulatory mechanisms that are fast, highly specific, tightly regulated and dynamic.
Figure 1.13 Calmodulin binds to its targets in a variety of modes

A. CaM bound to skeletal muscle myosin light chain kinase was the first complex determined (PDB 2BBM). B. CaM bound to CaMKII has a collapsed form onto its target similar to (A) (PDB 1CDM). C. CaM with no target bound. D. An unusual complex where 4 CaMs bridge two peptides that contain two CaM-binding regions within a voltage gated calcium channel (PDB 3OXQ). E. CaM binding a sodium channel peptide with only one lobe in an open conformation (PDB 4DJC). F. Two CaM molecules bound to a single peptide derived from a Ca$^{2+}$-ATPase (PDB 4AQR). G. CaM activating adenylyl cyclase by binding deep within the protein (PDB 1K9O). H. CaM bridging two peptides from a Ca$^{2+}$-activated potassium channel but with one lobe Ca$^{2+}$ bound and one Ca$^{2+}$ free (PDB 1G4Y). Ca$^{2+}$ are green spheres.
1.6.3.2 S100A1

The S100 family of proteins are also EF-hand containing proteins that appeared late in evolution and are only expressed in vertebrates. They were originally isolated from bovine brain and discovered to be soluble in 100% ammonium sulfate, hence their name ‘S100’. Like CaM, S100 proteins contain EF-hands that are able to bind Ca\(^{2+}\) followed by binding to target proteins. S100A1 is the first member of this family and forms small homodimers of 21 kDa. Unlike CaM lobes, the first EF-hand of each subunit is considered a “pseudo” EF-hand due to its extremely weak Ca\(^{2+}\) binding affinity (K\(_d\) ~ 100-500\(\mu\)M) while the second EF-hand binds with a more typical affinity with K\(_d\) = 1-50\(\mu\)M. The overall apparent Ca\(^{2+}\) affinity is increased by glutathionylation and the presence of target proteins.

S100A1 is highly abundant in skeletal muscle tissue at a concentration of 0.5 – 15\(\mu\)M, which is significantly higher than the concentration of CaM. In RyR1, S100A1 and CaM have been shown to compete for a common binding site, encoded by residues 3614-3643. S100A1 has a subtle effect on excitation-contraction coupling acting as an enhancer of the entire process. Knockouts of S100A1 have increased the incidence of heart failure and there is decreased S100A1 in cardiomyopathic hearts, suggesting S100A1 plays a critical role in muscle maintenance. In S100A1 knockout muscle fibres there are decreased Ca\(^{2+}\) transients upon electrical stimulation but this can be rescued by adenoviral S100A1 transfection. Due to the ability of S100A1 to enhance cardiac contractility, it has been suggested as a possible target for treatment of heart failure through gene therapy.
1.6.4 Calmodulin and Ryanodine Receptors

The first isolated preparations of RyR identified CaM as an important protein that was co-purified. Further biochemical characterizations showed that CaM binds directly to RyR and modulates the channel both in vivo and in vitro. As observed by cryo-EM, RyR is able to bind up to 4 CaMs (1/subunit), but it is not known how many are required to modulate the channel. Its modulatory effect depends on the free Ca\(^{2+}\) concentration and the RyR isoform. At high Ca\(^{2+}\) levels, CaM can inhibit both RyR1 and RyR2. At low Ca\(^{2+}\) levels it activates RyR1 but inhibits RyR2, although the inhibition at low Ca\(^{2+}\) concentrations has been controversial. In single channel recordings, the presence of 50 nM CaM at low Ca\(^{2+}\) levels (0.4\(\mu\)M) is sufficient to see an effect. In RyR1, the open probability (P\(_o\)) is doubled while in RyR2 it is almost halved. At higher Ca\(^{2+}\) levels (2\(\mu\)M), the P\(_o\) decreases for both isoforms. Recent experiments using RyR2 have shown that mutations to the N-lobe of CaM increase the threshold for termination of Ca\(^{2+}\) release, whereas mutations on the C-lobe decrease the threshold, suggesting individual roles and possibly distinct binding sites for the lobes on the full-length channel. Interestingly, mutations in CaM have also been associated with CPVT, indicating that a proper regulation of RyR2 by CaM is absolutely required. The mechanism of CaM modulation of RyR is currently unknown. Many sequences on the RyR have been found to bind CaM but not all have been thoroughly investigated. A crystal structure of CaM in complex with a RyR peptide (3614-3643; mouse RyR1) shows both lobes of CaM anchored. However, NMR data suggests that the Ca\(^{2+}/N\)-lobe is only weakly associated and could bind to a different site.
1.6.4.1 Binding Sites of Calmodulin on Ryanodine Receptors

When the full-length sequence of RyR was first determined, analysis revealed the presence of up to 9 different CaMBDs that consisted of a typical sequence (as discussed above) or a section that was predicted to be an amphipatic alpha helix.\textsuperscript{19,30,221,222} Several labs independently screened fusion protein fragments of RyR to see which can interact with CaM through pull-down experiments; however, results have been conflicting due to identification of different regions. One region that has been the topic of intense research is encoded by RyR1 residues 3614-3643 (3583-3603 in mouse RyR2).\textsuperscript{223-230} It has been identified as both an apoCaM and a Ca\textsuperscript{2+}/CaM binding site.\textsuperscript{223} Mutations in the site in RyR2 reduced the total amount of CaM bound on RyR and relieved CaM-dependent effects on the channel such as inhibition at high Ca\textsuperscript{2+}.\textsuperscript{230} This suggested that a single site was all that was necessary for CaM modulation of RyR. However, questions that are unresolved are: 1) how can a single site be responsible for the separate effects of ApoCaM and Ca\textsuperscript{2+}/CaM? 2) How can both CaM lobes have different functional effects? 3) How can apoCaM exert different effects on RyR1 and RyR2 when the proposed single binding site has a near-identical sequence in both isoforms?

Two views have been proposed by several groups. In one view, the 3614-3643 sequence is the site of action of CaM and all subsequent effects are due to downstream changes.\textsuperscript{215,229,231} Another view is that a second CaMBD site that is dissimilar between the two isoforms allows for site-specific modulation of the channel.\textsuperscript{94,225,226} The mechanism of how CaM exerts differential effects on the two isoforms is still unknown.

In addition, the RyR is regulated by S100A1 and other EF-hand proteins. There is high redundancy between CaMBDs with other EF-hand proteins suggesting that multiple players may compete for the same sites and play a role in RyR regulation. Unlike CaM,
S100A1 acts as an agonist of RyR activity at higher Ca\textsuperscript{2+} concentrations.\textsuperscript{95,199,204} The relative amounts of CaM and S100A1 thus may dictate channel activity when amounts of either protein are limiting. Finally, it has been suggested the EF-hand domain of RyRs is able to mimic Ca\textsuperscript{2+}/CaM and bind to the region 3614-3643 serving as a clamp to inhibit channel opening.\textsuperscript{123,228,232}

1.6.4.2 Structural Biology of CaMBDs

The most studied CaMBD of RyR is the 3614-3643 region. An X-ray crystal structure of the region in complex with Ca\textsuperscript{2+}/CaM reveals an arrangement similar to other ternary complexes with a few novelties.\textsuperscript{94} (Figure 1.14) Two aromatic anchors associate with the CaM lobes: W3620 for the C-lobe, and F3636 for the N-lobe. They have an unusual 1-17 spacing that had not been previously observed in other CaM:target complexes.\textsuperscript{123,228,232} 1H-15N RDCs (residual dipolar coupling) revealed that although the conformation of each lobe bound on the peptide is comparable between the crystal structure and in solution, the ordering and motions differ for the lobes in solution. This contrasts with other CaM complexes where solution NMR RDCs provided excellent fits with x-ray data.\textsuperscript{233,234} As such, it was interpreted that both lobes can bind their anchors independent from one another. With a shortened RyR1 peptide, 3614-3634, CaM is still able to bind with its C-lobe even with one anchor missing and without collapsing onto the peptide. The N-lobe is free to interact elsewhere.\textsuperscript{94} This opens up the possibility of state-dependent CaM regulation when the N-lobe anchor is conformationally inaccessible.
Figure 1.14 Ca\textsuperscript{2+} /CaM bound to amino acids 3614-3643 from RyR1.

The structure of Ca\textsuperscript{2+} /CaM bound to the peptide derived from amino acids 3614-3643 (PDB 2BCX) shows a closed compact conformation similar to other CaM complexes but with an atypical 1-17 spacing between the anchor residues Trp3620 and Phe3636. The peptide is bound antiparallel fashion. Ca\textsuperscript{2+} are green spheres.

The 3616-3627 segment in complex with S100A1 has also been determined by NMR.\textsuperscript{204} (Figure 1.15) In contrast with other S100A1 structures, the Ca\textsuperscript{2+} /S100A1 is arranged antiparallel to the peptide with respect to its 3\textsuperscript{rd} helix. It engages the same aromatic anchor, W3620, as the Ca\textsuperscript{2+} /C-lobe, suggesting a direct competition with CaM. Binding experiments suggest that both proteins are competitive at similar concentrations allowing for fine-tuning of RyR activity.\textsuperscript{204}
S100A1 also binds the same region as CaM on the RyR. Each half of the homodimer binds a short section of RyR (PDB 2K2F). The interaction is mediated by one of the anchor points that also binds CaM, Trp3620. Ca\(^{2+}\) are green spheres.

In addition to atomic models the locations of CaM have been localized on RyR1 and RyR2 by cryo-EM. ApoCaM and Ca\(^{2+}\)/CaM occupy overlapping regions on the surface of RyR1. There is a 30Å shift between the centre of mass of apoCaM to the Ca\(^{2+}\)/CaM position, suggesting that both states of CaM bind to different sites. However, one issue with this study is that there may have been intrinsic effects of Ca\(^{2+}\) on RyR conformation. Follow-up experiments using CaM\(_{1234}\), which is Ca\(^{2+}\)-binding deficient, at both low and high Ca\(^{2+}\) levels show no change in the position of apoCaM nor the overall structure of RyR. This suggests that the 30Å shift is likely due to CaM movement and not conformational changes of RyRs. Interestingly in RyR2, the apoCaM site coincides with the Ca\(^{2+}\)/CaM site in RyR1. The locations have yet to be correlated with the primary structure of RyR or specific functional effects on the channel.
GFP/YFP-insertions in RyR2 have attempted to map primary structure on to cryo-EM maps. Two insertions have been made at potential CaMBDs: 1) near R3595, which corresponds to the 3614-3643 region in RyR1 and 2) K4269. Both insertions localized near the CaM densities that were determined before, and the study suggest that CaM might be able to bridge the two segments. FRET measurements show that these two sites can undergo relative conformational changes upon channel opening, further suggesting that a bridging CaM may play a role in the transition between open and closed channels.

**Figure 1.16** Localization of CaM and potential CaMBD by difference Cryo-EM densities.

A. Difference density between RyR1 and RyR1 with apoCaM or Ca\(^{2+}\)/CaM reveal two distinct but overlapping locations for CaM. The centre of mass moves 30Å suggesting a large shift in the location of CaM upon binding Ca\(^{2+}\). B. GFP insertions at residues 3595 and 4269, near two putative CaMBD, show difference density near the location of CaM in A. The close proximity suggest that these two sites may be bridged by CaM. Adapted with permission from Samsó, M. and Wagenknecht, T. (2002) and from Huang et al. (2013).\(^{70,236}\)

### 1.6.5 Skeletal Muscle DHPR \(\alpha_{1S}\) Subunit

Excitation-contraction coupling is the process that allows a depolarizing electrical signal to be converted into a chemical signal, allowing for muscle contraction. In cardiac
muscle, entry of Ca\(^{2+}\) by activation of DHPR on T-tubules is sufficient to evoke Ca\(^{2+}\) release from RyR2. In skeletal muscle, however, the process is Ca\(^{2+}\)-independent as its entry is not required for RyR opening and Ca\(^{2+}\) release from the SR.\(^6\) It is thought that instead, conformational changes are evoked in the skeletal muscle DHPR \(\alpha_{1S}\) subunit upon depolarization, which directly activates RyR1 through mechanical coupling.\(^{11,237,238}\) This forward signal is termed 'orthograde', but it has also been found that 'retrograde' signals exist, whereby the state of RyR1 can affect the function of the DHPR.\(^{239}\) The different mechanism in E-C coupling between the two muscle types also translates to a physiological difference. In skeletal muscle, DHPRs form a group of four particles or “tetrads” which come into the contact with the corners of RyR. In cardiac muscle the DHPRs are arranged variably.\(^{65,66,240,241}\)

The regions of the skeletal muscle DHPR and of RyR1 that mediate this process have been investigated thoroughly but there is still little evidence to suggest that the two proteins are in direct physical contact. In the DHPR, a chimeric and biochemical approach has been used to identify cytosolic loops that may come into contact with RyR. These experiments have consistently shown that a single loop between domains II and II (II-III loop, 720-765) of the DHPR is required for E-C coupling to occur.\(^{242-247}\) Conversely, chimeric RyR1/RyR2 constructs expressed in dyspedic (lacking RyR1) myotubes indicate a wide variety of regions that may mediate signalling with the DHPR.\(^{248-250}\) Structurally, multiple regions of RyR1 also have been implicated for tetrad formation in skeletal muscle but not cardiac muscle.\(^{251}\) However, further analysis of these regions through both \textit{in vivo} and \textit{in vitro} methods have provided conflicting results, suggesting that the machinery mediating E-C coupling is complex and may involve multiple sites of contact or low affinity interactions.\(^{248}\)
1.6.6 β Subunit

The skeletal muscle DHPR is composed of multiple subunits, with the \( \alpha_{1S} \) forming the transmembrane subunit. The \( \beta \) subunit is an intracellular component and has been found to play a vital role in E-C coupling.\(^{252}\) \( \beta_{1A} \) null mice die perinatally due to asphyxia.\(^{253}\) \( \beta \) subunits interact with the I-II loop of the \( \alpha_{1S} \) and are known to promote membrane insertion, trafficking and proper DHPR activity.\(^{254}\) In addition, they are essential for the formation of tetrads that are necessary for skeletal muscle E-C coupling.\(^{252}\) However, direct interactions between this subunit and RyR1 have never been reported and currently it is thought that \( \beta \) subunits indirectly influence \( \alpha_{1S} \)-RyR1 interactions by stabilizing and tethering the DHPR in the membrane.

1.7 Research Question

The Ryanodine Receptor is a large and complex channel that is modulated by a wide assortment of proteins and ligands. Despite intense interest in certain aspects of RyR regulation, there are still many unanswered questions. Much of the underlying uncertainties are due to the lack of both structural and biochemical characterizations of the channel itself. The overall aim of this thesis is to shed light on two aspects of RyR regulation and structure.

The second chapter of the thesis focuses on the binding of CaM to RyR peptides that have been identified as potential effector sites on the channel. Although many sites have been reported in literature, there are still outstanding questions. (1) Which of these sites bind apoCaM and \( \text{Ca}^{2+}/\text{CaM} \)? (2) Are there differences in binding between homologous regions between the isoforms that may mediate isoform-specific functions? (3) Is CaM able to bind to CaMBDs that harbour disease-causing mutations?
In the third chapter, the focus is on a domain that has been implicated to mediate excitation-contraction coupling in RyR. Using X-ray crystallography and cryo-EM docking, the structure and its location within the full-length channel are revealed. Disease mutants were also characterized and crystallized. Finally, calorimetric binding experiments were used to test the possibility of interactions with other proteins.

My work has required the extensive use of calmodulin and isothermal titration calorimetry. I have been involved in several projects that are not covered within the scope of this thesis. A collaboration with the lab of Dr. Mate Erdélyi was initiated to probe the role of calmodulin binding to volatile anaesthetics. This was initiated to test the hypothesis that CaM could be the target of volatile anaesthetics, which prevents CaM from regulating target proteins such as RyR. This work is also published (Brath U., Lau, K., Van Petegem, F., Erdélyi. M. Mapping the sevoflurane-binding sites of calmodulin. (2014) Pharmacology Research and Perspectives. 2, e00025. Doi:10.10002/prp2.25).

I tested whether FKBP12 (Chapter 1.6.1) could bind to the RyR1 N-terminal region by calorimetry. This work was recently published as well (Kimlicka, L., Lau, K., Ching-Chieh, T., Van Petegem, F. Disease mutations in the ryanodine receptor N-terminal disease hot spot couple to a mobile intersubunit interface. (2013) Nature Communications. 4, Article 1506).

Together, the work presented represents an analysis of regions of RyR that have been implicated in modulating the channel. It provides much needed basic biochemical and structural information in regards to two processes that have been widely discussed and are controversial in the field of RyR regulation.
Chapter 2 - Lobe-Specific Calmodulin Binding to Different Ryanodine Receptor Isoforms

This chapter has been adapted from the original publication: Lau, K., Chan, M.M.Y, Van Petegem, F. Lobe-Specific Calmodulin Binding to Different Ryanodine Isoforms. *Biochemistry*. (2014) 53(5) 932-946
2.1 Abstract

Ryanodine Receptors (RyRs) are large ion channels that are responsible for the release of Ca\(^{2+}\) from the sarco/endoplasmic reticulum. Calmodulin (CaM) is a Ca\(^{2+}\) binding protein that can affect the channel open probability under conditions of both high and low Ca\(^{2+}\), shifting the Ca\(^{2+}\) dependencies of channel opening in an isoform-specific manner. Here we analyze the binding of CaM and its individual domains to three different RyR regions using isothermal titration calorimetry. We compared binding to skeletal muscle (RyR1) and cardiac (RyR2) isoforms, under both Ca\(^{2+}\)-loaded and Ca\(^{2+}\)-free conditions. CaM can bind to all three regions in both isoforms, but the binding modes differ appreciably in two segments. The results highlight a Ca\(^{2+}\)/CaM and apoCaM binding site in the C-terminal 1/5\(^{th}\) of the channel. This binding site is the target for malignant hyperthermia and central core disease mutations in RyR1, which affect the energetics and mode of CaM binding.
2.2 Introduction

The primary ligand to trigger RyR opening is cytosolic Ca\(^{2+}\). Under these circumstances, the RyR acts as a signal amplifier, in a process known as Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR).\(^{14,255}\) However, as Ca\(^{2+}\) levels in the cytosol rise, RyRs close, indicating that there are multiple Ca\(^{2+}\) binding sites with different affinities and binding kinetics. A plot of the open probability of the channel as a function of Ca\(^{2+}\) concentration therefore displays a bell-shaped curve.\(^{145}\) In addition, luminal Ca\(^{2+}\) levels can regulate RyR opening through the process of store overload induced calcium release (SOICR).\(^{256,257}\)

The exact timing of channel opening and closing is crucial. Several genetic diseases result from altered RyR activity, often increasing their sensitivity to cytosolic or luminal Ca\(^{2+}\) and leading to channels with enhanced open probabilities. RyR1 mutations are mostly associated with malignant hyperthermia (MH)\(^{258}\) and central core disease (CCD)\(^{259}\), whereas RyR2 mutations can cause catecholaminergic polymorphic ventricular tachycardia (CPVT).\(^{260}\) These mutations are found throughout the RyR genes, but many have been found to cluster at domain-domain interfaces.\(^{90-93,96,97,102,106}\) Multiple auxiliary proteins can further tune the sensitivity of RyRs to Ca\(^{2+}\).

A major player is Calmodulin (CaM), a 17-kDa protein with 4 EF-hands able to bind Ca\(^{2+}\). CaM directly binds to RyRs, but the effect is dependent on Ca\(^{2+}\) concentrations and the exact RyR isoform. At high Ca\(^{2+}\) levels, CaM can inhibit both RyR1 and RyR2. At low Ca\(^{2+}\) levels it activates RyR1 but inhibits RyR2\(^{212,216-218}\), although the inhibition at low Ca\(^{2+}\) concentrations has been controversial.\(^{219}\) Recent experiments using RyR2 have shown that mutations to the N-lobe of CaM increase the threshold for termination of Ca\(^{2+}\) release, whereas mutations on the C-lobe decrease the threshold\(^{220}\), suggesting individual roles and possibly distinct binding sites for the lobes on the full-length channel. Interestingly,
mutations in CaM have also been associated with CPVT\textsuperscript{57,172}, indicating that a proper regulation of RyR2 by CaM is absolutely required.

How exactly CaM exerts its stimulatory or inhibitory effects is currently unknown. Previously, a number of RyR peptides have been suggested to bind CaM based mainly on pull-downs, overlay assays, gel shifts, and fluorescence measurements.\textsuperscript{221,222,224-226,229,261-263} However, most of these have not been validated using a quantitative method, and multiple ones have been discarded because they have been shown to be inaccessible\textsuperscript{93} or do not agree with cryo-EM data, as recently discussed by Huang \textit{et al.}\textsuperscript{236} Thus far, only three RyR segments remain as candidates to be ‘true’ CaM binding sites. CaM has often been shown to use its individual lobes to bridge different peptide stretches in its target proteins, so it is likely that more than one of these segments is involved in binding either Ca\textsuperscript{2+}/CaM or apoCaM.

A crystal structure has shown that Ca\textsuperscript{2+}/CaM is able to bind to a peptide in the central region of RyR1 (residues 3614 to 3643 in rabbit RyR1), with both CaM lobes binding the segment simultaneously.\textsuperscript{94} However, NMR data have shown that the Ca\textsuperscript{2+}/N-lobe is only loosely associated with the peptide, suggesting it is likely to bind elsewhere in full-length channels.\textsuperscript{94} Cryo-EM studies have shown that only one CaM binds per RyR monomer\textsuperscript{70}, although this does not exclude the possibility that additional CaMs may bind to separate, low affinity sites. The cryo-EM studies also show that the binding site for CaM on RyR1 shifts by \textasciitilde 30 Å.\textsuperscript{70,235} This shift is too large to be attributed to a different binding mode onto the same peptide, and suggests that one or both CaM lobes shift to another sequence stretch upon binding Ca\textsuperscript{2+}. Curiously, these cryo-EM studies have also shown that the apoCaM binding site in RyR2 coincides with the Ca\textsuperscript{2+}/CaM binding site in RyR1. Together with the
different functional effects of CaM in the two isoforms, this indicates that the mode of binding in the two isoforms is different.

Because of the complexities by which CaM can bind its targets, and the inherent ability of the individual lobes to bridge different sequence stretches in other proteins, we used isothermal titration calorimetry (ITC) to analyze the binding of CaM and its individual lobes under both apo and Ca$^{2+}$-loaded conditions. We focused on the three remaining CaM binding candidates (CaM binding domains – CaMBDs 1-3; Figure 2.1) in both RyR1 and RyR2. The data show isoform-specific differences and suggest that the highest affinity for apoCaM resides in the C-terminal region of the RyR. The latter is also the target for disease mutations that affect CaM binding.

![CaMBD1](image1)

![CaMBD2](image2)

![CaMBD3](image3)

**Figure 2.1 Sequences of three CaM binding domains (CaMBDs).**

Residues that are strictly conserved among RyR1–RyR3 are highlighted in gray. Purple residues are the sites of mutation in RyR1CBD3, duplication of L4319–4321 and R4325D. The numbering is for rabbit RyR1, mouse RyR2, and human RyR3.
2.3 Methods and Materials

2.3.1 Cloning and Purification of CaMBDs

Sequences encompassing rabbit RyR1 (CaMBD1: residues 1975-1999; CaMBD2: 3614-3640) and rat RyR2 (CaMBD1: 1941-1965; CaMBD2: 3580-3606; CaMBD3: 4246-4276) were cloned in a pET24a vector containing a C-terminal hexahistidine tag using NdeI and XhoI cut sites. At the N-terminus, maltose binding protein (MBP) was attached, along with a cleavage site for tobacco etch virus (TEV) protease. The presence of affinity tags on both sides allows us to extract only peptides without degradation within the CaMBD sequence. RyR1 CaMBD3 (4295-4325) could not be cloned using routine PCR and instead synthetic peptides (Lifetein) were used for wild type and disease mutant versions. In order to protect the fusion proteins from proteolytic degradation, they were co-expressed with human CaM, which binds and prevents access to proteases. The CaM was co-expressed in a peGST vector, using *Escherichia coli* Rosetta (DE3) pLacI at 37°C, induced at an OD<sub>600</sub> of ~0.6 by addition of 0.2 mM IPTG for 4 hours. Cells were lysed by sonication in buffer A (250 mM KCl, 10 mM CaCl<sub>2</sub> and 10 mM HEPES, pH 7.4) supplemented with 25 mg ml<sup>-1</sup> DNase I, 25 mg ml<sup>-1</sup> lysozyme, 1 mM iodoacetamide and 1 mM phenylmethylsulphonyl fluoride. The lysate was applied to a 25 ml Poros MC column (Tosoh Bioscience), washed with 5CV of buffer A and 5CV of 2% (vol/vol) buffer B (250 mM KCl, 10 mM CaCl<sub>2</sub> and 500 mM imidazole, pH 7.4) and eluted with 30% (vol/vol) buffer B. The protein was diluted two-fold with buffer A and then applied to a 25 ml amylose column (New England Biolabs). The protein was then washed with 20CV of buffer A plus 10 mM EDTA, 2CV of buffer A and eluted with buffer C (buffer A plus 10 mM maltose). The wash step with EDTA completely removed the co-expressed CaM. The protein was diluted two-fold with buffer A and applied once again to a 25 ml Poros MC column, washed and eluted as before.
CaMBD2 constructs, which contain cysteines, 10 mM β-mercaptoethanol (BME) was present in all buffers except for ones used for the Poros MC column. The fusion proteins were confirmed to be monomeric using size exclusion chromatography on a Superdex200 column (GE Healthcare) (Figure 2.2). The molecular weights were confirmed by MALDI-TOF on a Voyager-DE STR (Applied Biosystems) as fusion proteins and as peptides after cleavage with TEV protease.
Figure 2.2 Size exclusion chromatograms and SDS-PAGE gels of purified and synthesized CaMBDs.

A,B. Size exclusion chromatograms of purified CaMBDs on a Superdex200 at 150 mM KCl, 10 mM HEPES 7.4, 0.1% NaN₃ and (A) 10 mM CaCl₂ or (B) 10 mM EDTA. C. 15% SDS-PAGE gel of purified CaMBDs. D,E. Size exclusion chromatograms of synthetic CaMBDs on a Superdex75 at 150 mM KCl, 10 mM HEPES 7.4, 0.1% NaN₃ and (D) 10 mM CaCl₂ or (E) 10 mM EDTA.
2.3.2 Cloning and Purification of Calmodulin and its Lobes

Full-length Human CaM, its N-terminal lobe (N-lobe, residues 1-78) and C-terminal lobe (C-lobe, residues 79-149) were cloned as described into a modified pET28 vector containing an N-terminal hexahistidine tag and a tobacco etch virus (TEV) protease cleavage site. Purification was like for the CaMBD constructs, with the following exception. After the first Poros MC column, the protein was cleaved with his-tagged TEV protease overnight at room temperature while dialyzed against buffer A. The tagged protease and cleavage product were removed with an additional PorosMC column. The flow through was applied to a Phenyl-Sepharose HP column (GE Healthcare) equilibrated with 150 mM KCl, 20 mM HEPES, pH 7.4, 10 mM CaCl₂. The protein was eluted with the same buffer containing 10mM EDTA instead of CaCl₂ and was applied to a HiLoad Q-Sepharose HP column equilibrated with 20 mM HEPES pH 7.4, 10 mM EDTA, and eluted with a gradient of 20% to 40% of buffer containing an additional 1M of KCl over 14 CV. Molecular weights for all proteins were confirmed by MALDI-TOF on a Voyager-DE STR (Applied Biosystems).

2.3.3 Isothermal Titration Calorimetry

The purified MBP-CaMBD fusion proteins were dialyzed against 150 mM KCl, 10 mM HEPES, pH 7.4, 10 mM 2-mercaptoethanol, 10 mM CaCl₂ or 10 mM EDTA at 4°C. Peptides that were synthesized and delivered as lyophilized powder (Lifetein) were dissolved directly in dialysis buffer. Peptide oligomeric state was confirmed to be monomeric on a Superdex75 column (GE Healthcare) (SI Figure 1). Concentrations were determined using the calculated extinction coefficient at 280nm in the presence of 6M Guanidine. Titrations consisted of 20 injections of 2µL titrant (CaM or lobes) into the cell containing a CaMBD at a 10-fold lower concentration. Typical concentrations for the titrant were between 100-
500\(\mu\)M for experiments in 10 mM CaCl\(_2\) and between 700-2000\(\mu\)M for experiments in 10 mM EDTA, depending on the affinity. Experiments were performed at 25\(^{\circ}\)C and a stirring speed of 1000 rpm on an ITC200 instrument (GE Healthcare). Control experiments titrating CaM or its lobes (2000\(\mu\)M) into the hexahistidine-MBP without CaMBD2 (200\(\mu\)M) in 10 mM CaCl\(_2\) or 10 mM EDTA showed heats that were indistinguishable from buffer injections (data not shown). In addition, control experiments titrating N-lobe (2000\(\mu\)M) into C-lobe (200\(\mu\)M) in the presence of 10 mM CaCl\(_2\) or 10 mM EDTA show heats that are indistinguishable from buffer injections (data not shown). The data were processed using Origin 7.0 and fit to a single- or two-site fitting model after background buffer subtraction.

### 2.4 Results

#### 2.4.1 Binding to CaMBD2 and its Mutants

A previous crystal structure has reported a complex between Ca\(^{2+}\)/CaM and a peptide in the central region of RyR1 (aa 3614 to 3643).\(^9\)\(^4\) We termed this ‘CaMBD2’ simply to reflect its position in the sequence relative to other CaM binding domains investigated in this study (Figure 2.1). We utilized ITC to explore the thermodynamic parameters that underlie the binding. Because physiological concentrations of Ca\(^{2+}\) would yield mixtures of Ca\(^{2+}\)-free and Ca\(^{2+}\)-occupied lobes, the experiments were performed in the presence of excess Ca\(^{2+}\) (10 mM) to saturate all Ca\(^{2+}\) binding sites and to isolate binding of a fully Ca\(^{2+}\)-occupied CaM. The interaction of Ca\(^{2+}\)/CaM is strong, with a \(K_d\) of 46 nM (Figure 2.3, Table 2.1). The binding is driven by enthalpy and has an unfavorable entropic contribution. In order to determine the contribution of each CaM lobe, we also investigated their binding individually. Ca\(^{2+}\)/C-lobe binds much stronger (\(K_d\) ~800 nM) than the Ca\(^{2+}\)/N-lobe (\(K_d\)
The $\text{Ca}^{2+}$/C-lobe is driven by both favorable entropy and enthalpy while $\text{Ca}^{2+}$/N-lobe binding is driven by enthalpy alone.

### Table 2.1 Thermodynamic Parameters of Binding between $\text{Ca}^{2+}$/CaM and its Lobes and CaMBDs in 10 mM $\text{CaCl}_2$

<table>
<thead>
<tr>
<th>construct</th>
<th>lobe</th>
<th>no. of sites (N)</th>
<th>$K_d$ ($\mu$M)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$ (cal mol$^{-1}$ deg$^{-1}$)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>RYR1</td>
<td>CaMBD1</td>
<td>CaM</td>
<td>0.81 ± 0.22</td>
<td>2.05 ± 0.96</td>
<td>−9.36 ± 1.25</td>
<td>−5.21 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.79 ± 0.33</td>
<td>5.95 ± 2.07</td>
<td>−8.81 ± 1.82</td>
<td>−5.58 ± 5.4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.88 ± 0.35</td>
<td>7.88 ± 0.74</td>
<td>−1.49 ± 0.04</td>
<td>18.4 ± 0.4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>CaMBD2</td>
<td>CaM</td>
<td>0.75 ± 0.14</td>
<td>0.046 ± 0.032</td>
<td>−16.8 ± 2.9</td>
<td>−18.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.87 ± 0.11</td>
<td>33.3 ± 10.7</td>
<td>−10.7 ± 2.1</td>
<td>−15.4 ± 7.7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.80 ± 0.03</td>
<td>0.80 ± 0.29</td>
<td>−5.94 ± 0.57</td>
<td>8.09 ± 1.83</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CaMBD3</td>
<td>CaM</td>
<td>1.02 ± 0.19</td>
<td>0.052 ± 0.022</td>
<td>−5.00 ± 1.19</td>
<td>16.7 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>1.07 ± 0.08</td>
<td>4.19 ± 1.44</td>
<td>−4.18 ± 2.27</td>
<td>10.7 ± 8.0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1.16 ± 0.27</td>
<td>2.62 ± 1.22</td>
<td>−2.77 ± 1.95</td>
<td>16.5 ± 7.1</td>
<td>6</td>
</tr>
<tr>
<td>RYR2</td>
<td>CaMBD1</td>
<td>CaM</td>
<td>0.71 ± 0.04</td>
<td>7.59 ± 1.05</td>
<td>−6.09 ± 0.14</td>
<td>3.01 ± 0.76</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.75 ± 0.11</td>
<td>26.7 ± 8.7</td>
<td>−5.52 ± 0.74</td>
<td>2.47 ± 2.66</td>
<td>2</td>
</tr>
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<td></td>
<td>C</td>
<td>binding detected but not quantifiable</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CaMBD2</td>
<td>CaM</td>
<td>0.77 ± 0.13</td>
<td>0.047 ± 0.017</td>
<td>−17.2 ± 1.1</td>
<td>−24 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>1.13 ± 0.23</td>
<td>18.9 ± 7.9</td>
<td>−7.25 ± 0.47</td>
<td>−4.44 ± 4.13</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1.16 ± 0.41</td>
<td>1.03 ± 0.75</td>
<td>−5.09 ± 0.18</td>
<td>10.6 ± 6.1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>CaMBD3</td>
<td>CaM</td>
<td>complex binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N, Kd</td>
<td>1.06 ± 0.11</td>
<td>0.052 ± 0.015</td>
<td>−1.28 ± 0.16</td>
<td>29.1 ± 0.9</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>N, Kd</td>
<td>1.14 ± 0.08</td>
<td>4.6 ± 0.4</td>
<td>3.79 ± 0.18</td>
<td>37.2 ± 0.5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1.04 ± 0.01</td>
<td>3.09 ± 1.12</td>
<td>1.99 ± 0.18</td>
<td>31.7 ± 0.5</td>
<td>4</td>
</tr>
</tbody>
</table>

*Values are averages of n measurements. Errors are standard deviations.*
Figure 2.3 Ca$$^{2+}$$/CaM and its lobes bind to all three RyR1 CaMBDs.

A-I. ITC binding isotherms show the interaction between CaM or its lobes titrated into each CaMBD in the presence of 10 mM CaCl$_2$. The columns indicate the titrant, the rows show the CaMBD in the cell. Solid lines represent the fit. Concentrations used are (titrant into cell) A: 500µM in 50µM, B: 1330µM in 133µM, C: 1330µM in 133µM, D: 150µM in 15µM, E: 1000µM in 80µM, F: 800µM in 80µM, G: 223µM in 22.3µM, H: 490.9µM in 31.5µM, G: 315µM in 31.5µM. Affinity and thermodynamic parameters are shown in Table 1.
As it is possible that both lobes compete for an overlapping binding site, we performed competition experiments with the Ca\(^{2+}\)/C-lobe pre-mixed with RyR1 CaMBD2 (Figure 2.4, Table 2.2). These show that the affinity of CaMBD2 for the Ca\(^{2+}\)/N-lobe, once the Ca\(^{2+}\)/C-lobe has been bound, is even lower (\(K_d\sim60\mu\text{M}\)). The ability of the weaker Ca\(^{2+}\)/N-lobe to still bind in the presence of excess Ca\(^{2+}\)/C-lobe indicates that the binding sites are not mutually exclusive, but the weaker apparent affinity shows there are some unfavourable interactions when both lobes bind.

### Table 2.2 Thermodynamic Parameters of Binding between Ca\(^{2+}\)/CaM and its Lobes in Competition Experiments and CaMBDs in 10 mM CaCl\(_2\)^a

<table>
<thead>
<tr>
<th>construct</th>
<th>lobe</th>
<th>no. of sites (N)</th>
<th>(K_d) ((\mu\text{M}))</th>
<th>(\Delta H) (kcal/mol)</th>
<th>(\Delta S) (cal mol(^{-1}) deg(^{-1}))</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>RYR1</td>
<td>CaMBD1</td>
<td>N &gt; C</td>
<td>0.71 ± 0.18</td>
<td>22.4 ± 6.8</td>
<td>−6.44 ± 0.17</td>
<td>−0.27 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C &gt; N</td>
<td>heats indistinguishable from background</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CaMBD2</td>
<td>N &gt; C</td>
<td>0.67 ± 0.05</td>
<td>60.7 ± 27.2</td>
<td>−6.40 ± 1.94</td>
<td>−2.04 ± 7.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C &gt; N</td>
<td>0.44 ± 0.02</td>
<td>8.00 ± 1.35</td>
<td>−1.08 ± 0.07</td>
<td>19.7</td>
</tr>
<tr>
<td>RYR2</td>
<td>CaMBD3</td>
<td>N &gt; C</td>
<td>0.51 ± 0.20</td>
<td>5.86 ± 4.54</td>
<td>−2.50 ± 0.80</td>
<td>16.0 ± 4.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C &gt; N</td>
<td>heats indistinguishable from background</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^aValues are averages of \(n\) measurements. Errors are standard deviations (for \(n = 1\), errors are deviations of the fit from data).
Figure 2.4 Competition between N-lobe and C-lobes of CaM in all 3 CaMBDs.

A-F. ITC binding isotherms show the interaction between either N-lobe or C-lobe titrated into CaMBD with pre-incubated C-lobe or N-lobe, respectively, in the presence of 10 mM CaCl₂. The columns indicate the experiment, the rows show the CaMBD in the cell. Solid lines represent the fit. Concentrations used are (titrant in cell with pre-incubated lobe) A: 1330µM in 133µM with 222µM, B: 1330µM in 133µM with 222µM, C: 1000µM in 80µM with 133µM, D: 800µM in 80µM with 167µM, E: 1000µM in 100µM with 167µM, F: 661µM in 63µM with 185µM. Affinity and thermodynamic parameters are shown in Table 2.2.
As Ca\textsuperscript{2+}/CaM has different effects on RyR1 and RyR2, we wondered whether there are any differences in the binding affinities or energetic signatures of either lobe between both isoforms. However, the corresponding peptide in RyR2 binds with a very similar affinity, enthalpy and entropy, and as for RyR1, the Ca\textsuperscript{2+}/C-lobe forms the major interaction (Fig. 2.5, Table 2.1). Thus, in agreement with the high amount of sequence conservation among the different RyR isoforms, the overall binding mode on CaMBD2 is likely to be very similar.
Figure 2.5 Ca\(^{2+}\)/CaM and its lobes bind to all three RyR2 CaMBDs.

A-I. ITC binding isotherms show the interaction between CaM or its lobes titrated into each CaMBD in the presence of 10 mM CaCl\(_2\). The columns indicate the titrant, the rows show the CaMBD in the cell. Solid lines represent the fit. Concentrations used are (titrant into cell) A: 1100\(\mu\)M in 110\(\mu\)M, B: 1132\(\mu\)M in 110\(\mu\)M, C: 1132\(\mu\)M in 110\(\mu\)M, D: 150\(\mu\)M in 15\(\mu\)M, E: 788\(\mu\)M in 79\(\mu\)M, F: 674\(\mu\)M in 67\(\mu\)M, G: 630\(\mu\)M in 63\(\mu\)M, H: 1109\(\mu\)M in 63\(\mu\)M, G: 661\(\mu\)M in 63\(\mu\)M. Affinity and thermodynamic parameters are shown in Table 2.1. Binding isotherm in Panel C could not be accurately fit due to the low affinity.
In the crystal structure of the RyR1 CaMBD2 complex, a Trp residue forms the main anchor point for the Ca\(^{2+}\)/C-lobe, whereas a Phe residue provides the major contacts with the Ca\(^{2+}\)/N-lobe. We used individual lobes with mutant CaMBD2s to further dissect this interaction. In agreement with this, the W3586A mutation in RyR2 completely abolishes Ca\(^{2+}\)/C-lobe binding to CaMBD2, while the F3602A mutation has no effect (Figure 2.6, Table 2.3). However, both W3586A and F3602A still allow for Ca\(^{2+}\)/N-lobe binding, but with different affinities and enthalpic components, suggesting that the Ca\(^{2+}\)/N-lobe can bind to two different sites. When one Ca\(^{2+}\)/N-lobe site is abolished, it can still bind another, a type of behavior that has been observed in other CaM targets. These data are in agreement with previous studies showing that neither the equivalent W3586A or F3602A mutations individually can knock out Ca\(^{2+}\)/CaM binding in full-length RyR2. Overall, these data suggest that CaMBD2 primarily forms a high affinity Ca\(^{2+}\)/C-lobe binding site, whereas the binding site for Ca\(^{2+}\)/N-lobe, as observed in the crystal structure, is weak and mobile.

Table 2.3 Thermodynamic Parameters of Binding between Ca\(^{2+}\)/CaM and its Lobes in Mutant CaMBDs in 10 mM CaCl\(_2\)^a

<table>
<thead>
<tr>
<th>construct</th>
<th>lobe</th>
<th>no. of sites (N)</th>
<th>(K_d) (μM)</th>
<th>(\Delta H) (kcal/mol)</th>
<th>(\Delta S) (cal mol(^{-1}) deg(^{-1}))</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaMBD2W3586A</td>
<td>CaM</td>
<td>0.91 ± 0.16</td>
<td>1.01 ± 0.43</td>
<td>-11.9 ± 2.6</td>
<td>-12.3 ± 9.3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.69 ± 0.39</td>
<td>18.9 ± 7.6</td>
<td>-12.8 ± 0.7</td>
<td>-21.3 ± 2.0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td>heats indistinguishable from background</td>
<td>4</td>
</tr>
<tr>
<td>CaMBD2F3602A</td>
<td>CaM</td>
<td>1.10 ± 0.02</td>
<td>0.32 ± 0.09</td>
<td>-12.7 ± 0.4</td>
<td>-13.0 ± 1.0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.96 ± 0.02</td>
<td>6.86 ± 1.43</td>
<td>-1.86 ± 0.02</td>
<td>17.4 ± 1.8</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.78 ± 0.50</td>
<td>0.44 ± 0.29</td>
<td>-7.66 ± 0.09</td>
<td>3.8 ± 3.6</td>
<td>3</td>
</tr>
<tr>
<td>CaMBD3LRRdup</td>
<td>CaM</td>
<td>0.79 ± 0.14</td>
<td>0.014 ± 0.008</td>
<td>-10.3 ± 1.4</td>
<td>1.6 ± 3.9</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.82 ± 0.05</td>
<td>2.35 ± 0.67</td>
<td>-7.37 ± 0.31</td>
<td>1.1 ± 1.6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.88 ± 0.25</td>
<td>1.21 ± 0.16</td>
<td>-8.21 ± 2.18</td>
<td>-0.5 ± 7</td>
<td>3</td>
</tr>
<tr>
<td>CaMBD3R4325D</td>
<td>CaM</td>
<td>0.64 ± 0.02</td>
<td>0.12 ± 0.008</td>
<td>-9.03 ± 0.06</td>
<td>1.4 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>0.83 ± 0.03</td>
<td>5.11 ± 0.46</td>
<td>-6.35 ± 0.17</td>
<td>2.9 ± 0.7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1.01 ± 0.09</td>
<td>1.54 ± 0.11</td>
<td>-6.04 ± 0.03</td>
<td>6.4 ± 0.12</td>
<td>3</td>
</tr>
</tbody>
</table>

^aValues are averages of \(n\) measurements. Errors are standard deviations (for \(n = 1\), errors are deviations of the fit from data).

59
Figure 2.6 RyR2 CaMBD2 W3586A and F3602A mutants alter lobe binding.

A-F. ITC binding isotherms show the interaction between CaM or its lobes titrated into each mutant CaMBD in the presence of 10 mM CaCl₂. The columns indicate the titrant, the rows show the CaMBD in the cell. Solid lines represent the fit. Concentrations used are (titrant into cell) A: 500 µM in 50 µM, B: 1000 µM in 100 µM, C: 521 µM in 52 µM, D: 500 µM in 50 µM, E: 788 µM in 79 µM, F: 674 µM in 67 µM. Affinity and thermodynamic parameters are shown in Table 2.33.

We also analyzed apoCaM binding to CaMBD2, by repeating the ITC experiments in the absence of Ca²⁺ and the presence of excess EDTA (Figure 2.7 and 2.8, Table 2.4). ApoCaM is able to bind CaMBD2 from either isoform, but significantly weaker than Ca²⁺/CaM (Kₐ 50-70 µM). The individual lobes are also capable of binding independently,
but the binding was too weak to be quantified reliably. These data show that both apo-lobes contribute to the binding of apoCaM to CaMBD2.

Table 2.4 Thermodynamic Parameters of Binding between apoCaM and its Lobes and CaMBDs in 10 mM EDTA

<table>
<thead>
<tr>
<th>construct</th>
<th>lobe</th>
<th>no. of sites (N)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$ (cal mol$^{-1}$ deg$^{-1}$)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>RYR1</td>
<td>CaMBD1 apoCaM</td>
<td>heats indistinguishable from background</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>apo-N-lobe</td>
<td>heats indistinguishable from background</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>apo-C-lobe</td>
<td>not performed</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CaMBD2 apoCaM</td>
<td>1</td>
<td>46.5 ± 22.5</td>
<td>1.59 ± 0.09</td>
<td>26 ± 2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>apo-N-lobe</td>
<td>1.09 ± 0.10</td>
<td>55.6 ± 36.6</td>
<td>0.34 ± 0.09</td>
<td>20.8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>apo-C-lobe</td>
<td>binding detected but not quantifiable</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CaMBD3 apoCaM</td>
<td>0.38 ± 0.09</td>
<td>27.1 ± 14.6</td>
<td>1.5 ± 0.5</td>
<td>26 ± 1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>apo-N-lobe</td>
<td>0.90 ± 0.40</td>
<td>22.7 ± 7.7</td>
<td>0.90 ± 0.46</td>
<td>24.3 ± 2.4</td>
<td>3</td>
</tr>
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<td></td>
<td>apo-C-lobe</td>
<td>heats indistinguishable from background</td>
<td>2</td>
<td></td>
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<tr>
<td>RYR2</td>
<td>CaMBD1 apoCaM</td>
<td>heats indistinguishable from background</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>apo-N-lobe</td>
<td>heats indistinguishable from background</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>apo-C-lobe</td>
<td>heats indistinguishable from background</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CaMBD2 apoCaM</td>
<td>0.89 ± 0.82</td>
<td>72.3 ± 28.6</td>
<td>2.2 ± 2.2</td>
<td>26.3 ± 4.7</td>
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</tr>
<tr>
<td></td>
<td>apo-N-lobe</td>
<td>heats indistinguishable from background</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>apo-C-lobe</td>
<td>binding detected but not quantifiable</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CaMBD3 apoCaM</td>
<td>0.87 ± 0.26</td>
<td>5.11 ± 2.69</td>
<td>13.2 ± 7.8</td>
<td>68.8 ± 26.7</td>
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<tr>
<td></td>
<td>apo-N-lobe</td>
<td>1.25 ± 0.07</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>apo-C-lobe</td>
<td>1.01 ± 0.13</td>
<td>62.1 ± 33</td>
<td>1.7 ± 0.2</td>
<td>25.2 ± 1.7</td>
<td>2</td>
</tr>
</tbody>
</table>

"Values are averages of n measurements. Errors are standard deviations (for n = 1, errors are deviations of the fit from data).$^b$This value was forced to be 1 to allow for fitting. $^c$This lower value suggests that one calmodulin binds two peptides (i.e., N = 0.5). This is possible as apo-C-lobe binding is enthalpically silent and undetectable and overlaps with the apo-N-lobe site."
Figure 2.7 Binding of apoCaM and its lobes to RyR1 CaMBDs.

A-G. ITC binding isotherms show the interaction between CaM or its lobes titrated into each CaMBD in the presence of 10 mM EDTA. The columns indicate the titrant, the rows show the CaMBD in the cell. Solid lines represent the fit. Concentrations used are (titrant into cell) A: 1000µM in 82µM, B: 1500µM in 150µM, C: 2264µM in 200µM, D: 1435µM in 200µM, E: 1500µM in 150µM, F: 1500µM in 150µM, G: 1500µM in 150µM. Affinity and thermodynamic parameters are shown in Table 2.4.
Figure 2.8 Binding of apoCaM and its lobes to RyR2 CaMBDs.

A-G. ITC binding isotherms show the interaction between CaM or its lobes titrated into each CaMBD in the presence of 10 mM EDTA. The columns indicate the titrant, the rows show the CaMBD in the cell. Solid lines represent the fit. Concentrations used are (titrant into cell) A: 760 µM in 60 µM, B: 1000 µM in 100 µM, C: 1000 µM in 100 µM, D: 1000 µM in 100 µM, E: 250 µM in 25 µM, F: 750 µM in 75 µM, G: 750 µM in 75 µM. Affinity and thermodynamic parameters are shown in Table 2.4.
2.4.2 Binding to CaMBD1

As the Ca\textsuperscript{2+}/N-lobe affinity for the CaMBD2 is low, especially in the presence of a prebound Ca\textsuperscript{2+}/C-lobe, we tested the binding to another segment, CaMBD1 (aa 1975 to 1999 in RyR1), which was previously suggested to form a binding site based on gel shift assays.\textsuperscript{226} Cross-linking studies also imply that it is close to CaMBD2.\textsuperscript{226} This site is less conserved than CaMBD2 between the isoforms (Figure 2.1), with 64% sequence identity between RyR1 and RyR2. In RyR1, CaMBD1 binds Ca\textsuperscript{2+}/CaM with a $K_d$ of $\sim$2\textmu M. Both the Ca\textsuperscript{2+}/N-lobe and Ca\textsuperscript{2+}/C-lobe can bind, but their affinities are weaker than for full-length Ca\textsuperscript{2+}/CaM, indicating that both contribute to the avidity (Figure 2.3, Table 2.1). In the presence of pre-bound Ca\textsuperscript{2+}/C-lobe, the affinity of Ca\textsuperscript{2+}/N-lobe for CaMBD1 is weakened $\sim$3.5 fold (Figure 2.4, Table 2.2), suggesting that the binding of both lobes simultaneously induces some strain.

The CaMBD1 sequences of RyR1 and RyR2 differ in 9 positions (Figure 2.1), and this affects the binding of Ca\textsuperscript{2+}/CaM. In RyR2, Ca\textsuperscript{2+}/CaM binds $\sim$4-fold weaker, with an affinity of $\sim$7.6\textmu M. Ca\textsuperscript{2+}/N-lobe binds with a $\sim$27\textmu M $K_d$, but Ca\textsuperscript{2+}/C-lobe titrations yielded a flat binding isotherm that could not be fit reliably, suggesting the interaction is extremely weak (Figure 2.5, Table 2.1). As in RyR1, the affinity of Ca\textsuperscript{2+}/CaM is higher than for either lobe, indicating that both lobes can still contribute to the binding. The main difference between RyR1 and RyR2 thus seems to be a higher affinity for CaMBD1 of RyR1, and only a very small contribution of the Ca\textsuperscript{2+}/C-lobe to RyR2 CaMBD1 binding. Unlike CaMBD2, apoCaM was not observed to bind to CaMBD1 from either isoform (Figure 2.7 and 2.8, Table 2.4).
In summary, CaMBD1 forms a Ca\(^{2+}\)/CaM binding site that is mainly driven by the Ca\(^{2+}\)/N-lobe. This lobe binds CaMBD1 stronger than it binds CaMBD2 (when a Ca\(^{2+}\)/C-lobe is prebound to it), suggesting that Ca\(^{2+}\)/CaM could bridge these 2 segments.

### 2.4.3 Binding to CaMBD3

Yet another segment, located in the C-terminal 1/5\(^{th}\) of the channel, has been suggested to form a putative CaM binding site.\(^{221,262}\) The analyzed segment, termed CaMBD3, spans residues 4295 to 4325 in RyR1. There is only \(~26\%\) sequence identity between RyR1 and RyR2 in this region (Figure 2.1).

In RyR1, CaMBD3 forms a high affinity Ca\(^{2+}\)/CaM binding site (\(K_d \sim 52\) nM) with favorable enthalpy and entropy (Figure 2.3, Table 2.1). The individual lobes bind as well, but with weaker affinities (2-4\(\mu\)M \(K_d\)), indicating that both contribute to Ca\(^{2+}\)/CaM binding.

The corresponding region in RyR2 interacts with Ca\(^{2+}\)/CaM in a completely different manner as it exhibits a complex binding isotherm that cannot be fit using a simple 1:1 binding model (Figure 2.5, Table 2.1). Such complex isotherms have been observed before for CaM-peptide interactions in other ion channels and is likely due to a single CaM binding two peptides at the initial stage of the ITC experiment (when sufficient free peptide is available), followed by unbinding of one peptide and a 1:1 stoichiometry at the later stages (when free peptide is scarce).\(^{265}\) Despite our inability to fit this isotherm, the sharp transition suggests that the affinity is very high. In order to deconstruct this complex curve, we also analyzed the binding of the individual CaM lobes. The Ca\(^{2+}\)/C-lobe binds with a \(K_d \sim 3\mu\)M, similar to RyR1-CaMBD3. However, the Ca\(^{2+}\)/N-lobe titrations show two transitions, revealing a high-affinity site with a \(K_d\) of 52nM driven by both favorable entropy and enthalpy and a second low-affinity site (\(K_d \sim 4.6\mu\)M) with unfavorable enthalpy. We used a
competition experiment to determine whether both sites are available in the presence of Ca$^{2+}$/C-lobe (Figure 2.4, Table 2.2). In the presence of Ca$^{2+}$/C-lobe, only one exothermic Ca$^{2+}$/N-lobe binding event was observed with a $K_d$ of 5.9µM. Overall, the binding mode and energetics of Ca$^{2+}$/CaM binding to CaMBD3 in both isoforms thus appears to be very different. (Figure 2.9)

Figure 2.9 Overview of Ca$^{2+}$/CaM and apoCaM binding to CaMBDs.
Bar graphs comparing the $K_d$ and $\Delta G$ of binding for CaM (A) and the individual Ca$^{2+}$/lobes (B) to the three CaMBDs. Error bars indicate S.D.
Importantly, the CaMBD3 also forms a binding site for apoCaM in both RyR1 \( (K_d \approx 27 \mu M) \) and RyR2 \( (K_d \approx 5 \mu M) \) (Figures 2.7 and 2.8, Table 2.4). These values show that apoCaM can bind CaMBD3 significantly stronger than CaMBD2, suggesting that CaMBD3 may form the primary binding site for apoCaM. The two isoforms differ appreciably in their apoCaM binding. In RyR1, the binding seems to involve only the apo-N-lobe \( (K_d \approx 23 \mu M) \), whereas in RyR2, both lobes contribute to apoCaM binding \( (K_d \approx 50 \mu M \) and \( \approx 60 \mu M \) for apo-N-lobe and apo-C-lobe, respectively).

In conclusion, CaMBD3 forms the strongest apoCaM binding site of the peptides tested, and forms an additional high-affinity site for Ca\(^{2+}\)/CaM. Both the sequence and binding mode differ appreciably between both isoforms.

### 2.4.4 Disease Mutations in RyR1-CaMBD3

Interestingly, two disease mutations map into the CaMBD3 sequence of RyR1 (Figure 2.1). R4325D has been linked to CCD and multiminicore disease. The second mutation involves a duplication event from L4319 - R4321, which repeats the sequence LRR and has been linked to increased serum creatine kinase and potentially causes MH.

Compared to wild type CaMBD3, the R4325D mutant weakly affects Ca\(^{2+}\)/CaM binding \( (K_d 120 \text{ nM} \) versus \( 52 \text{ nM} \) (Figure 2.10, Table 2.3). However, apoCaM binding appears stronger \( (K_d \approx 7 \mu M \) versus \( \approx 27 \mu M \) (Figure 2.11).
Figure 2.10 R4325D and LRR duplication mutants in RyR1 CaMBD3

A-F. ITC binding isotherms show the interaction between CaM or its lobes titrated CaMBD3 mutants in the presence of 10 mM CaCl₂. The columns indicate the titrant, the rows show the CaMBD in the cell. Solid lines represent the fit. Concentrations used are (titrant into cell) A: 200µM in 20µM, B: 400µM in 40µM, C: 400µM in 40µM, D: 400µM in 35µM, E: 400µM in 35µM, F: 400µM in 35µM. Affinity and thermodynamic parameters are shown in Table 2.3.
Figure 2.11 ApoCaM and its lobes bind to RyR1 CaMBD3 LRRdup and R4325D mutants with different energetics than wild-type.

A-F. ITC binding isotherms show the interaction between CaM or its lobes titrated into RyR1 CaMBD3 mutants in the presence of 10 mM EDTA. The columns indicate the titrant, the rows show the CaMBD in the cell. Solid lines represent the fit. Concentrations used are (titrant in cell) A: 1500µM in 150µM, B: 2000µM in 200µM, C: 1500µM in 150µM, D: 1500µM in 150µM, E: 2000µM in 200µM, F: 2000µM in 200µM. Affinities are B: 57±9µM, C: 65.4±9.8µM, D: 7±2µM, E: 101±19µM. Binding isotherm in Panel A could not be fit due to complex binding.

In contrast, the LRR duplication mutant binds Ca²⁺/CaM ~3.5 fold stronger than wild type (14 nM versus 52nM), and the affinities of each lobe individually have changed (Figure 2.10, Table 3). Under Ca²⁺-free conditions, the LRR duplication mutant produces a
complex binding isotherm with both endothermic and exothermic components (Figure 2.11). Experiments with the individual lobes show that apo-N-lobe binding is endothermic, whereas apo-C-lobe binding is exothermic. This is different from the WT CaMBD3, which shows a single endothermic binding event, and where direct apo-C-lobe binding could not be detected.

In conclusion, both disease mutations have direct effects on the binding of both apoCaM and Ca$^{2+}$/CaM to RyR1 CaMBD3.

2.5 Discussion

CaM has been identified as a resident Ca$^{2+}$ sensor for multiple ion channel families, including voltage-gated calcium and sodium channels, small conductance Ca$^{2+}$ activated potassium channels, KCNQ potassium channels and many more.$^{270-273}$ In many cases, the primary role of CaM seems to be affecting the gating properties (opening, closing, inactivation) of these channels, but it has also been involved in channel trafficking.

RyRs are sensitive signal amplifiers: increases in cytosolic Ca$^{2+}$ concentrations trigger their opening, through a phenomenon known as Ca$^{2+}$-dependent Ca$^{2+}$ release. Although the skeletal muscle isoform (RyR1) can be activated through direct mechanical coupling with L-type Ca$^{2+}$ channels,$^{11,66,237,274}$ they are also gated by Ca$^{2+}$ ions. CaM can fine-tune the precise sensitivity to Ca$^{2+}$ concentrations, in a manner that appears to be isoform-specific. How exactly this happens remains unknown, but cryo-EM images have shown that the cytoplasmic cap of RyRs undergoes large conformational changes during opening and closing. Both apoCaM and Ca$^{2+}$/CaM have been shown to bind to the lateral side of the cap,$^{70}$ and are likely to interfere with these allosteric motions. For example, by stabilizing the
closed state or destabilizing the open state, Ca²⁺/CaM would exert an inhibition. In both RyR1 and RyR2, Ca²⁺/CaM seems to inhibit the channel.²¹²,²¹⁶-²¹⁸

2.5.1 Ca²⁺/N-lobe is Loosely Associated with CaMBD2

In order to understand the mechanisms that underlie CaM regulation of RyRs, it is necessary to know which regions of the RyR sequence can bind CaM and under which conditions. Several RyR regions have been proposed as CaM binding sites²²¹,²²²,²²²-²²⁶,²²⁹,²⁶¹-²⁶³, but in many cases these sites were not validated using a quantitative method. In fact, most of the initially identified sites have been shown to be inaccessible within a folded domain⁹³, or were found to be located too far from the CaM binding site identified in cryo-EM studies.²³⁶ A crystal structure of Ca²⁺/CaM bound to the CaMBD2 of RyR1 has shown that this area forms a strong Ca²⁺/C-lobe binding site. However, the Ca²⁺/N-lobe is only loosely associated, and is likely to bind elsewhere.⁹⁴ We confirm these observations using ITC as the Ca²⁺/C-lobe binds significantly stronger than Ca²⁺/N-lobe, with the latter affinity decreasing even further once the Ca²⁺/C-lobe is already bound. Therefore it is possible that the Ca²⁺/N-lobe is associated with a different segment.⁹⁴,²²⁶ Here we also analyzed the binding of apoCaM and Ca²⁺/CaM to the two remaining candidates, which we named CaMBD1 (corresponding to RyR1 residues 1975-1999) and CaMBD3 (RyR1 4295-4325).

2.5.2 CaMBD1 Only Binds Ca²⁺/CaM

CaMBD1 was first identified as a potential apoCaM binding site in RyR1.²²⁶ However, using our ITC experiments we could not detect any apoCaM binding to CaMBD1 for either RyR1 or RyR2. Instead, CaMBD1 only forms an alternative Ca²⁺/CaM binding site, with a higher affinity for the Ca²⁺/N-lobe than for the Ca²⁺/C-lobe. Our results may suggest that
previous pull-downs may have been false positives due to improperly folded protein. This is especially true in RyR2, where Ca\(^{2+}\)/C-lobe binding to CaMBD1 individually could not be detected. A simple interpretation would then be that Ca\(^{2+}\)/CaM could bridge CaMBD1 and CaMBD2 via its N-lobe and C-lobe, respectively.

2.5.3 CaMBD3 Binds Strongly to Both ApoCaM and Ca\(^{2+}\)/CaM

However, CaMBD3, a poorly conserved segment in the C-terminal 1/5\(^{th}\) of the channel, forms an additional binding site for both Ca\(^{2+}\)/CaM and apoCaM. In fact, it forms the highest affinity binding site we detected for CaM in the absence of Ca\(^{2+}\), suggesting that it may form the primary apoCaM binding site. In both RyR1 and RyR2, CaMBD3 strongly bound apoCaM and is mainly mediated by the apo-N-lobe. The Ca\(^{2+}\)/CaM binding is almost as strong as for CaMBD2, but the energetic profile differs substantially between RyR1 and RyR2, with even 2 binding sites existing for the Ca\(^{2+}\)/N-lobe in RyR1.

2.5.4 Mutations in RyR1-CaMBD3 Affect the Energetics and Mode of Binding

We also investigated physiological mutations in RyR1-CaMBD3 which have been linked to CCD, mmCD (R4235D) and to increased creatine kinase levels/MH (LRR duplication). Neither mutation abolishes CaM binding, but both affect the energetics and likely also the binding mode, suggesting that interfering with CaM regulation at this site may be the primary cause of the disease for these specific mutations.

2.5.5 Multiple CaMBDs are Compatible with Known Structural Data

A crucial question is whether the CaMBDs described here also bind CaM when present in their native context of a full-length channel. In the absence of a high-resolution
structure of the full-length protein, this is a difficult question to answer. CaM binds to RyR subunits with a 1:1 stoichiometry, which would seem at odds with the presence of 3 sites that can all bind Ca\(^{2+}\)/CaM. Two sites could bind Ca\(^{2+}\)/CaM simultaneously through the ability of individual CaM lobes to bind targets separately, a feature that has been observed in several CaM:ion channel complexes.\(^{270,273}\) In addition, the sites could also be mutually exclusive, whereby binding to one site occludes the other one through direct steric hindrance or allosterically. This allows redundancy in CaM binding, without affecting the 1:1 stoichiometry. Cryo-EM studies have shown that the center of mass of CaM undergoes a \(~30\text{Å}\) shift on RyR1. Because CaMBD2 is able to bind both apoCaM and Ca\(^{2+}\)/CaM, it has been suggested that CaM shifts along this helix upon binding Ca\(^{2+}\).\(^{223,224,275}\) However, mere shifts within the CaMBD2 are unlikely to create the large movements observed in the cryo-EM studies. Instead, it is more likely that either one or both lobes of CaM shift to another CaMBD upon associating with Ca\(^{2+}\). FRET measurements between CaM and FKBP on RyRs however do not reveal a large shift in apoCaM and Ca\(^{2+}\)/CaM.\(^{276}\) To reconcile these differences it has been proposed that CaM moves in an arc with constant radius relative to the donor FKBP in the FRET measurements.

The concentration of free CaM in the cytosol of cardiac myocytes has been measured at \(~50-75\text{nM}\).\(^{175}\) In addition, the affinity of CaM for intact RyR2 near cardiac myocyte Z-lines has been found to have a \(K_d\sim10-20\text{nM}\), comparable to the \(K_d\) values obtained by measuring \[^{35}\text{S}]\text{CaM binding to RyRs in SR vesicles (}~20-30\text{nM}\), even at Ca\(^{2+}\) concentrations below 0.01\(\mu\text{M}.\(^{278}\) One could therefore question the relevance of individual CaMBDs with \(K_d\) values higher than 1\(\mu\text{M}.\) However, functional studies with voltage-gated calcium channels have shown that CaM concentrations in the immediate vicinity of some targets can be enriched several orders of magnitude.\(^{279}\) More importantly, due to the inherent
ability of the CaM lobes to bind segments individually, it is likely that higher affinity sites are formed by allowing the lobes to bridge non-contiguous CaMBDs, capitalizing on additive affinities. This would be most important for apoCaM, since the highest affinity we observed is ~5µM for RyR2 CaMBD3. Since CaMBD1 does not bind apoCaM in our experiments, an attractive possibility is thus that apoCaM bridges CaMBD2 and CaMBD3.

2.5.6 CaMBD2 and CaMBD3 may be Accessible

Importantly, for the CaMBDs to be ‘true’ CaM binding sites, they also have to be solvent accessible within full-length RyR. Cryo-EM reconstructions using GFP insertions in full-length RyR2 localized CaMBD3 to the cytosolic surface, near the side of domain 3 and close to the cleft formed by domains 7 and 8a. This is in proximity to the Ca²⁺/CaM binding site of RyR1 and the apoCaM site in RyR2. Importantly, the GFP insertion did not create any visible conformational changes, suggesting that the site could be solvent exposed. Similarly, GFP insertion studies show that CaMBD2 is located near domains 3 and 8a, again close to both apoCaM and Ca²⁺/CaM sites of RyR1, and to the apoCaM site of RyR2. On the other hand, two GFP insertions near CaMBD1, at positions T1874 and T2023 of RyR2, localized to domains 9 and 4, respectively. These were interpreted to be too far from the identified CaM binding sites in cryo-EM studies. Although this would argue against the involvement of CaMBD1 in CaM binding, no insertion was done directly within the CaMBD1 sequence (residues 1941-1965 in RyR2), and the longer linker length used for inserting GFP (nine residues on either side of the insertion) may have created difference densities further away from the insertion site. Importantly, the insertions in CaMBD2 and CaMBD3 had used shorter linkers, so the locations of these sites are more reliable.
In addition to cryo-EM studies, the functional effect of CaM on full-length RyRs has been analyzed on a series of chimeras, deletion mutants and point mutations. Introduction of mutations in RyR2 CaMBD2 has been shown to cause early cardiac hypertrophy in mice and in neonatal cardiomyocytes, as well as an impaired inhibition by CaM. This study highlights a clear role for CaMBD2 in mediating CaM regulation.

2.5.7 Calmodulation of RyR May Be Involved in More than Just Channel Activity

For CaMBD1, no deletion or CaM binding knockout mutations have been generated, so a direct test of this segment in modulation by CaM has not been performed yet. The CaMBD1 sequences of RyR1 and RyR2 differ in 10 positions, but swapping these did not have an effect on the channel open probability at either high or low Ca\(^{2+}\). The difference in modulation of RyR1 and RyR2 by apoCaM is therefore unlikely to be due to any sequence differences in CaMBD1, but this does not preclude a role for CaMBD1 in binding and modulation by CaM.

No deletion has been made for CaMBD3 individually, but deletion experiments have been performed on a much larger area (RyR1 4274-4535), which encompasses CaMBD3. Overlay binding assays showed that apoCaM and Ca\(^{2+}\)/CaM can still bind to this deletion mutant. Ca\(^{2+}\)/CaM inhibited, and apoCaM activated this mutant with potencies similar to wild type RyR1. These observations argue against an involvement of CaMBD3 in mediated CaM regulation of RyR1. However, CaMBD3 may still have a function. First, no deletion experiments have been performed on CaMBD3 in RyR2, and given the differences in both binding and functional effects of CaM in RyR1 and RyR2, it may still be directly involved in CaM regulation of RyR2. Second, similar to other ion channels, there may be additional functions for CaM beyond regulation of the open probability, such as mediating proper
folding and trafficking, and these may involve CaMBD3. CaM may also be able to further affect $P_o$ in the presence of additional RyR post-translational modifications or auxiliary proteins, and this may be dependent on particular CaMBDs.

### 2.5.8 CaMBDs Present Additional Platforms for Binding to Other EF-hand Proteins

Finally, one should also consider the possibility of an EF-hand redundancy. It is frequently observed that CaM target peptides are also able to bind other EF-hand containing proteins. For example, the IQ domain of voltage-gated calcium channels forms a known binding site for CaM, but is also able to bind other members of the CaBP family, and even an EF-hand containing region of RyRs. Within RyRs, CaMBD2 has also been found to associate with S100A1, a dimeric EF-hand containing protein that is thought to compete with CaM for binding RyRs. In addition, CaMBD2 has been found to bind the same RyR EF hand-containing region. Many other EF-hand containing proteins simply have not been tested yet. It is therefore possible that one or several of the RyR CaMBDs are general EF-hand binding domains, with the exact binding depending on $Ca^{2+}$ levels and relative availabilities of the various EF-hand containing proteins.
Chapter 3 - Crystal Structures of Wild Type and Disease Mutant Forms of the Ryanodine Receptor SPRY2 Domain

A version of this chapter has been accepted for publication: Lau, K., Van Petegem, F.

Crystal structures of wild type and disease mutant forms of the Ryanodine Receptor SPRY2 domain. Nature Communications (September 2014)
3.1 Abstract

Ryanodine receptors (RyRs) form channels responsible for the release of Ca$^{2+}$ from the endoplasmic and sarcoplasmic reticulum. The SPRY2 domain in the skeletal muscle isoform (RyR1) has been proposed as a direct link with L-type calcium channels (Ca$v_{1.1}$), allowing for direct mechanical coupling between plasma membrane depolarization and Ca$^{2+}$ release. Here we present the crystal structures of the SPRY2 domain from RyR1 and RyR2 at 1.34-1.84Å resolution. They form two antiparallel $\beta$ sheets establishing a core, and four additional modules of which several are required for proper folding. A buried disease mutation, linked to hypertrophic cardiomyopathy and loss-of-function, induces local misfolding and strong destabilization. Isothermal titration calorimetry experiments negate the RyR1 SPRY2 domain as the major link with Ca$v_{1.1}$. Instead, docking into full-length RyR1 cryo-electron microscopy maps shows that the SPRY2 domain forms a link between the N-terminal gating ring and the clamp region.
3.2 Introduction

Ryanodine receptors represent the pinnacle of ion channel complexity: with molecular weights exceeding 2.2 MDa, they form docking sites for tens of auxiliary proteins and small molecules that can regulate their activities. Cryo-electron microscopy (cryo-EM) studies have shown that the RyRs form tetrameric assemblies that resemble the shape of a mushroom⁶⁹,⁷⁵,⁷⁶,⁸¹: the stalk region is thought to traverse the membrane of the ER or SR, and the large cap is located entirely in the cytosol. The cap regulates the ability of the channel to open or close, turning RyRs into enormous allosteric membrane proteins. A number of crystal structures have been reported, focusing mostly on the N-terminal three domains⁹⁰-⁹²,⁹⁶,⁹⁷,¹⁰¹,¹⁰², and a single domain containing a phosphorylation hot spot loop.⁹³,¹⁰⁶

The primary signal for opening of RyRs is Ca²⁺, the very same ion it permeates.¹⁴ In cardiac myocytes, the triggering Ca²⁺ primarily originates from opening of DHPR located in the plasma membrane. In skeletal muscle, however, it has been shown that depolarization of the plasma membrane can trigger Ca²⁺ release even in the absence of extracellular Ca²⁺. Many studies suggest that RyR1 and Caᵥ₁.1 form direct physical contacts, with Caᵥ₁.1 acting as the voltage sensor for RyR1 through direct mechanical coupling.¹¹,⁶⁶,²³⁸,²⁷⁴,²⁸² In addition, ‘retrograde’ signalling has been reported, whereby changes in RyR1 can affect the function of Caᵥ₁.1.²⁴²,²⁸³

Understanding how Caᵥ₁.1 and RyR1 can influence one another’s behaviour is one of the major puzzles in the field of excitation-contraction coupling. On the Caᵥ₁.1 side, several studies have shown the importance of the cytosolic loop connecting transmembrane repeats II and III (‘II-III loop’).²³⁷,²⁴³,²⁸⁴ Within RyR1, several regions of RyR1 that may bind this loop have been proposed through the use of pull-downs and chimeras between RyR1
and RyR2. Several interaction sites have been suggested and a major focus has been on the SPRY2 domain.

SPRY domains lend their name to splA kinase and RyRs, where they were first identified. RyRs contain three such SPRY domains, conserved among vertebrates and invertebrates. Although they are founders for this family, no high-resolution structure has been reported for any of them. Here we describe crystal structures of the SPRY2 domain of both RyR1 and RyR2, showing they consist of additional modules not previously predicted. Their location in full-length RyR1 suggests a role for linking motions in the central gating ring, formed by the N-terminal domains, with the mobile corner region. We also analyse the effect of disease-causing mutations on the structure and stability.

3.3 Methods and Materials

3.3.1 Cloning, Expression, Purification and Crystallization of SPRY2

Rabbit RyR1 (1070-1246, 1085-1208) and mouse RyR2 (1080-1253) were cloned into the pET28 HMT vector as previously described. Mutations were introduced by the Quikchange method (Stratagene). Human Ca\textsubscript{v}1.1 II-III loop (666-787) was cloned in a pET24a vector containing a C-terminal hexahistidine tag using NdeI and XhoI restriction sites. At the N-terminus, maltose-binding protein (MBP) was attached along with a Tobacco Etch Virus (TEV) protease cleavage site.

Cultures were grown in 2xYT broth to an OD\textsubscript{600} ~ 0.8-1 at 37°C. The temperature was then lowered to 18°C, cells were induced with 0.2 mM IPTG and allowed to grow for a total of 22-24 hours. Two liter cell pellets were frozen at -20°C.

Cell pellets were lysed by sonication after addition of 5 mL glycerol, 40 mL Buffer A (250 mM KCl, 10 mM HEPES 7.4) supplemented with 25 µg/mL DNase I, 25 µg/mL.
lysozyme, 1 mM PMSF and 10 mM β-mercaptoethanol (bME). The lysate was centrifuged for 30 minutes at 35,000xg and a clarified supernatant was loaded onto a PorosMC (Tosoh Biosep) or TALON (GE Healthcare) column. The column was washed with 5-10 column volumes (CV) of Buffer A followed by 5 CV of 2% Buffer B (250 mM KCl, 500 mM imidazole pH 7.4). The 2% Buffer B wash was omitted for all RyR2 constructs. Protein was eluted with 30% Buffer B, TEV protease added and immediately placed in a dialysis containing Buffer A at room temperature for 4 hours or overnight. TEV protease was removed by loading the cleavage mixture onto a second PorosMC or Talon column with the flow-through collected. Excess MBP was removed by passing the flow-through over an amylose column. The flowthrough was diluted three-fold and further purified by anion-exchange chromatography with a Hiload-Q (GE Healthcare) equilibrated with Buffer C (20 mM HEPES pH 7.4) then on a gradient from 15 to 30 % Buffer D (1M KCl, 20 mM HEPES pH 7.4) over 12 column volumes. The protein was concentrated and polished by gel-filtration chromatography on a Superdex 200 (GE Healthcare).

Protein crystals were grown by the hanging-drop method at 4°C for RyR1 and at room temperature for RyR2. RyR1 SPRY2 domain (5-10 mg/mL) was crystallized in 0.9-1.1 M sodium citrate pH 6.5. RyR2 SPRY2 domain (4 mg/mL) and its mutant were crystallized in 0.2 M sodium formate, 18%-25% PEG3350.

3.3.2 Data Collection and Structure Determination

Crystals were harvested and flash-frozen in solutions containing the original growth conditions supplemented with 30% glycerol (RyR1) or 25% isopropyl alcohol (RyR2). Diffraction data were collected at the Canadian Light Source (CLS) beamline 08ID-1 and the Advanced Photon Source (APS) beamline 23-ID-D-GM/CA. Data were processed with
XDS. Initial phases were calculated by Sulfur-SAD with Autosol using native sulfur atoms on the RyR2 SPRY2 WT structure collected at 6.5 keV. The initial structure was further refined against a 1.34 Å dataset using PHENIX and manual model building using COOT. The RyR2 SPRY2 structure was used as a search model for molecular replacement to solve the RyR1 structure and RyR2 SPRY2 A1107M mutant. Table 3.1 contains the statistics for data collection and refinement.

Table 3.1 Data collection and refinement statistics

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<th>RyR1 1070-1246</th>
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<th>RyR2 1080-1253 (SAD)</th>
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3.3.3 Cryo-EM Docking

The ‘colores’ program in the SITUS suite was used to dock the RyR1 SPRY2 domain, containing the C-terminal tail in the antiparallel configuration, into four different RyR1 cryo-EM maps with EMDB entries 1606, 1607, 1275 and 5014. Six dimensional
searches were performed with a rotational sampling of 3 degrees and with off-lattice Powell optimization. No density cutoff was applied, and a Laplacian filter was used throughout.

### 3.3.4 Thermofluor Thermal Shift Assays

Protein stability was measured by fluorescence as described before. 10 μL of 1.5 mg/mL protein was mixed with 5 μL of a 1/500 dilution of SYPRO orange (Invitrogen) and diluted to 50 μL with buffer (150 mM KCl, 10 mM sodium phosphate pH 7.4, 14.3 mM bME). Thermal melts were performed eight fold using a DNA Engine Opticon 2 real-time PCR machine (Biorad) with the SYBR green filter. The temperature was increased from 25°C to 95°C in 0.5°C increments with each step held constant for 15 seconds. Curves were normalized and the midpoints were taken as the melting points.

### 3.3.5 Isothermal Titration Calorimetry

Proteins were dialyzed against 150 mM KCl, 10 mM HEPES (pH 7.4), 10 mM bME and 1 mM NaN₃ at 4°C. Concentrations were determined by the method of Edelhoch. Titrations consisted of 20 injections of 2 μL RyR1 SPRY2 domain into the cell containing either buffer or II-III loop. Concentrations used are noted in the figure legends. Experiments were performed at 25°C and a stirring speed of 1000 rpm on an ITC200 instrument (GE Healthcare). All data were processed using Origin 7.0.

### 3.4 Results

#### 3.4.1 The SPRY2 Domains in RyR1 and RyR2 Consist of Modular Subdomains.

We solved the crystal structures of the SPRY2 domains of two RyR isoforms, encoded by residues 1070-1246 in rabbit RyR1 and 1080-1253 in mouse RyR2. The RyR2
SPRY2 structure was solved via sulphur-SAD phasing, which was then used as a molecular replacement model for the RyR1 SPRY2 domain structure. The final resolutions were 1.84 Å (RyR1) and 1.34 Å (RyR2). Three molecules are present in the asymmetric unit for the RyR1 structure. As these are nearly identical (Figure 3.1), we performed all analyses with chain A.

![Figure 3.1 Superposition of chains A, B and C (blue, green, purple, respectively) of RyR1 SPRY2.](image)

The chains look nearly identical, with RMSD values for Cα atoms corresponding to: A vs B: 0.09Å; A vs C: 0.13Å; B vs C: 0.12Å.

Figure 3.2 shows the overall structures of the SPRY2 domains. It is immediately clear that these consist of several individual structural elements that make them substantially different from previously predicted SPRY2 domain structures. The core of the protein
consists of a stretch of 10 β strands (β₁-β₁₀), arranged in two antiparallel sheets, which was previously assumed to constitute the entire SPRY2 domain. However, at the N-terminus an extension is present, adding a supplementary beta strand (β₁₀) and a short 3₁₀ helix. A 10-residue loop (‘insertion loop’) is inserted in what is normally strand β₈, splitting it up into two smaller strands β₈a and β₈b. C-terminal to the last β strand of the core is a region we named the ‘lid’, containing two 3₁₀ helices and a short β strand that forms β sheet interactions with the first strand of the core. The lid makes substantial hydrophobic interactions with both the N-terminal extension and the core, burying ~820 Å² of surface area. The core, lid and insertion loop are in the exact same position in RyR1 and RyR2 (Figure 3.2C). Because an identical arrangement is found in two structures for which the crystallization conditions and crystal contacts are entirely different, these arrangements appear stable and likely also occur in full-length RyRs.
Figure 3.2 Overall structure of the SPRY2 domain.

A. Two views of the RyR1 SPRY2 domain. Secondary structure elements are labelled. The core of the structure is shown in purple. Extra modules are shown in different colours (blue: N-terminal extension; yellow: lid; cyan: insertion loop; green: tail). B. Two views of the RyR2
SPRY2 domain with colouring and labels as in panel A.  C. Superposition of the SPRY2 domains from RyR1 (colours) and RyR2 (grey).  D. Surface representation of the RyR1 SPRY2 domain with each module in a different color, showing the extensive interactions between the modules.  E. Sequence alignment of the SPRY2 domains in RyR1, RyR2, and RyR3, with the secondary structures indicated. Colours represent the modules. Loop regions with structures are indicated by a solid line. Dotted lines represent parts of the construct that lack interpretable electron density. The red letters indicate positions of disease-causing mutations.

Immediately following this lid is an extended region ('tail'). This unusual conformation in both structures is the direct result of crystal contacts (Figure 3.3A,B). In both RyR1 and RyR2, the tail interacts with the same surface of a neighbouring molecule, although the orientation is different: parallel in RyR1, but forming an additional antiparallel β strand in RyR2. Despite these extensive crystal contacts, the constructs behave as monomeric species using size exclusion chromatography, suggesting that in solution, the tail is more probable to form an intramolecular, rather than intermolecular interaction. Only the antiparallel arrangement can be formed, extending the antiparallel β sheet (Figure 3.3C). A parallel intramolecular arrangement is sterically not allowed due to the limited length of the tail. The tail contains a Trp that forms extensive interactions and that is highly conserved among RyRs (Figure 3.4).
**Figure 3.3 Swapping of the tail region in the crystal lattice.**

Two neighbouring chains in the crystal lattice are shown for the SPRY2 domain of A. RyR1 and B. RyR2, indicating that the tail mediates crystal packing contacts with strand $\beta_0$. In RyR2, the tail extends an antiparallel sheet. C. Likely intramolecular arrangement for the tail in solution, shown here for the RyR2 SPRY2 domain.

| RyR1 Rabbit | VTTWF |
| RyR1 Human | VTTWF |
| RyR2 Mouse | ITMWL |
| RyR2 Human | ITMWL |
| RyR3 Human | VAMWF |
| RyRβ Chicken | VAMWF |
| RyR Drosophila | VTHYW |
| RyR Sea Urchin | MPLHW |

**Figure 3.4 Interactions between the tail and the N-terminal extension strand in RyR2.**

A. Sequence alignment between selected RyR sequences shows the conservation of a Trp residue in the tail. B. Detail of the structure showing that the tail Trp resides in a small pocket formed by the remainder of the SPRY2 domain. The tail peptide (green) extends one of the $\beta$ sheets through interactions with $\beta_0$ of the N-terminal extension. Colours are as in Figure 3.2.
In comparison with SPRY domains from other proteins, several elements appear unique to the SPRY2 domain (Figure 3.5). The closest structural homolog is the SPRY domain from Ash2L, which also contains a ‘lid’, but still misses the insertion loop and contains two extra β sheets that replace the N-terminal and C-terminal extension strands. Two other SPRY domains are present within the RyR sequence. Both share low sequence identity with SPRY2 (~23% and 17% for SPRY1 and SPRY3, respectively) and several insertions and deletions are present (Figure 3.6). Neither of them seems to contain the insertion loop and the sequences within the additional modules are particularly dissimilar, suggesting that the three SPRY domains within RyRs have diverged substantially.
Figure 3.5 Comparison with other SPRY structures.

Shown are superpositions of SPRY2 (colours) with (white) SPRY domains of (A) Trim5α (PDB 4B3N), (B) SPRYD3 (PDB 2YYO) and (C) Ash2L (PDB 3TOJ). The labeled portions indicate parts that are different in the superposed structures. Compared to all existing SPRY structures, the insertion loop, which cuts SPRY2 strand β8 in two halves, is not observed in any other SPRY structure. No other SPRY domain has a C-terminal extension (shown here in the extended conformation, but likely to form an additional beta strand interaction with the SPRY2 core). A lid is observed in Ash2L, but obtains a different conformation. Many other SPRY structures contain an extra β sheet at the N-terminus, also known as a PRY domain that is absent in SPRY2.
Figure 3.6 Sequence alignment of the three predicted SPRY domains in rabbit RyR1.

The shaded regions show strictly conserved residues. The secondary structure of rabbit RyR1 SPRY2 is shown on top for reference. Overall, the sequence identities between the three domains are very low, with multiple insertions and deletions.
3.4.2 Loss-of-Function Mutations in the RyR2 SPRY2 Domain

The SPRY2 domain of RYR2 harbours the position of a very unusual disease mutation (T1107M) that has been linked to hypertrophic cardiomyopathy. Importantly, functional experiments have indicated a loss-of-function phenotype for the T1107M mutant, with a reduction in fractional Ca$^{2+}$ release and an increase in the threshold for its termination. Since most mutations characterized in the cytosolic portions of RyRs are instead linked to gain-of-function phenotypes, we decided to take a closer look at this loss-of-function mutation.

The corresponding residue in the mouse RyR2 (A1107) is buried, with the C$\alpha$ atom pointing towards a hydrophobic core (Figure 3.7A,C). There is space for a Thr at this position, but a Met side chain would completely clash with Trp1156 (Figure 3.8). Surprisingly, the A1107M mutation is tolerated within the individual domain, as indicated by the absence of any aggregation in size exclusion chromatography (Figure 3.9). However, the substitution causes a significant destabilization of the domain, with the melting temperature decreasing ~9°C compared to wild type (Fig. 3.7B). At a physiological temperature of 37°C, ~22% of the domain is unfolded. Although the actual melting temperature will be different within full-length RyR2, where domain interactions may confer additional stability, there is a clear trend towards destabilization of the fold.
Figure 3.7 Disease mutations within SPRY2.

A. Relative positions of disease-causing mutations (black sticks) in the RyR1 and RyR2 SPRY2 domains. Labels indicate the mutation in human RyRs, with the corresponding rabbit RyR1 and mouse RyR2 residue in brackets. B. Thermal melting curves, as determined by thermofluor assays, for WT and mutant SPRY2 domains. The curves are the average of 8 independent measurements. The obtained melting temperatures are (with standard deviations): RyR1 SPRY2: 43.2±0.3°C, RyR2 SPRY2: 47.9±0.3°C, RyR1 R1076W: 40.2±0.5°C, RyR2 A1107M: 39.1±0.2°C. C. Close-up of the superposition of WT (colours) and A1107M SPRY2 (white) in RyR2. Ala1107 is shown in black. The arrows show the relative positional changes. D. Close-up of Arg1076 (black) in RyR1 SPRY2. A Trp side chain (white) at this site would cause a steric clash with neighbouring hydrophobic residues.
Figure 3.8 Clash induced by the A1107M mutation in RyR2 SPRY2.
Shown is the wild type RyR2 SPRY2 crystal structure with the Ala1107 residue replaced by a Met residue. This would cause a steric clash with Trp1156. The Van der Waals surfaces for Met1107 and Trp1156 are shown transparent to highlight the clash.

Figure 3.9 SPRY2 mutants are monomeric.
Superposed size exclusion chromatograms (Superdex200 analytical, GE) for wild-type and disease mutant forms of the RyR1 and RyR2 SPRY2 domains.
We crystallized the corresponding A1107M mutant and solved its structure at 1.44 Å. In order to allow the Met side chain to fit in the hydrophobic core, the backbone of residue 1107 is shifted over 1.6 Å. This shift now results in several changes at the surface. It affects the main chain positions of the neighbouring residues Glu1106 and Val1108, whose side chains adopt different conformations, resulting in positional shifts up to 3.1 Å (Fig. 3.7C). In the wild type structure, Glu1106 forms a salt bridge with Arg1214, but in the mutant this salt bridge is lost and the electron density for the Arg1214 is absent, indicating that this residue has become flexible. In conclusion, the mutation induces a local misfolding resulting in surface changes at low temperatures, and has a substantial degree of unfolding at 37°C. These combined effects likely underlie the loss-of-function phenotype associated with this mutation.

3.4.3 Mutations in the RyR1 SPRY2 Domain

The RyR1 SPRY2 domain contains locations for 5 disease mutations (Fig. 3.7A). This includes R1075W (human numbering), identified in two patients from one family who died shortly after birth. Muscle biopsies indicated central core disease (CCD). The residue is strictly conserved, and is located at the end of the N-terminal extension strand (β₀), making multiple interactions with neighbouring residues. The stalk portion of the side chain is in hydrophobic contact with several hydrophobic residues (Fig. 3.7D), whereas the positively charged guanidinium group is exposed to the surface.

In a model of the RyR1 SPRY2 domain where the tail is added in the antiparallel, intramolecular arrangement, the Arg1075 is also in contact with the tail (Figure 3.10). Without substantial rearrangements, there would be no room for a bulkier Trp side chain,
even in the absence of the tail strand. We tested the impact of the corresponding R1076W mutation in the rabbit RyR1 SPRY2 domain. The protein can still fold, as it does not cause aggregation as measured by size exclusion chromatography (Figure 3.9). However, thermofluor experiments show that the mutation destabilizes the domain, reducing the melting temperature by ~3°C (Fig. 3.7B). Although the effect on thermal stability is thus smaller than for the A1107M mutation in RyR2, the RyR1 SPRY2 domain is already less stable than in RyR2, and the melting temperature of the R1076W mutant SPRY2 is very similar to the RyR2 A1107M SPRY2. Our attempts to crystallize the mutant failed, suggesting that the structural rearrangements are substantial. Based on these observations, we predict that the human R1075W mutant may lead to a loss-of-function phenotype, consistent with CCD.
The human RyR1 disease mutation R1075W corresponds to position Arg1076 in rabbit RyR1. This residue (black) is part of strand $\beta_6$ in the N-terminal extension (blue) and forms interactions with the core (purple) and the tail (green, modelled for the RyR1 SPRY2 domain based on the antiparallel interaction seen for the RyR2 SPRY2 domain). A. and B. The structure without and with the tail, respectively. Both in the absence and presence of the tail, several residues would have to rearrange to accommodate the bulky Trp side chain.

Two other mutations in the RyR1 SPRY2 domain have also been identified in patients with CCD. However, in both cases, an additional mutation was found, so the phenotype could be due to either one or their combination. The G1165D is located in a turn
preceding strand $\beta_{8a}$ in a region that interacts with the lid module (Figure 3.7A and 11). An Asp residue at this position is expected to interfere with the interactions, as it would cause clashes with 2 hydrophobic residues of the lid (Figure 11). The mutation is therefore highly likely to affect function of RyR1. In contrast with this, the R1179W mutation is exposed at the surface of the protein, and is thus not expected to interfere with folding (Figure 3.7A). It can only be responsible for a disease phenotype if it is located at an interface with an auxiliary protein or another domain.

Figure 3.11 Effect of the G1165D disease mutation.

The human RyR1 mutation G1165D corresponds to residue Gly1166 in rabbit RyR1, and is located at the interface between the SPRY2 core (purple) and the lid module (yellow). A simple introduction of an Asp residue would cause clashes with hydrophobic residues (Phe1213 and Ile1216) in the lid. The modelled aspartic acid is shown in black sticks, with its Van der Waal's surface transparent.

In addition, two mutations in the SPRY2 domain have been linked to malignant hyperthermia, a disorder linked to RyR1 gain-of-function.\textsuperscript{49,297} The R1127H and R1140C
mutations (human numbering) are both exposed at the surface of the protein (Figure 3.7A). The substitutions are not predicted to create any steric hindrance, and therefore would only affect the surface properties of the domain. Similar to the R1179W mutation, they can only create a disease phenotype if they are located at an interface. Finally, the P1144L mutation was found in exome sequencing but it is unclear whether it is causative of MH. Its corresponding residue is a Ser in the rabbit RyR1 sequence, and is exposed at the surface.

In order to consider the further impact of these disease mutations, we explored the potential role of the SPRY2 domain by analysing its proposed binding and its location in full-length RyRs.

### 3.4.4 Does the RyR1 SPRY2 Domain Form an Interaction Site with the II-III Loop?

Several studies have supported a role for the RyR1 SPRY2 domain in direct interactions with the cytosolic II-III loop of Ca\textsubscript{v}1.1. With a very soluble and well-behaved RyR1 SPRY2 domain in hand, we tested its ability to bind the II-III loop using isothermal titration calorimetry (ITC). However, even at high concentrations, the heat signals were indistinguishable from background titrations (Figure 3.12A), indicating that any binding between the two, if at all, would be extremely weak (K\textsubscript{d} > 1 mM), or proceed with extremely small enthalpic changes. However, pull-down experiments we performed also did not indicate any interaction (results not shown).
Figure 3.12 Absence of interactions between the RyR1 SPRY2 domain and the CaV1.1 II-III loop.

A. Raw ITC data comparing the titrations of RyR1 SPRY2 (887 µM) into buffer or the II-III loop (89 µM), showing no significant difference in heat signals. B. Size exclusion chromatograms (analytical Superdex200) of MBP fusions of RyR1 1070-1246 and 1085-1208. Removal of the MBP tag from the 1085-1208 construct led to complete loss of the protein. C. Structure of the RyR1 SPRY2 domain, highlighting residues 1076-1112 (human numbering, green) and the ‘F-loop’, encoded by residues 1107-1121 (human numbering, orange). Alternating colours show regions of overlap. Both are involved in extensive interactions with the remainder of the domain and are unlikely to adopt a native conformation in isolation. D. Detail of the RyR1 F-loop (orange), indicating its hydrophobic side chains in green sticks. These interact extensively with hydrophobic residues on the rest of the SPRY2 domain (purple sticks).

Previous biochemical experiments were performed using only the ‘core’ of the SPRY2 domain, consisting of residues 1085-1208 in RyR1.\textsuperscript{120,285,286} This represents the major module in the structure, but upon expression and purification of such a construct, it proved to be poorly soluble and caused major aggregation as indicated by size exclusion.
chromatography (Fig. 3.12B). This aggregation is observed even despite the presence of a stabilizing maltose binding protein (MBP) fused at the N-terminus. Proteolytic removal of the MBP (see Methods) caused complete loss of the protein. Even constructs in which only the N-terminal extension or lid was deleted separately appeared to be misfolded. This is in agreement with the extensive amount of hydrophobic surface that is buried by these extra modules.

As such, any previous studies using only the SPRY2 core or portions thereof are likely to yield unfortunate false positives because these missed major elements required for stability of the SPRY2 domain. We conclude that the RyR1 SPRY2 domain is unlikely to be a major determinant of the RyR1-CaV1.1 interaction. As a result, the disease-causing mutations also are unlikely to affect binding to CaV1.1 directly. We next explored a possible role for the SPRY2 domain by analyzing its location.

### 3.4.5 Location of the SPRY2 Domain in Full-length RyR1

We attempted to locate the position of the RyR1 SPRY2 domain in available cryo-EM maps of full-length RyR1. We used an unbiased approach, employing systematic 6-dimensional searches (3 rotational and 3 translational parameters) as implemented in Situs.291 We analysed and ranked the cross-correlation coefficients for each of the tested locations. As noted before, the addition of a Laplacian filter appeared crucial to obtain sensible docking results.92 In three of the tested maps (EMDB accession numbers 5014, 1606, and 1607)69,131, the top hit consistently docked to the same location and, reassuringly, in the same orientation (Figure 3.13A). Plotting the correlation coefficients of the top hits shows that there is some contrast, especially in the EMD5014 map where the top hit is separated by the mean of the next 9 hits by up to ~8 standard deviations.
Figure 3.13 Location of the SPRY2 domain in intact RyR1

A. Top docking solutions for the RyR1 SPRY2 domain in three different cryo-EM maps of full-length RyR1. The colours for the SPRY2 domain are as in Figure 3.2. The positions of the N-terminal three domains (blue, green, red) are also shown for reference. The bar graphs below show the normalized correlation coefficients for the top 10 unique hits in each map. Due to the four-fold symmetry, each solution comes up 4 times, but is only shown once in the bar graphs. The filled circles in the top left panel represent the locations for difference density observed for RyR2-GFP insertions near RyR2 residues 846 (yellow circle) and 1366 (orange circle). The purple dotted circle represents the area where difference densities were observed for cryo-EM studies of anti-SPRY2 antibody bound to RyR1. B) 'side view' of the docked SPRY2 domain, showing its solvent accessibility at the periphery of the RyR (arrow). C) Close-up showing that 2 solvent channels (arrows) line the docked SPRY2. The loop encoded by residues 1107-1121 is shown in orange. It is accessible from both solvent channels.

Searches in a fourth map (EMDB 1275) only yielded nonsense positions, such as the column positions, transmembrane area, and the position where the N-terminal area was
found to be located. As expected with nonsense solutions, there was no contrast between the top hit and the following solutions (Figure 3.14).

Figure 3.14 Docking of the RyR1 SPRY2 domain into the RyR1 cryo-EM map with EMDB entry 1275.

A. and B. 2 views of the RyR1 cryo-EM map (mesh). The positions of the N-terminal three domains, which have been validated, are shown in black. The different coloured domains correspond to the top 10 ranked docking results. Because of the 4-fold symmetry, each hit would appear 4 times, but is only shown once for clarity. All top 10 solutions dock to nonsense regions located either within the central columns, or clashing with the N-terminal domains. None of these are compatible with previous GFP insertion studies in areas close to the SPRY2 domain. C) Normalized correlation coefficients for these top 10 unique hits. There is no large ‘contrast’ between these hits, which is typical of spurious docking results.

Because there are two other SPRY domains within the RyR polypeptide, there is the inherent possibility that the top hit could correspond to either SPRY1 or SPRY3. In the
absence of crystal structures for these, this cannot be formally tested, but docking of homology models in the EMDB 5014 map yielded entirely different positions. In addition, docking of other known SPRY structures also yielded different positions, suggesting that there is sufficient resolution in the maps to discriminate between proteins of the same fold.

We therefore propose the location shown in Figure 3.13 as the most likely position for the SPRY2 domain. This setting is strategically located between the corner, an area shown to undergo large conformational changes during opening and closing\textsuperscript{69}, and the central rim, formed by the N-terminal three domains establishing a ‘gating ring’ that is thought to modulate channel open probability.\textsuperscript{97} The SPRY2 domain is likely required to couple the conformational changes in both areas, and disease-causing mutations on its surface may interfere with this process.

Importantly, this location is in agreement with several previous studies. First, several antibodies raised against RyR1 SPRY2 have been shown to access their epitopes in native, folded RyR1.\textsuperscript{88} In the proposed dock, the SPRY2 domain is accessible via 2 solvent channels and from the periphery (Figure 3.13B,C). Cryo-EM reconstructions of antibody-bound complexes showed difference density in the clamp region, in an area compatible with our proposed SPRY2 position, especially given the \textasciitilde160Å dimensions of antibodies (Figure 3.13A, left panel, dotted circles). One of the antibodies was raised against residues 1107-1121, and this loop is accessible via 2 solvent channels. Second, several cryo-EM studies have been performed on GFP fusions of RyR2. In one of these, GFP was inserted after RyR2 residue Thr1366, \textasciitilde110 residues downstream of the SPRY2 sequence, and difference density was shown in subregion 6.\textsuperscript{84} Another study inserted GFP near residue Tyr846, \textasciitilde200 residues upstream of the SPRY2 domain, yielding difference density in the top portion of subregion 9.\textsuperscript{299} So although no GFP insertion studies have been reported for the SPRY2 domain,
insertion studies in the neighbouring domains within the sequence appear compatible with the proposed SPRY2 position, which is located directly in between these two difference densities (Fig. 3.13A, left panel).

3.5 Discussion

3.5.1 RyR SPRY2 Contains Modules that are Required For Folding and Not Present in Other SPRY Domains.

The Ryanodine Receptor is a large allosteric membrane protein. Thanks to the presence of multiple domains located in the cytosolic cap, this membrane protein giant can receive input from tens of proteins and small molecules. One type of domain, the so-called ‘SPRY’ domain is repeated three times in the RyR sequence, and these are conserved from mammalian species all the way down to Drosophila and C. elegans. However, up to now, no structure from any SPRY domain of any RyR had been reported. Here we show high resolution crystal structures of the SPRY2 domain of both RyR1 and RyR2. Contrary to previous predictions, this domain consists of several distinct modules (N-terminal extension, lid, insertion loop, and tail) that add to what was previously thought to contain the SPRY2 domain. At least two of these modules, the N-terminal extension and lid, are absolutely required for proper folding of the domain.

3.5.2 Mutations in SPRY2 Induces Local Misfolding or Alter Interdomain Interactions.

RyRs are the target for disease-causing mutations that mostly result in Malignant Hyperthermia or Central Core Disease for RyR1 or in Catecholaminergic Polymorphic Ventricular Tachycardia for mutations in RyR2. A common theme is that most mutations
on the cytosolic cap have been linked to a gain-of-function, resulting in premature or prolonged release of Ca\textsuperscript{2+} into the cytosol. The SPRY2 structures allowed us to map six mutations. Interestingly, they segregate into two groups, with three mutations either fully or partially buried within the domain, and three fully exposed at the surface. The buried T1107M mutation targets RyR2 and has been linked to hypertrophic cardiomyopathy\textsuperscript{293}, as well as CPVT.\textsuperscript{303} It is unclear how it could result in two different phenotypes, but functional experiments have clearly shown it to confer a rare loss-of-function phenotype with early termination of Ca\textsuperscript{2+} release.\textsuperscript{294} The mutation causes a significant thermal destabilization, resulting in 22% unfolding at physiological temperatures. In addition, it induces local misfolding, which results in conformational changes at the surface. Although the folding in intact RyR2 can be stabilized, the 9°C shift in melting temperature stability suggests a trend towards significant destabilization, suggesting that misfolding of the SPRY2 domain may generally lead to loss-of-function phenotypes.

Two RyR1 mutations (G1165D, R1075W) also affect partially buried residues. Interestingly, both of them have been linked to CCD, a disorder that can be associated with loss-of-function phenotypes in RyR1. Although both mutations were also found in conjunction with mutations elsewhere in RyR1, we predict they interfere with proper folding and either contribute or are fully responsible for the disease phenotype.

Three other mutations are found at the surface. Two of these have been linked to MH, a disorder characterized by gain-of-function of RyR1, and another has been linked to CCD. Since CCD can be linked to both gain and loss of function, a possible interpretation here is that mutations interfering with folding of SPRY2 create loss-of-function, whereas mutations on the surface create gain-of-function phenotypes. For the three mutations at the surface to be disease-causing, they have to lie at an interface with an auxiliary protein or
other domain. In order to identify what this might be, we analysed its interaction with a proposed binding partner and its location in full-length RyR.

3.5.3 The RyR1 SPRY2 Domain Binding to the Ca\textsubscript{v}1.1 II-III Loop is Undetectable by Isothermal Titration Calorimetry.

In the absence of a high-resolution structure, it was previously assumed that the SPRY2 domain only consisted of a smaller region, encoded by human RyR1 residues 1085-1208. However, it is now clear that the SPRY2 domain consists of additional portions that add ~43\% of extra sequence, and our data show that the shorter construct causes aggregation, in stark contrast with the high solubility and monomeric nature of the crystallized 1070-1246 construct. In the presence of these additional modules, our ITC experiments fail to recapitulate the interaction with the Ca\textsubscript{v}1.1 II-III loop, indicating that the SPRY2 domain cannot be the major determinant of the RyR1-Ca\textsubscript{v}1.1 interaction. Previous experiments also suggested that an even smaller portion of the SPRY2 domain, encoded by residues 1076-1112, could bind to the II-III loop.\textsuperscript{119} However, it is clear from the structure that such a fragment would not be able to fold in its native conformation, as it isolates single \(\beta\) strands from two different \(\beta\)-sheets (Figure 3.12C). Other experiments have further narrowed down the interaction to a loop encoded by residues 1107-1121, and showed that this peptide, in isolation, can affect the activity of RyR1 in planar lipid bilayers.\textsuperscript{118} However, it is clear that this loop, which previously was thought to be extended,\textsuperscript{285} makes substantial interactions with the remainder of the structure, burying hydrophobic side chains (Fig. 3.12D). The loop is highly unlikely to be adopting this stabilized conformation in isolation. Most likely, the interactions between RyR1 and Ca\textsubscript{v}1.1, whether direct or via an intermediate
protein, involve one or multiple RyR1 segments that have previously been suggested to be important for E-C coupling.\textsuperscript{249-251,304}

A popular method for studying RyR structure-function is the use of isolated RyR peptides and to measure their effect on RyR function. It was shown that the ‘F-loop’, encoded by RyR1 residues 1107-1121, could increase the activity of RyR1 in planar lipid bilayers.\textsuperscript{118} However, this loop forms extensive hydrophobic interactions with the remainder of the SPRY2 domain (Figure 3.12D), and is thus very unlikely to adopt a native structure in isolation. It is unclear then why such a peptide could cause a functional effect. The same is true for a peptide in the N-terminal region of RyR2 (residues 163-195), which was shown to increase leak of Ca\textsuperscript{2+} through RyR2,\textsuperscript{305} but is largely an integral part of the β-trefoil core of domain A\textsuperscript{91,96}, showing that it is impossible to adopt the same conformation in isolation. It may simply be that RyRs are sensitive to several unfolded peptides that do not necessarily mimic a native structure found in intact RyRs.

3.5.4 SPRY2 is a Domain in the Clamp Region of RyR

Obtaining a high-resolution structure of the entire RyR has thus far been unsuccessful. The next best thing is to obtain high-resolution structures of individual domains or domain clusters, and then to locate these by finding the best position in full-length RyR cryo-EM maps. RyR1 reconstructions are available in the 10-12 Å range\textsuperscript{69,74-76}, and these have allowed locating the N-terminal three domains of RyR1 with high accuracy to the central rim.\textsuperscript{92,97} Thanks to the larger size, locating a three-domain structure appeared straightforward, with high contrast between the correlation coefficient of the top hit compared to the next hits in the ranking. The position of the N-terminal region was also confirmed via difference cryo-EM and FRET measurements.\textsuperscript{98} However, with single-domain
structures, the contrast is typically lower.\textsuperscript{93} Locating the SPRY2 domain yielded consistent results among three out of four RyR1 cryo-EM maps (Figure 3.13A). Given that the refined positions appear to adopt the exact same relative orientation of the domain in all three maps, we propose this location as the most likely site for the SPRY2 domain.

There currently is no report of any cryo-EM study using the SPRY2 domain as a position for insertions. However, the proposed location of the SPRY2 domain is in agreement with previous cryo-EM studies, which used insertions of GFP ~200 residues upstream\textsuperscript{299} or ~110 residues downstream\textsuperscript{84} of the SPRY2 domain. The proposed location of SPRY2 is directly in between the two difference densities (Figure 3.13A, left panel). In addition, cryo-EM reconstructions of RyR1 bound to antibodies raised against the SPRY2 domain are also consistent with this location.\textsuperscript{88} One antibody was raised against the ‘F-loop’, which is accessible in the docked SPRY2 via two solvent channels. However, some structural changes may be necessary to allow access to the antibodies, which may explain why most of the RyR1 particles did not have antibodies bound to each subunit simultaneously.\textsuperscript{88} Interestingly, antibodies raised against the SPRY2 domain were found to increase RyR1 activity in planar lipid bilayers, which would be expected if conformational changes are required for antibody binding.

Importantly, this location is directly in between the corner region and the central rim. Both areas have been shown to undergo large conformational changes during channel opening.\textsuperscript{69,97} The N-terminal three domains have been shown to form a ring near the four-fold symmetry axis\textsuperscript{92}, and opening of the channel is coupled to disruption of an intersubunit interface.\textsuperscript{97} The corner positions have been shown to move ‘downward’ towards the SR membrane during channel opening.\textsuperscript{69} The SPRY2 domain, together with other domains, is likely involved in linking these transitions and the disease-causing mutations may then
interfere with this process. One of the contacts seems to involve the insertion loop, a loop that cuts strand β₈, normally found in canonical SPRY folds, into two smaller strands. Interestingly, two of the mutations located at the surface (R1179W,R1140C) flank this loop, and may thus interfere directly with this domain-domain interaction.

The identities of the domains directly contacting SPRY2 remain to be found. Importantly, the RyR sequence contains two additional predicted SPRY domains. Homology models for these domains did not dock to the proposed SPRY2 location, which is not surprising given their low degree of sequence identity with SPRY2 (23% and 17% for SPRY1 and SPRY3, respectively), and the multiple insertions and deletions (Supplementary Fig. S4). Similarly, we have previously shown that the cryo-EM maps can discriminate between domains A and B, which both form β-trefoil folds with twelve β strands each. Despite the same fold, and a near perfect superposition of the strands, the loop regions of these domains largely determine the overall shape, which determines their docked location.

In order to further confirm the SPRY2 location, FRET experiments and further difference cryo-EM studies will be invaluable for experimental verification and to suggest possible interacting domains.
Chapter 4 – Future Directions and Conclusions
Ever since the Ryanodine Receptor was first cloned it has proven to be a major challenge for structural. Due to its large size, teasing apart the intricate web of interactions between its domains and modulators is a tough puzzle to solve. In addition, its membrane protein nature and size make it a difficult protein to overexpress and purify.

Studying the modulation and control of RyR has been of interest because its dysfunction leads to multiple diseases. Currently, the mechanism of action of triggers that activate RyRs, such as volatile anaesthetics, or compounds that can inhibit the channel, such as dantrolene, are not known. In a small protein, one can assay for a functional endpoint or try to detect binding via biochemical methods such as fluorescence, NMR, calorimetry and surface plasmon resonance amongst others. For RyRs, most of these methods prove impossible due the limited availability of recombinant protein. Even with known ligands that modulate the channel it has been a challenge to locate their exact binding sites. Initial attempts have been made to overcome this limitation by expressing portions of the channel with fusion proteins. Although this methodology is quick and easy, it is undoubtedly prone to false positives. As no structural data was available, and often no quality control of the proteins is performed, these fusion proteins may have contained only parts of domains. The resulting misfolded protein constructs then often expose hydrophobic regions that would otherwise be buried, causing non-specific interactions with several RyR modulators.

A more elegant method that has been used in other membrane proteins that can be used on RyR is through its limited proteolysis. Some domains that are part of the receptor are inherently stable and treatment with proteases can provide soluble domains. Sequencing of these fragments can then lead to the identity of these islands of protein stability.

Even with the current domain structures of the N-terminal region, phosphorylation domain and SPRY2, the binding sites of RyR ligands, including: Ca\(^{2+}\), Mg\(^{2+}\), ruthenium red,
FKBP12/12.6, ATP, dantrolene, ryanodine, caffeine and DHPR II-III loop are still not known. Sites that have previously been proposed have also never been independently confirmed. Calmodulin remains one exception where there is both structural and biochemical evidence. However, the field is still debating about its exact binding site almost 20 years after the discovery of its role in RyR modulation.

4.1 Calmodulin Can Bind More than One Site, but What Site?

The work presented in this thesis covered the binding analysis of three separate CaMBDs that are located within the RyR. The rationale was to provide clear biochemical evidence of either binding or absence of binding by CaM to these regions. There has been great controversy within the field as to the number of sites that CaM might act on within RyR. Although cryo-EM suggests that 4 CaM molecules bind to equivalent sites within an intact channel, previous reports have shown that there are up to 9 different possible binding sites.

I determined that indeed more than one site could bind both apoCaM and Ca\(^{2+}/\text{CaM}\). Importantly, there are differences between CaM binding to homologous regions of different isoforms, suggesting that these could underlie the different regulation of RyR1 and RyR2. Although it is well established now that the 3614-3643 site, or CaMBD2, is an important site, it does not exclude the potential contributions of other CaMBDs. A key question is now whether the identified CaMBDs can also bind within the context of intact channels. In isolation, peptides and RyR domains that contain CaMBDs are made accessible and can interact with CaM. However, within the folded channels these sites may not be available. This is already apparent in the phosphorylation domain, which contains three putative CaMBDs, none of which are accessible when mapped on the structure. During the
preparation of Chapter 2, I tested CaM binding to this domain which could not be detected by ITC, in agreement with the fact that they are buried within the domain.\textsuperscript{93}

One way to answer this question is through the use of purified channels. Recombinant RyR channels with mutations or deletions in CaMBDs can be generated. By making specific mutants within CaMBDs that prevent CaM binding, we can eliminate known sites (CaMBD2) and examine the contributions to binding by other CaMBDs. Furthermore, mutations can also be made in CaMBD3 to analyze its contribution to CaM binding, since it is the strongest apoCaM binder and because it is adjacent to a region that has recently been shown to be surface exposed.\textsuperscript{236} These channels would then have to be purified in high quantity for binding experiments through ITC or a complementary technique like SPR. However, the yields from heterologous expression are typically very low, so an alternative option is to monitor the functional effect of CaM on the CaMBD mutants. Using planar lipid bilayer electrophysiology, measurements of RyR channel opening and closing can be observed.

Due to RyR’s high affinity for both apoCaM and Ca\textsuperscript{2+}/CaM one could try photo-crosslinking experiments to further localize its binding sites. Affinity tagged-CaM could be labelled with widely available compounds such as biotin-hydrazone and bound to RyR at different Ca\textsuperscript{2+} levels. Regions close to CaM would be cross-linked to it upon UV light exposure. The channel can then be subjected to limited proteolysis and the fragments that are cross-linked to CaM enriched using the biotin affinity tag. CaM is extremely stable even in the presence of proteases. The fragment can then be sequenced by mass spectrometry to provide a map of nearby residues. This would further aid the localization of CaM.
4.2 Calmodulin CPVT Mutants and CaMBDs

Recently, CaM missense mutants, two of which are N98S and N54I, have been isolated that cause CPVT.\textsuperscript{57} There are conflicting reports showing that the CPVT mutants have either impaired Ca\textsuperscript{2+} binding or improved affinity compared to WT-CaM.\textsuperscript{56,57} Functionally, the mutants become stimulatory instead of inhibitory towards RyR at high Ca\textsuperscript{2+} levels. This presents a conundrum as it poses the question as to whether CaM’s binding affinity to RyR2 translates to either an activating or inhibiting effect. To test whether binding affinity has changed due to the CaM mutants, a recent undergraduate, Stephanie Ng, and I have cloned and expressed the CaM mutants and their associated lobes (N-lobe for N54I and C-lobe for N98S).

In preliminary experiments, we could not detect a difference in binding affinity to CaMBD2 between the mutants versus the wild-type. However, further follow-up experiments are needed. If binding affinity is affected, it would be critical to determine the binding mode to CaMBD2. As our mutational analysis with CaMBD2 revealed, Ca\textsuperscript{2+}/N-lobe is able to bind both of the anchor residues. It has also been proposed that the lobes themselves may be responsible for the different effects of CaM: the C-lobe is inhibitory while the N-lobe is activating.\textsuperscript{225} CPVT mutations in CaM may be affecting the way the lobes are interacting with CaMBD2’s anchor points. As one group also reported no difference in mutant CaM binding affinity to CaMBD2, the mutations may instead be tuning CaM’s affinity for the other CaMBDs.

4.3 Calmodulin and S100A1

It is accepted that at high Ca\textsuperscript{2+} levels CaM inhibits and S100A1 enhances RyR activity. Both of these proteins are also thought to bind the same regulatory site, CaMBD2. Little
work has been done to further understand how these proteins, both of which have high affinities for CaMBD2, can compete for the same site. A criticism of current S100A1 experiments with CaMBD2 is that they do not use the entire region, but just a fragment that contains a canonical S100 binding sequence. What is crucial is the fact that S100A1, as shown from NMR structures, only binds via the Trp in CaMBD2 as the Phe anchor is not present. Using ITC, CaMBD2 mutants that lack the Trp or Phe anchor could be used to test if S100A1 prefers binding to the Trp as observed by NMR. How the two proteins are able to compete for the same site can also be tested through competition experiments where S100A1 or CaM is pre-incubated with CaMBD2 and is titrated with the competing protein to determine the apparent affinity. Finally, an alternative possibility is that both proteins can bind simultaneously, with the S100A1 binding via the Trp and CaM binding through the Phe.

4.4 Do CaMBDs Serve as Internal EF-Hand Binding Sites?

An overlooked component in CaM modulation of RyR is the functional role of the predicted EF-hands in the RyR. Classically, these domains have been thought of as simple Ca^{2+} sensors that would undergo conformational changes upon binding Ca^{2+} increasing RyR channel activity. Only recently has it been proposed that EF-hands may transduce this effect through more CaM-like means. The EF-hands are predicted to be folded and functional and bind CaMBD2. Two reports suggest an interplay between the EF-hands, CaM and CaMBDs. They suggest that the interactions between EF-hands and CaMBD2, depending on channel state, promote either its inhibition or activation. CaM competition with the EF-hands for CaMBD2 would disrupt this link. In addition, the EF-hands are capable of binding to additional CaMBDs in other proteins.
A previous graduate student in the lab, Paolo Lobo, has tested an EF-hand construct in RyR1, 4071-4138, and tested binding to the CaMBD3LRR mutant. Surprisingly, in high Ca\(^{2+}\) the EF-hand showed binding to the peptide with a \(K_d \sim 7 \mu\text{M}\). I have now cloned and purified additional EF-hand constructs in RyR2 and RyR3 for further biochemical characterization. The proteins are highly soluble but do not crystallize. Presently, others are attempting to determine the structure through mutagenesis to reduce the flexibility of surface residues and to eliminate charged residues that may reduce protein solubility. Further binding experiments of the EF hands with CaMBDs are also forthcoming.

4.4 SPRY1 and 3 Domains of RyR

With the successful determination of the structure of SPRY2, another significant piece of the RyR puzzle has been obtained. SPRY2 being one of three repeats can provide clues in solving the other two domains. Structural alignments of SPRY2 with the predicted stretches of SPRY1 and SPRY3 reveal large sequence differences within the N-terminus region and the loops connecting \(\beta\)-strands. (Figure 3.6) These differences in structural elements suggest that multiple approaches may need to be used to crystallize these remaining domains. As there are insertions in loops, which extend long stretches in SPRY2, constructs that delete portions of the loops predicted to be highly disordered may aid in crystallization.

The N- and C- terminus structural elements, which are novel in the SPRY2 structure, may also be present in the SPRY1 and SPRY3 domains. Evolutionarily it is much more likely that the three repeats were all derived through duplication from an initial SPRY domain. It is possible that through time significant changes have occurred in these modules due to the different pressures faced by SPRY1 and SPRY3. However, the structural feature with the addition of a short \(\beta\) strand and an \(\alpha\) helix at the N-terminus and a lid covering hydrophobic
residues at the C-terminus may still be present in these SPRYs. New constructs that have variable termini may aid in finding a well-behaved domain amenable to crystallization.

### 4.5 Tandem Domains

Having defined boundaries of a well-behaved SPRY2 construct, one can systematically add increasing amounts of sequence to either terminus to attempt to generate larger constructs. These tandem domains are beneficial in many ways. First, RyRs are built up by independent domains that can have intricate interdomain interactions and understanding their arrangement in the superstructure is crucial. An example is the N-terminal region, consisting of three domains that adopt a compact triangular arrangement. Second, having a larger construct increases the reliability of docking into cryo-EM maps. Although SPRY2 was placed with a high docking contrast for a protein of its size, there are concerns that proteins with similar folds (ie. SPRY1 or SPRY33) may also dock to the same location. With Laplacian filtering, the edges of an image or body are enhanced. The more discrete a protein structure is, the more reliable the dock. For example, a small globular protein with limited surface features may resemble many domains observed on a cryo-EM map resulting in a low docking contrast. A small protein with a unique extension or a large multi-domain construct would provide enough details to fit the contours of a map more reliably. Finally, larger constructs may provide the necessary interdomain surfaces that mediate the plethora of RyR interactions that have not been confirmed. In the case of SPRY2, a larger construct may allow for detection of binding to the DHPR II-III loop by ITC.

Attempts have been made to extend SPRY2 further from both ends. Holding the C-terminus fixed, I have extended it N-terminally to include the first predicted tandem repeat
and SPRY1 domain that are present before SPRY2 to create Repeat1-SPRY2 or SPRY1-Repeat1-SPRY2 constructs (Figure 1.3). The region C-terminal to SPRY2 is predicted to have the propensity to form a small domain of approximately 50-70 amino acids that is composed of \( \beta \)-strands. I have systematically added residues in groups of 10. Beyond this subdomain lies a region of divergence, divergent region 2 (DR2). DR2 may play a role in EC coupling, DHPR II-III loop binding and tetrad formation.\(^84,251,310\) I have attempted to express this region alone but have been unsuccessful at obtaining well-behaved pure protein. I am now extending SPRY2 into the DR2 region with the hope of obtaining a stable longer construct.

The next domain after DR2 is SPRY3 and attempts are also being made into successfully expressing a SPRY2-DR2-SPRY3 construct. If this is unsuccessful an interesting possibility to generate a deletion mutant is available. In RyR3, which is expressed widely in all tissues, there is no DR2 suggesting that it is not crucial at least in this isoform. Constructs in RyR3 or deletions of the DR2 region in RyR1 and RyR2 may be possible in producing a soluble SPRY2-SPRY3 tandem domain construct.

### 4.6 FRET Localization of SPRY2

A recent development in the field is using FRET to confirm the location of docked atomic structures of RyR.\(^311\) Based upon a docking, His insertions into the flexible loops of a protein domain are made in full-length RyR. The His insertions can bind to an NTA dye which can participate in FRET with a donor fluorophore attached to a protein at a known location on RyR such as FKBP.\(^311\) Through the FRET efficiencies and the known locations of the donor fluorophore, distance restraints can be calculated to triangulate the location of the acceptor on the domain. The docking solution provides several locations on SPRY2 that
may be amenable to the insertion of a His sequence. We are currently planning for insertions
to be placed in loops facing the two solvent channels (Gly1185 and His1127) according to
our proposed SPRY2 docking site, as well as an insertion in a loop exposed to the lateral
surface (Arg1212).

4.7 Conclusions

The work here has only shone a small laser light on to the vast Ryanodine Receptor.
Using ITC, thermodynamics of binding of CaM have now been described to three different
potential CaMBD on the channel. Importantly, it reveals a possible new apoCaM binding
site that is the target for disease-causing mutations. It remains to be seen what the roles are
for each of these sites in regulating RyR.

The SPRY2 structure from RyR1 and RyR2 provides the first evidence that it may
play a role in channel gating as it is located between the central vestibule formed by the N-
terminal region (domains ABC) and the clamp region. The lack of binding to the DHPR II-
III loop also suggests that the interaction is located elsewhere or requires a surface that is
formed by multiple domains.
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