LUMINESCENCE RESONANCE ENERGY TRANSFER-BASED MODELING OF TROPONIN IN THE PRESENCE OF MYOSIN AND TROPONIN/TROPOMYOSIN
DEFINING MYOSIN BINDING TARGET ZONES IN THE RECONSTITUTED THIN FILAMENT

Dipesh A. Patel, B. S.

Dissertation Prepared for the Degree of
DOCTOR OF PHILOSOPHY

UNIVERSITY OF NORTH TEXAS
May 2009

APPROVED:

Douglas Root, Major Professor
Robert Benjamin, Committee Member
Kent D. Chapman, Committee Member
Rebecca Dickstein, Committee Member
Pamela Padilla, Committee Member
Arthur J. Goven, Chair of the Department of Biological Sciences
Michael Monticino, Interim Dean of the Robert B. Toulouse School of Graduate Studies

Mechanistic details on the regulation of striated muscle contraction still need to be determined, particularly the specific structural locations of the elements comprising the thick and thin filaments. Of special interest is the location of the regulatory component, troponin, on the actin filament and how its presence influences the behavior of myosin binding to the thin filament. In the present study: (1) Luminescence resonance energy transfer was used to monitor potential conformational changes in the reconstituted thin filament between the C-terminal region of troponin T and myosin subfragment 1; (2) Location of troponin in previously derived atomic models of the acto-myosin complex was mapped to visualize specific contacts; and (3) Shortened tropomyosin was engineered and protein binding and ATPase assays were performed to study the effect of myosin binding close to the troponin complex.

Analysis of the results suggest the following: (1) Irrespective of calcium levels, the C-terminal region of troponin T is located close to myosin loop 3 and a few actin helices that may perturb strong acto-myosin interactions responsible for force production. (2) Atomic models indicate myosin subfragment 1 cannot attain the post-powerstroke state due to the full motion of the lever arm being sterically
hindered by troponin. (3) A shortened tropomyosin with five actin binding modules (instead of the native seven in muscle cells) binds actin contiguously in a head-to-tail manner and serves to increase the periodicity of troponin complexes on the actin filament. Such behavior eliminates the structure of the actin filament being responsible for the binding location of tropomyosin. (4) Differential behavior of myosin subfragment 1 i.e. (a) binding adjacent to troponin and (b) binding further away from troponin, is apparent as tropomyosin and troponin appear to govern the regions or “target zones” where myosin can bind productively along the actin filament. Physiologically, myosins able to bind close to troponin, but not participate in force production may function as mechanical sensors to attenuate or dampen the force generated from the so-called “target zones”. Therefore, this could be a pseudo-regulatory mechanism that functions to protect the contractile apparatus from damage.
Copyright 2009

by

Dipesh A. Patel
ACKNOWLEDGEMENTS

I owe my success to Dr. Douglas Root, my mentor and advisor who has given me tremendous support and without whom this project would not have been possible. His immense commitment to science and research along with his knowledge has inspired me in my work. I would also like to thank Dr. J.-P. Jin from the Molecular Cardiology Department at Northwestern University, for providing the opportunity to gain vital experience in his lab and for his boundless support and advice. I express my deepest gratitude to him, without whom this project would not have been completed.

Additionally, I greatly appreciate the support and mentoring received by my doctoral committee members: Dr. Robert Benjamin, Dr. Kent Chapman, Dr. Rebecca Dickstein, and Dr. Pamela Padilla. I would like to thank Dr. Zhiling Zhang for preparing chicken troponin T. I would also like to recognize all my co-workers, colleagues, family and friends in supporting me throughout the project.

Finally, I gratefully acknowledge the funding agencies responsible for the success of this project: National Institutes of Health (NIH), National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) and the American Heart Association (AHA).
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS........................................................................................................ iii
LIST OF TABLES................................................................................................................ viii
LIST OF FIGURES............................................................................................................ ix
ABBREVIATIONS............................................................................................................... xi

## CHAPTER

1  INTRODUCTION............................................................................................................. 1
   1.1 Muscle Contraction and Ultrastructure................................................................. 1
   1.2 Model of the Contraction Mechanism: ATPase Cycle and Calcium Regulation................................................................. 8
   1.3 ATP Hydrolysis Transition State Analogues......................................................... 12
   1.4 Objective............................................................................................................. 15
   1.5 Broader Applications.......................................................................................... 20

2  MATERIALS AND METHODS.................................................................................... 23
   2.1 Isolation and Purification of Skeletal Myosin, Actin, Troponin and Tropomyosin................................................................. 23
   2.2 Synthesis of the Tb$^{3+}$ Chelate and its Attachment to Chicken Troponin T............................................................................... 23
   2.3 Reconstitution of Skeletal Muscle Contractile Proteins................................. 24
   2.4 Preparation of Myosin Subfragment-1 by α-Chymotrypsin or Papain and its Purification................................................................. 25
   2.5 Labeling of Myosin S1 with Acceptor Fluorophores........................................ 26
2.6 Isolation and Acceptor Labeling of the RLC and Exchange into Papain S1 .........................................................28
2.7 Exchange of Chicken TnT-chelate with Endogenous TnT in Rabbit Skeletal Myofibrils......................................................29
2.8 Preparative Equipment ..................................................................................................................................................31
2.9 Sample Preparation and LRET Data Collection ..............................................................................................................32
2.10 LRET Data Analysis: Curve Fitting and Calculation of Distance Values .............................................................................34
2.11 3-D Mapping of C-TnT into Acto-Myosin Atomic Models .....35
2.12 Conformational Search of the Tb$^{3+}$ Chelate Bound to Chicken Troponin T ................................................................37
2.13 Engineering of α-Tropomyosin Deletion Mutant ..................38
2.14 Enzyme Linked Immunosorbant Assay (ELISA) of Muscle Proteins .........................................................................................41
2.15 F-actin Cosedimentation Assays ............................................43
2.16 Myosin S1 ATPase Assay ..........................................................43
2.17 S1 ATPase Data Analysis ..........................................................44
2.18 Confocal Microscopy of Labeled Rabbit Skeletal Myofibrils .........................................................................................46

3 EXPERIMENTAL STRATEGY .........................................................47

3.1 Fluorescence and Competing Processes ....................................47
3.2 Principle of Fluorescence Resonance Energy Transfer (FRET) and Förster’s Theory .............................................................50
3.3 Measuring FRET via Steady State Methods ..............................60
3.4 Measuring FRET via Time-Resolved Fluorescence Methods ............................................................................................64
3.5 Advantage of Time-Resolved Fluorescence ...............................67
3.6 Luminescence Resonance Energy Transfer (LRET) and
Lanthanide Probes........................................................................68
3.7 Lanthanide Chelates..................................................................71
3.8 Summary of Measuring FRET/LRET.........................................75
3.9 Conformational Searching of the Tb\(^{3+}\) Chelate.................75

4 RESULTS...................................................................................82
4.1 Labeling of Myosin S1 and RLC with Acceptor
Fluorophores.............................................................................82
4.2 LRET in the Reconstituted Thin Filament and Myofibrils:
Emission and Lifetime Decay Curves........................................86
  4.2.1 LRET-Derived Distances between Donor and
    Acceptor Probes: A) S1-Cy5-ATP..............................90
  4.2.2 B) S1-TMR............................................................92
  4.2.3 C) S1-FHS.............................................................93
  4.2.4 D) S1-RLC-AmrB.................................................93
  4.2.5 E) Myofibril-Cy5-ATP...........................................94
4.3 Myosin Binds Preferentially near Troponin............................95
4.4 3-D Mapping of C-TnT in Three Acto-Myosin Atomic
    Models................................................................................98
4.5 Tb\(^{3+}\) is Held in Close Proximity to Troponin T...............105
4.6 Binding Assays of Tropomyosin, Troponin and F-actin......107
4.7 Cosedimentation Assay of dAc23 Tropomyosin.................115
4.8 Myosin S1 ATPase Assay....................................................120
4.9 Fluorophore Labeling of Rabbit Myofibrils.........................126

5 DISCUSSION............................................................................128
5.1 The Effect of Labeling Myosin S1........................................128
5.2 Atomic Models suggest Interaction between Myosin Loop 3
5.3 Troponin may Sterically Hinder Completion of the Myosin Powerstroke.................................135
5.4 Tropomyosin and Troponin Determines Myosin Binding Target Zones along the Actin Filament...........141
5.5 Myosin Binding adjacent to Troponin may Function to Attenuate the Overlap of Thick and Thin Filaments in Muscle Contraction.......................................................145
5.6 Justification of using dAc23 Tropomyosin and Effect of removing other Actin Binding Modules.....................147
5.7 Relationship between Tropomyosin Actin Binding Sites and Actin Monomers.........................................................150
5.8 Cy5-ATP Localizes with the Myosin Thick Filament........151
5.9 Implications of Myosin Binding near Troponin...............154
5.10 Potential Future Projects.................................................157
5.11 Conclusion........................................................................158

REFERENCES.............................................................................160
LIST OF TABLES

1. LRET-derived distances in myosin S1 and myofibrils.................................91
2. Comparison of distances from each acto-myosin atomic model................102
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Myosin and the muscle ultrastructure</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Myosin ATPase cycle</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>Calcium regulates muscle contraction via Tn and Tm</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>A double-stranded cDNA map of chicken skeletal α-Tm</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>Overlap of the Tb$^{3+}$ donor emission spectrum and acceptor excitation/emission spectra</td>
<td>52</td>
</tr>
<tr>
<td>6</td>
<td>The exponential relationship between distance and efficiency of energy transfer</td>
<td>57</td>
</tr>
<tr>
<td>7</td>
<td>Eliminating unwanted fluorescence signals</td>
<td>63</td>
</tr>
<tr>
<td>8</td>
<td>Time-domain method of measuring fluorescence lifetime</td>
<td>66</td>
</tr>
<tr>
<td>9</td>
<td>The photophysics of chelates</td>
<td>73</td>
</tr>
<tr>
<td>10</td>
<td>15% SDS-PAGE of purified and labeled myosin S1</td>
<td>83</td>
</tr>
<tr>
<td>11</td>
<td>Atomic model of labeled myosin S1</td>
<td>85</td>
</tr>
<tr>
<td>12</td>
<td>Emission spectra of Tb$^{3+}$-labeled Chicken TnT</td>
<td>87</td>
</tr>
<tr>
<td>13</td>
<td>Fluorescence lifetime decay of Tb$^{3+}$-labeled chicken TnT</td>
<td>89</td>
</tr>
<tr>
<td>14</td>
<td>Tn fraction located close to acceptor-labeled myosin S1</td>
<td>97</td>
</tr>
<tr>
<td>15</td>
<td>Location of C-TnT in three acto-myosin atomic models</td>
<td>99</td>
</tr>
<tr>
<td>16</td>
<td>Potential contact between myosin S1, TnT and actin</td>
<td>104</td>
</tr>
</tbody>
</table>
17. Lowest energy structure of the Tb\textsuperscript{3+} chelate................. 106
18. CH1 antibody titration with Tm............................................................... 108
19. Cardiac TnT binding to Tm................................................................. 110
20. Tm binding to F-actin................................................................. 112
21. cTnT binding to F-actin-Tm............................................................ 114
22. Cosedimentation of F-actin with WT and dAc23 Tm......................... 116
23. Saturating F-actin with each Tm form........................................... 118
24. ATPase phosphate standard curve............................................... 121
25. S1 ATPase activity with WT Tm reconstituted with actin and Tn......... 123
26. S1 ATPase activity with dAc23 Tm reconstituted with actin and Tn...... 124
27. Localization of Cy5-ATP and Alexa 647-ATP in myofibrils.............. 127
28. Effect of Tm on the alignment of Tn along the actin filament............. 144
29. Myosin binding close to Tn.............................................................. 156
ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>( \lambda_{\text{max}} )</td>
<td>Absorbance maximum at wavelength, ( \lambda )</td>
</tr>
<tr>
<td>ABTS</td>
<td>2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AlF₄</td>
<td>Aluminum fluoride</td>
</tr>
<tr>
<td>AmrB</td>
<td>Aminorhodamine B</td>
</tr>
<tr>
<td>A-band</td>
<td>Anisotropic band</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>( \alpha )-Tm</td>
<td>( \alpha )-tropomyosin</td>
</tr>
<tr>
<td>BD</td>
<td>Brownian Dynamics</td>
</tr>
<tr>
<td>BeFx</td>
<td>Beryllium fluoride</td>
</tr>
<tr>
<td>( \beta )-me</td>
<td>( \beta )-mercaptoethanol</td>
</tr>
<tr>
<td>B_max</td>
<td>Binding maximum</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CCD</td>
<td>Cooled coupled device</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CM</td>
<td>Carboxymethyl</td>
</tr>
<tr>
<td>CS124</td>
<td>Carbostyril 124, 7-amino-4-methyl-2(1H)-quinolinone</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxyl terminal of a protein</td>
</tr>
<tr>
<td>C-TnT</td>
<td>Carboxyl terminal of TnT</td>
</tr>
<tr>
<td>cTnT</td>
<td>Cardiac TnT</td>
</tr>
<tr>
<td>Cy5-ADP</td>
<td>Cyanine 5-conjugated adenosine diphosphate</td>
</tr>
<tr>
<td>Cy5-ATP</td>
<td>Cyanine 5-conjugated adenosine triphosphate</td>
</tr>
<tr>
<td>dAc23 Tm</td>
<td>Deleted actin binding sites 2 and 3 of Tm (mutant Tm)</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential Interference Contrast</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DTNB</td>
<td>5', 5'-dithiobis-(2-nitrobenzoic acid), Ellman’s reagent</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E</td>
<td>Efficiency of energy transfer</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglycoltetraacetic acid</td>
</tr>
<tr>
<td>( \varepsilon_{\lambda} )</td>
<td>Molar extinction coefficient at a specific wavelength (( \lambda ))</td>
</tr>
<tr>
<td>ELC</td>
<td>Essential light chain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbant Assay</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>( E_{\lambda_{\text{max}}} )</td>
<td>Maximum emission wavelength</td>
</tr>
<tr>
<td>ES</td>
<td>Enzyme-Substrate complex</td>
</tr>
<tr>
<td>( E_T )</td>
<td>Total enzyme concentration</td>
</tr>
<tr>
<td>E-64</td>
<td>Trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FHS</td>
<td>Fluorescein-5(6)-carboxamidocaoric-N-hydroxysuccinimide</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>HMM</td>
<td>Heavy meromyosin</td>
</tr>
<tr>
<td>I-band</td>
<td>Isotropic band</td>
</tr>
<tr>
<td>J</td>
<td>Overlap integral</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>( k^2 )</td>
<td>Orientation factor</td>
</tr>
<tr>
<td>( k_1 )</td>
<td>Association rate constant</td>
</tr>
<tr>
<td>( k_2 )</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>( k_{\text{cat}} )</td>
<td>Catalytic rate constant</td>
</tr>
<tr>
<td>( K_d )</td>
<td>Dissociation equilibrium constant</td>
</tr>
<tr>
<td>( K_m )</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>LD</td>
<td>Langevin Dynamics</td>
</tr>
<tr>
<td>LMM</td>
<td>Light meromyosin</td>
</tr>
<tr>
<td>LRET</td>
<td>Luminescence resonance energy transfer</td>
</tr>
<tr>
<td>MCMM</td>
<td>Monte Carlo Molecular Mechanics forcefield</td>
</tr>
<tr>
<td>MD</td>
<td>Molecular Dynamics</td>
</tr>
<tr>
<td>MDa</td>
<td>Megadalton</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>( \eta )</td>
<td>Refractive index</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino terminal of a protein</td>
</tr>
<tr>
<td>NTPase</td>
<td>Nucleotide triphosphatase</td>
</tr>
<tr>
<td>( \Phi_D )</td>
<td>Relative emission intensity of the donor</td>
</tr>
<tr>
<td>OPLS-AA</td>
<td>Optimized Potential for Liquid Simulations – All Atom forcefield</td>
</tr>
<tr>
<td>OS</td>
<td>Oxygen scavenging</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate ion</td>
</tr>
<tr>
<td>PIPES</td>
<td>Piperazine-1,4-bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>P-loop</td>
<td>Phosphate binding loop</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>PRCG</td>
<td>Polak-Ribiere Conjugate Gradient energy minimization method</td>
</tr>
<tr>
<td>( Q_D )</td>
<td>Donor quantum yield ((\tau_{\text{da}}/\tau_d))</td>
</tr>
<tr>
<td>( Q_{\text{Ln}} )</td>
<td>Lanthanide quantum yield</td>
</tr>
<tr>
<td>( Q_{\text{Total}} )</td>
<td>Total quantum yield</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>Q_{Transfer}</td>
<td>Quantum yield of energy transfer</td>
</tr>
<tr>
<td>R</td>
<td>Distance between donor and acceptor pair</td>
</tr>
<tr>
<td>R_0</td>
<td>Critical transfer distance at which E is 50%</td>
</tr>
<tr>
<td>RLC</td>
<td>Regulatory light chain</td>
</tr>
<tr>
<td>R_{rms}</td>
<td>Root mean square</td>
</tr>
<tr>
<td>S</td>
<td>Substrate</td>
</tr>
<tr>
<td>SD</td>
<td>Stochastic Dynamics</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>S1</td>
<td>Subfragment 1 of myosin</td>
</tr>
<tr>
<td>S2</td>
<td>Subfragment 2 of myosin</td>
</tr>
<tr>
<td>Tb^{3+}</td>
<td>Terbium ion</td>
</tr>
<tr>
<td>\tau_d</td>
<td>Lifetime decay signal of the donor only</td>
</tr>
<tr>
<td>\tau_{da}</td>
<td>Lifetime decay signal of the donor in presence of acceptor (quenched donor) is also equal to the long-lived apparent lifetime of the acceptor in presence of the donor (sensitized emission)</td>
</tr>
<tr>
<td>Tm</td>
<td>Tropomyosin</td>
</tr>
<tr>
<td>TMR</td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td>5-TMRIA</td>
<td>Tetramethylrhodamine-5-iodoacetamide dihydroiodide</td>
</tr>
<tr>
<td>Tn</td>
<td>Troponin, complex includes TnT, TnI and TnC</td>
</tr>
<tr>
<td>TnC</td>
<td>Troponin C</td>
</tr>
<tr>
<td>TnI</td>
<td>Troponin I</td>
</tr>
<tr>
<td>TnT</td>
<td>Troponin T</td>
</tr>
<tr>
<td>Tris</td>
<td>Trishydroxymethylaminomethane</td>
</tr>
<tr>
<td>Vi</td>
<td>Vanadate ion</td>
</tr>
<tr>
<td>WT Tm</td>
<td>Wild-type tropomyosin</td>
</tr>
<tr>
<td>XRC</td>
<td>X-ray crystallography/crystallographic</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Muscle Contraction and Ultrastructure

Myosins, kinesins and dyneins comprise the three major eukaryotic molecular motor families responsible for ATP (adenosine triphosphate)-dependent movement. These so called ATPases (adenosine triphosphatases) function in a variety of eukaryotic cell processes including cytokinesis, cell cargo trafficking and organelle transport in the case of kinesins and dyneins binding to microtubules, while myosins bind to actin filaments to produce force via muscle contraction (Leipe et al., 2002). Other important cellular functions that depend on these motors include cytoskeletal dynamics, cell motility, morphogenesis and signal transduction. Here, we focus on myosin, a diverse superfamily comprised, to date, of about 20 classes that function in organisms including invertebrates, vertebrates and plants. The fact that molecular motors can generate movement is attributed to their ability to bind and hydrolyze ATP, which remarkably results in the amplified movements often visualized in a microscope at the cellular level, or more obviously when animals move at the macroscopic level. ATPases are thus a class of enzymes that catalyze the conversion of ATP to ADP (adenosine diphosphate) and Pi (inorganic phosphate ion).
The free energy from the phosphoanhydride bond can be utilized in several ways. Perhaps the most obvious is its use in driving biochemical reactions that would otherwise be thermodynamically unfavorable.

The energy of ATP can be used to induce conformational rearrangements in the protein, where the nucleotide binding initiates for example, pumping of ions in transport ATPases, polymerization of actin and tubulin monomers into their respective filaments, or even sliding of thick and thin filaments in muscle contraction. Finally, the free inorganic phosphate cleaved from ATP can be transferred by a protein kinase to another target macromolecule mediating its activation/inactivation (Vetter and Wittinghofer, 1999). The fact that nucleotides in general bind to their respective NTPases (nucleotide triphosphatases) is attributed to a common sequence motif called the phosphate binding loop or P-loop (Saraste et al., 1990). Initially identified in the α- and β-subunits of the F$_1$-ATPase, it is also termed the Walker A motif (Walker et al., 1982), which for the purine (adenine and guanine) nucleotide binding proteins at least, is an eight amino acid consensus sequence (Gly/Ala-X-X-X-Gly-Lys-Ser/Thr). This is therefore used to identify new nucleotide binding proteins (Bairoch et al., 1997). The structure of this nucleotide binding motif, first observed in adenylate kinase, forms a loop between an α-helix and N-terminal β-strand (Schulz et al., 1974) stabilized by several hydrogen bonding interactions via a conserved lysine (Dreusiche et al., 1988). Though many purine nucleotide binding proteins e.g. thymidine kinase (Fry et al., 1986), elongation factor Tu (Berchtold et al., 1993),
G-proteins (Coleman et al., 1994) and even myosin (Rayment et al., 1993a) demonstrate a wide array of functions, they remarkably show a similar three dimensional structure of the P-loop despite differences in the amino acid sequence (Smith and Rayment, 1996b).

Of specific interest are the class II myosins responsible for the contraction of three types of muscle: smooth, skeletal and cardiac. Microscopic inspection of smooth muscle reveals a lack of visible striations due to the irregular packaging of the muscle fibers, while the more uniform arrangement in skeletal and cardiac muscle results in a striated appearance. The striations are formed due to the relatively less dense actin thin filaments and more dense myosin thick filaments, which, under a conventional light microscope appear as light (Isotropic, I-bands) and dark (Anisotropic, A-bands) regions, respectively. Though the focus here is on the skeletal muscle system, all three muscle types possess the myosin II class molecule - a heterotrimeric dimer i.e. two molecules each comprised of a heavy chain, essential light chain (ELC), and regulatory light chain (RLC). The N-terminal of the heavy chain forms the head domain and interacts with actin, while the C-terminal tail is responsible for the formation of bipolar thick filaments (Goodson and Spudich, 1993) (Figure 1A).
Figure 1. Myosin and the muscle ultrastructure. A) The myosin II molecule is comprised of a two-headed structure held together via the long tail portion. The ELC and RLC stabilize the lever arm and help regulate myosin activity. Fragmentation by trypsin yields heavy meromyosin (HMM, 350 kilodaltons, kDa) and light meromyosin (LMM, 150 kDa), while further digestion via papain or chymotrypsin yields single-headed subfragment 1 (S1, ~110 kDa), with both light chains bound and only the ELC present, respectively. S2, subfragment 2.
Some non-myosin components in the thick filament include myosin binding protein H, C, X and titin, with each playing a specific structural role in the muscle ultrastructure. For example, myosin binding protein C functions to maintain thick filaments in bundles of 200-400 molecules and is located in the middle third of each half of the A-band, while titin spans the sarcomere from the M- to the Z-line and is thought to direct assembly of contractile elements and provide elasticity (Houmeida et al., 1995). The main binding partner for myosin that allows muscle contraction to occur is the actin filament. This filament is formed by an array of actin monomers that assemble into a single (proto) filament, two of which then intertwine to form a right-handed coiled-coil, thus forming a groove near which tropomyosin (Tm) binds (Holmes et al., 1990). Both Tm and troponin (Tn) comprise the regulatory proteins that mediate muscle contraction and along with the actin filament constitute the thin filament (Gordon et al., 2000). Tm, is also a coiled-coil filament comprised of a dimer arranged in head-to-tail fashion i.e. the N-terminal of one dimer binds to the C-terminal of the next and so on, with each dimer long enough to bind about 7 actin monomers (Ebashi et al., 1969). Tn however, assumes a more globular shape and is a complex composed of three proteins: TnT, TnI and TnC (Greaser and Gergley, 1971) that binds along the actin filament about every 7 actin monomers. The arrangement of the thick and thin filament proteins in a typical skeletal muscle, otherwise known as the ultrastructure is shown in Figure 1B and 1C.
Figure 1B. The region between two Z lines, the sarcomere, comprises the basic contractile unit (typically 2.2 µm at rest). Actin thin filaments (blue) are anchored via their plus end at the Z lines and emanate in a bipolar fashion towards the myosin thick filaments (green) where they terminate at the minus end. Myosin heads protrude from the thick filament in a bipolar fashion where they are anchored about the M-line. Note, for clarity, the myosins are drawn as single heads, but actually occur as two headed structures. The region of the sarcomere that contains only thick filaments is called the H-zone, within which resides a bare-zone that contains only myosin tails and no myosin heads. The I-band refers to the non-overlapping actin filaments, while the A-band refers to the thick filament including the region that overlaps the actin filaments. The regulatory components, Tm (yellow) and Tn (red) operate to mediate acto-myosin interactions. Contraction occurs when actin activated myosin head ATPase activity allows for strong binding to the actin filament followed by conformational changes that causes the sliding of the filaments past one another.
Figure 1C. Myosin heads protrude in a helical fashion such that each head is separated by 14.3 nm and the axial repeat distance is 42.9 nm. Also, note how Tm wraps around the actin filament covering myosin binding sites on the actin molecules.
1.2 Model of the Contraction Mechanism: ATPase Cycle and Calcium Regulation

At the molecular level, muscle contraction can otherwise be described as the interaction between myosin thick and actin thin filaments (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954). More specifically, it occurs as a result of the cyclic formation and disruption of myosin crossbridges with the actin filament, which also correlates with the hydrolysis of ATP (Figure 2).
Figure 2. Myosin ATPase cycle. Myosin bound to the actin filament in the absence of nucleotide (rigor) detaches once ATP (orange star) binds to the nucleotide binding site. ATP hydrolysis to ADP + Pi causes a structural change in the myosin favoring a weak binding interaction with the actin filament. Ejection of the hydrolysis products Pi (yellow circle) and ADP (red square) strengthens the binding between the two filaments and causes the swinging of the lever arm or powerstroke to proceed. Here, the actin filament slides in the direction opposite (straight dashed arrow) to the motion of the lever arm (curved dashed arrow), before the myosin assumes the rigor state. The continuous cycling of ATP binding, hydrolysis and product release leads to the maximum overlap between thick and thin filaments and thus force production.
While contraction of muscles results in the production of force, which is typically associated with locomotion, such events are only productive if they are initiated and terminated at the appropriate time. In other words, this regulation of contraction is mediated by the calcium ion (Ca$^{2+}$) concentration in muscle cells, where an increase in Ca$^{2+}$ allows more myosin heads to bind the actin filament. The regulatory process can be more clearly explained at the molecular level, which involves a series of conformational changes by the regulatory proteins Tn and Tm. Beginning in the absence of Ca$^{2+}$, a segment of TnI, the inhibitory component of Tn binds actin and blocks the acto-myosin interaction. Then upon initiation of contraction by Ca$^{2+}$ ions, the Ca$^{2+}$ transducer segment (switch) of TnI shifts away from actin and binds the N-terminal of Ca$^{2+}$-activated TnC (Pearlstone et al., 1997; Perry, 1998). Thus, in an accepted model of thin filament regulation (McKillop and Geeves, 1993) where TnT contacts Tm, TnI and TnC (Gordon et al., 2000; Stefancsik et al., 1998) and where, TnI with Tm blocks myosin binding sites on actin (Van Eyk et al., 1997), Ca$^{2+}$ relieves the attachment of the TnI inhibitory segment on actin, which in turn, allows Tm to uncover more myosin binding sites and facilitate cooperative binding of myosin to the actin filament (Ebashi et al., 1969; Gorga et al., 2003) (Figure 3).
Figure 3. Calcium regulates muscle contraction via Tn and Tm. A cross section of the thin filament proteins, actin, TnT, TnI, TnC and Tm show how myosin binding to actin is regulated. TnI functions to bind a region of the myosin binding site on actin (black circle), however, this inhibition is relieved once calcium binds TnC. This causes a series of structural rearrangements in TnI and TnT, which results in an azimuthal shift or roll of Tm. The movement of Tm closer to the groove formed between the two actin protofilaments uncovers additional myosin binding sites to allow contraction to occur (not shown). The motion of proteins is shown by dashed arrows.
Though many studies collectively appear to have deciphered how for example myosin binds to the actin filament and how muscle contraction is regulated, specific details are still unclear and thus need to be addressed as section 1.4 describes.

1.3 ATP Hydrolysis Transition State Analogues

As described in the previous section, myosin catalyzes the hydrolysis of ATP accompanied with a series of transitional structural states governed by the specific catalysis intermediate products present. For instance, when the initial binding of ATP to myosin occurs, the strong rigor interaction between myosin and actin is relieved with ATP still intact, suggesting that nucleotide binding triggers a conformational change that lowers the affinity of myosin for actin. Then hydrolysis of the ATP terminal phosphate (γ-phosphate) to ADP.Pi results in a “cocking” of the lever arm, with the products still complexed within the myosin active site. This state has a much higher affinity for actin (Rayment et al., 1996) thus rebinding of myosin to actin accelerates the loss Pi. It is this phosphate release that is thought to trigger the conversion of the pre-powerstroke state to the post-powerstroke state, ultimately leading to force generation (White and Taylor, 1976; Siemankowski et al., 1985; Goldman, 1987). Next, the dissociation of ADP causes myosin to complete the ATPase cycle and return to the rigor conformation. Thus, in all, five myosin intermediate or transitional structural states have been described in relation to the actin filament: 1) actin.myosin (rigor:
ADP release, no nucleotide), 2) myosin.ATP, 3) myosin.ADP.Pi, 4) actin.myosin.ADP.Pi (pre-powerstroke) and 5) actin.myosin.ADP (post-powerstroke). These intermediate myosin states that are also tightly coupled to muscle contraction, can fortunately be studied with a class of chemicals called phosphate analogues. This provides a way to further characterize the intermediate states and their role in the regulatory process of muscle contraction.

Phosphate analogues have previously been shown to be effective phosphate substitutes that help stabilize specific myosin transition states, that otherwise would be too short-lived to obtain meaningful data from (Chabre, 1990). More specifically, aluminum and beryllium fluoride (AlF₄ and BeFₓ, respectively) (Phan and Reisler, 1992; Phan et al., 1993; Werber et al., 1992; Kagawa et al., 2004; Finnazi et al., 1994; Bigay et al., 1997) and vanadate ion (ViO₄⁻³) (Goodno, 1979; Muhlrad et al., 1991; Smith and Rayment, 1996a) have successfully been used to mimic the ATP γ-phosphate in a variety of studies. In particular, the half-life of the pre-hydrolysis (myosin.ATP) and post-hydrolysis state (myosin.ADP.Pi) is increased to where structural analysis is possible (Reynoso Jr. et al., 2000). Here, potential conformational changes can be compared in addition to the post-powerstroke state (actin.myosin.ADP), thus providing important structural information in the fully reconstituted thin filament system. Before discussing the effects of these phosphate analogues in further detail, it is first necessary to summarize the first relevant X-ray crystallographic (XRC) myosin structures used to visualize the cross-bridge cycle intermediate
states. The first myosin structure was solved using chicken skeletal myosin, which included both the ELC and RLC (Rayment et al., 1993a). The second structure originated from Dictyostelium discoideum, a slime mold, in which the lever arm and light chains were genetically truncated at residue 762 (Fisher et al., 1995). The third structure from chicken smooth muscle included the ELC, but not the RLC (Dominguez et al., 1998). Finally, the structure of striated scallop muscle S1 was solved with both light chains present (Houdusse et al., 1999).

Analysis of these structures along with other studies on skeletal muscles (Phan and Reisler, 1992), Dictyostelium discoideum (Gullick et al., 1997), smooth muscles (Maruta et al., 1993) and scallop (Houdusse et al., 2000), reveal three conformational states thought to be present during the myosin ATPase cycle: 1) in the absence of nucleotide the rigor state is induced, 2) addition of BeF₆ to ADP induces the myosin·ATP (pre-hydrolysis) state and 3) addition of AlF₄ or Vi to ADP induces the myosin·ADP·Pi state. However, discrepancies in matching these kinetic states with the appropriate phosphate analogue are apparent when using smooth muscle myosin. In this case ADP·BeF₆ and ADP·AlF₄ induce an identical smooth muscle myosin structure (Dominguez et al., 1998) corresponding to the ADP·Pi bound state observed in Dictyostelium discoideum complexed with ADP·AlF₄ or ADP·Vi (Smith and Rayment, 1996a). Reasons for such structural discrepancies are thought to be attributed to different crystallization methods including the ionic strength (Peyser et al., 2001) and the trapping of different forms of the S1 in the crystal lattice. For instance, the
myosin.ATP and myosin.ADP states may co-exist in S1 complexes with nucleotide and phosphate analogues present due to a low energy barrier favoring a dynamic equilibrium between the two states (Reynoso Jr. et al., 2000). This may occur in the structure of scallop myosin.ADP because it resembles the myosin.ATP structure (Houdusse et al., 1999). Other factors that can cause induction of either the pre-hydrolysis, post-hydrolysis or possibly even a novel myosin conformation include: the type of phosphate analogue, the use of different recombinant or truncated myosin isoforms, the presence or absence of light chains, and the presence or absence of the full complement of sarcomeric proteins (Kraft et al., 2005; Dantzig et al., 1992).

In the present study, both BeFx and AlF₄ are used in conjunction with ADP to mimic the transition states of ATP binding and hydrolysis to ADP.Pi. The effect of the phosphate analogues in increasing the half-life of these myosin intermediates then makes it feasible to investigate and monitor structural changes occurring between the induced intermediates of the ATPase cycle. The spectroscopic method used to detect potential structural changes is discussed in more detail in the following section and Chapter 3.

1.4 Objectives

Biochemical and computational structural studies have revealed insights into the mechanism of force generation in skeletal muscle, but specific details on the location and orientation of Tn on the actin filament and associated
conformational changes in response to calcium and myosin binding remain to be
determined. Despite the recent completion of the human cardiac (Takeda et al.,
2003) and chicken skeletal Tn (Vinogradova et al., 2005) crystal structure
providing vital structural data, several missing segments have made it difficult to
establish exact contacts with actin, Tm and within each component of the Tn
complex. While a variety of studies have shown or suggested the location of Tn
on the actin filament via 3-D reconstruction using electron micrographs (Flicker et
al., 1982; Lehman et al., 2001; Narita et al., 2001; Murakami et al., 2005; Pirani
et al., 2006) and fluorescence resonance energy transfer (FRET) methods
(Kimura et al., 2002; Kimura et al., 2008; Ferguson et al., 2003; Sun et al., 2006),
more detailed information is required on the position and orientation of Tn in
response to calcium and myosin binding to the regulated thin filament. To date,
various XRC models have been used to study the structural relationship between
various sites in the acto-myosin complex (Holmes et al., 2004; Labbé et al.,
1993; Labbé et al., 1995; Schröder et al., 1993). However, domain shifts of the
heavy chain, flexible surface loops and modeling errors makes XRC an
inaccurate method to suggest specific binding interactions (Root, 2002a).
Arguably, the most significant concern of XRC protein structures is that they may
not represent the true physiological form due to the conditions used to prepare
them and/or because of innate properties of the protein/amino acids that yield
poor crystals for X-ray diffraction. A similar problem is apparent in electron
microscopy (EM) studies where derived structures suffer from poorer resolution
than XRC models. In addition, because of the limiting factor imposed by the inherent structure of the thin filament with Tn bound at every seventh actin monomer, EM cannot accurately determine the shape, orientation, position and contact sites involving proteins (Flicker et al., 1982; Lehman et al., 2001; Milligan and Flicker, 1987). Consequently, there is a need to provide a more accurate high resolution 3-D atomic model showing the exact position of the Tn complex, more so upon myosin binding to the reconstituted thin filament.

In order to understand the physical protein interactions fundamental to the regulatory mechanism of striated muscle contraction, an alternative to the conventional optical techniques must be employed. The expected spatial resolution from such methods are, at best, about 0.2 µm as governed by the diffraction limit, so the proximity between proteins must be determined more accurately by another higher resolution method. A tool that allows for sub-nanometer resolution, while also providing a physiological environment under which proximity relationships between specific sites can be studied is FRET. FRET is a spectroscopy-based technique and phenomenon based on the radiationless transfer of energy between an excited state donor fluorophore (or fluorescent probe) and a second acceptor probe that are both often chemically attached to a specific site on the molecule(s) of interest. The technique is highly sensitive to the distance between the probe pairs and can typically resolve distances on the order of 1-10 nm - ideal for monitoring critical nanoscale structural changes. In addition, a wide range of applications have utilized this
technology including the investigation of membrane ion channels, protease activity, calcium metabolism, fluorescence imaging within live cells and has even claimed a niche in high-throughput screening assays, such as quantification of gene expression. The principles of this technique are discussed in more detail in section 3.1.

The distances reported in atomic models (via XRC studies) of actin and acto-myosin (Holmes et al., 1990; Milligan et al., 1990; Lorenz et al., 1993; Rayment et al., 1993b) correlate well with most of the corresponding distances measured by FRET. In particular, previous FRET studies have revealed the distance between specific sites on actin/TnT (Kimura et al., 2002), actin/Tn (Miki et al., 1998a) and actin/Tm (Miki et al., 1998b, 2004), but measurements between other protein pairs are lacking. With the purpose of identifying new, yet significant sites for FRET labeling that could potentially suggest novel acto-myosin interactions, analysis of the Tn and myosin XRC structure revealed a specific site on Tn and several on myosin that warranted further investigation. In particular, the extent of contacts exhibited by TnT suggests it may play an essential role in communicating Ca\(^{2+}\) dependent structural changes (initiated at TnC after Ca\(^{2+}\) binding) to Tm. The atomic structure of the Tn complex reveals a location at the C-terminal end of TnT (C-TnT) that is juxtaposed to TnC and TnI, which would serve as an ideal donor probe location to potentially monitor the movement of all three regulatory components of the Tn complex. Three acceptor sites on myosin S1 were selected that represent key regions well known to
influence myosin function: the ATPase site, the actin binding site and the junction of the lever arm and head domain (converter domain).

In the grand scheme, we investigate: 1) the potential dynamic changes of the C-terminal region of TnT and/or those labeled myosin S1 sites upon binding of myosin S1 to the reconstituted thin filament, in the presence and absence of calcium using luminescence resonance energy transfer (LRET), a modified version of FRET. Specifically, three kinetic states of myosin S1; i.e., the rigor, pre- and post-powerstroke state and distances between donor/acceptor pairs are analyzed. 2) The effect of calcium on the structural relationship between the myosin head and Tn bound to actin, where the three-dimensional position of C-TnT is visualized using previous acto-myosin atomic models derived by XRC (Rayment et al., 1993a; Rayment et al., 1993b; Holmes et al., 1993), EM (Lorenz et al., 1993), and computational docking (Mendelson and Morris, 1997). 3) The likely conformation of the donor probe attached to a modified XRC Tn complex (Colowick and Kaplan, 1982) using computational simulations. 4) The effect of Tn on myosin S1 binding to the actin filament by constructing a deletion mutant Tm to increase the periodicity/density of Tn along the actin filament.

This study presents significant findings that provide: 1) more accurate physiological measurements versus XRC/EM studies, 2) an atomic resolution view of the position of C-TnT with respect to myosin S1 in the regulated thin filament, 3) insight into the specific amino acids involved in potentially critical interactions with implications on the contraction mechanism, 4) evidence that Tn
and Tm dictate where myosin binds productively on actin thin filaments, perhaps implying a novel regulatory function.

### 1.5 Broader Applications

Collectively, this research will help lay the foundation for the development of diagnostic tools to identify muscle defects. Besides evaluating genetic profiles from affected individuals to confirm the presence of a muscle disease-causing mutation, the FRET/LRET technique could be applied to a muscle biopsy sample from which a molecular model can be generated and compared with that from an unaffected individual. Though initially numerous measurements would be required to develop such a model, once in place, only a few measurements would be required on a biopsy sample to conclude any structural deviation compared with the unaffected muscle model.

One of the long-term goals for the muscle research community is to generate a universal 3-D model of the acto-myosin filaments using all the available research data, which can ultimately be used to correlate molecular changes with specific diseases/conditions including hypertrophic cardiomyopathy, nemaline myopathy and muscular dystrophy. This study will contribute vital structural information and help achieve this future universal model that can also potentially illustrate the mechanics and molecular basis of any muscle-related condition. Additionally, the model can provide a basis for design of therapeutic agents where for instance, any identified defect can potentially be
corrected using chemical agents and/or proteins that interact at key regions associated with the specific muscle disorder or condition. In particular, a 3-D molecular model of the contractile machinery will allow us to observe the effect of any mutation(s) on the contractile process e.g. in myosin, actin, Tn, Tm or any muscle component for that matter. Consequently, molecular targets for therapy can be easily and efficiently identified, which would result in more effective treatments bearing minimal side-effects or potentially eliminating them completely. Since this study is based on findings using a skeletal muscle system, comparisons with the cardiac and smooth muscle system can be made to observe similarities in structure and perhaps function. If common aspects are found between, for example the skeletal and cardiac system, then it could be possible to use the same therapeutic approach to correct defects in both these muscle types.

Another application for this model is that it can be used in computational studies that will significantly reduce resource costs and increase the output of data, limited only by the processing power of the computer and the software. The completion of the human genome, along with the recent estimate of the DNA containing ~20,000 genes has provided the scientific community a wealth of opportunities to carry out similar research to potentially uncover the mechanism of any disease. From a general perspective, an understanding of the regulation, structure and function of proteins is paramount to characterizing disease
conditions, which will ultimately benefit those individuals affected and those genetically pre-disposed to the disease.
MATERIALS AND METHODS

2.1 Isolation and Purification of Skeletal Myosin, Actin, Troponin and Tropomyosin

Rabbit psoas (back) muscles were extracted according to Colowick and Kaplan (1982) to obtain the necessary skeletal muscle proteins. Ion-exchange column chromatography was then used to purify the main contractile proteins including a diethylaminoethyl (DEAE)-Sepharose A-50 column for initial Tn subunit separation and isolation of TnC and a carboxymethyl (CM)-Sephadex C-5-120 column to purify TnT and TnI. Purified chicken breast TnT was used instead of the rabbit form because it possesses a unique cysteine residue that allows for specific labeling by a fluorescent probe.

2.2 Synthesis of the Tb^{3+} Chelate and its Attachment to Chicken Troponin T

Synthesis of the terbium (Tb^{3+}) chelate, DTPA-CS124-NEM was carried out as follows: 10 mM diethylenetriaminepentaacetic acid (DTPA) and 10 mM carbostyril 124 (CS124, 7-amino-4-methyl-2(1H)-quinolinone) was agitated in 1 ml dimethylformamide (DMF) for 3 hours at room temperature. Then, N-(2-
aminoethyl) maleimide (NEM) was added at an equivalent molar concentration and allowed to react for 1 hour. Contaminants were removed using a DEAE-Sepharose column. The sample product was slowly added to 20 ml 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.5 (Buffer A), which was also used to equilibrate the column. Elution was carried out by a linear gradient of 1 M NaCl, 10 mM MES, pH 6.5 (Buffer B). The molar extinction coefficients ($\varepsilon$) are: CS124 $\varepsilon_{337} = 12,000$ M$^{-1}$cm$^{-1}$, Chicken TnT $\varepsilon_{280} = 26,000$ M$^{-1}$cm$^{-1}$ (denatured), 12,000 M$^{-1}$cm$^{-1}$ (native). Tb$^{3+}$ was added to the purified chelate in a 1:1 ratio (~260 $\mu$M) before being mixed with chicken TnT in a 5:1 molar ratio (chelate:protein). Excess chelate was then removed by dialyzing twice against 1 L 6 M urea, 50 mM trishydroxymethylaminomethane (Tris), pH 8.

2.3 Reconstitution of Skeletal Muscle Contractile Proteins

First, labeled chicken TnT, TnI, TnC and Tm monomer was mixed in a 1:1:1:2 molar ratio, respectively, because this is the stoichiometry observed physiologically in muscle cells. Next, dialysis was performed sequentially, first in 6 M urea, 1 M NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris and 1 mM dithiothreitol (DTT), then in 1 M NaCl, 2 mM EDTA, 50 mM Tris and 1 mM $\beta$-mercaptoethanol ($\beta$-me), and finally in 0.15 M NaCl, 50 mM Tris, all at pH 8 and 4 °C for ~15 hours (overnight). Tn $\varepsilon_{280} = 0.45$ ml·mg$^{-1}$cm$^{-1}$ and Tm $\varepsilon_{280} = 0.24$ ml·mg$^{-1}$cm$^{-1}$. Next, actin was reconstituted with Tn-Tm at a ratio of 7:1, respectively. The actin:Tn:Tm complex was dialyzed overnight in 0.15 M NaCl,
50 mM Tris at pH 8 and 4 °C, then ultracentrifuged and analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to confirm reconstitution of the thin filament regulatory proteins with actin.

2.4 Preparation of Myosin Subfragment-1 by α-Chymotrypsin or Papain and its Purification

Typically, α-chymotrypsin was used to cleave myosin between the ELC and RLC binding domains, thus generating myosin S1 with only the ELC bound. In this case, three acceptor fluorophores (described in the next section) are able to bind to the head domain of myosin S1. The only case where papain-generated myosin S1 was used was when fluorescently labeled RLC was exchanged with the endogenous RLC. This is possible because cleavage occurs on the C-terminal side of the RLC binding domain of myosin, thus resulting in myosin S1 with both the ELC and RLC present. In either case, about 1-2 ml (10-20 mg/ml) of rabbit myosin stored at -80 °C was dialyzed against 1 L of 0.5 M KCl, 10 mM imidazole, pH 7.0 for ~15 hours at 10 °C, then the product was ultracentrifuged at 100,000 g (47,000 RPM) in a TLA 100.3 rotor using a Beckman Coulter, Inc. TL-100 Ultracentrifuge for 1 hour at 4 °C. The supernatant was dialyzed against 1 L of 0.1 M KCl, 10 mM imidazole, pH 7.0 for ~15 hours at 10 °C. Then, either A) α-chymotryptic S1 or B) papain S1 was generated:

A) EDTA from a buffered concentrated stock (e.g. 0.3 M EDTA in 0.32 M Tris, pH 7.0) was added to 2 mM EDTA and warmed to room temperature (~22 °C). Next,
a fresh 5 mg/ml stock of α-chymotrypsin was added to the sample to 0.05 mg/ml and agitated gently for 7 minutes. Proteolysis was inhibited by adding 30 μl/ml of a stock of 100 mM phenylmethylsulphonyl fluoride (PMSF) dissolved in methanol.

B) MgCl₂ was added to 2 mM before a fresh 5 mg/ml stock of papain was added to 0.05 mg/ml. The reaction was stopped after 7 minutes of gentle shaking with 50 μl/ml of a stock of 1 mg/ml trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64). Both α-chymotryptic S1 and papain S1 digests were dialyzed for ~10 hours at 10 °C in 1L of 0.04 M KCl, 10 mM imidazole, pH 7.0, before being ultracentrifuged as before. The concentration of the supernatant was measured using ε₂₈₀ = 0.75 ml·mg⁻¹cm⁻¹. α-chymotryptic S1 (no RLC) and papain S1 (ELC and RLC) MW = ~110 kDa and ~130 kDa, respectively. A 1 ml Hi-Trap DEAE-Sepharose column equilibrated with 0.04 M KCl, 10 mM imidazole, 0.1 mM DTT pH 7.0 (Buffer A), was eluted with Buffer A + 1 M KCl (Buffer B) to yield pure S1. Fractions were analyzed using SDS-PAGE and pooled and concentrated using polyethyleneglycol (PEG) and dialyzed against 0.04 M KCl, 10 mM Imidazole, pH 7.0.

2.5 Labeling of Myosin S1 with Acceptor Fluorophores

Three labels A) Cy5-ATP, B) TMR and C) FHS were used to label the myosin head domain in independent experiments. Each was prepared in the following way:
A) Cy5-ATP (Cyanine 5-conjugated adenosine triphosphate) was synthesized similarly to Cy3-ATP as previously described by Xu and Root (1998) and purified using column chromatography. Because the label binds to the myosin nucleotide binding site with high affinity via the ATP group, labeling was achieved simply by adding the dye to the acto-myosin contractile complex immediately prior to acquisition of LRET data. Cy5-ATP $\varepsilon_646 = 250,000$ M$^{-1}$cm$^{-1}$ and maximum emission emission wavelength ($\text{Em}_{\lambda_{\text{max}}}$) = 670 nm. Since the ATP moiety of Cy5-ATP is hydrolyzed by myosin S1, it is also referred to as Cy5-ADP (Cyanine 5-conjugated adenosine diphosphate), which indicates myosin having undergone the powerstroke step.

B) 5-TMRIA (tetramethylrhodamine-5-iodoacetamide dihydroiodide, Molecular Probes ®) was dissolved in 1 ml DMF, added to myosin S1 in a 1:1 molar ratio and incubated for 30 minutes. The reaction was then terminated by adding 10-fold molar excess DTT and dialyzed in 0.04 M KCl, 10 mM imidazole, pH 7.0 at 4 °C (Root et al., 1991). $\varepsilon_543 = 87,000$ M$^{-1}$cm$^{-1}$, $\text{Em}_{\lambda_{\text{max}}} = 567$ nm and S1-TMR absorbance maximum wavelength ($A_{\lambda_{\text{max}}}$) = 550 nm.

C) FHS (fluorescein-5(6)-carboxamidocaoroic N-hydroxysuccinimide ester, Sigma ®) was incubated with myosin S1 as described by Bertrand et al. (1995) and glycine was used to quench the reaction. $\varepsilon_495 = 75,000$ M$^{-1}$cm$^{-1}$, $\text{Em}_{\lambda_{\text{max}}} = 520$ nm and S1-FHS $A_{\lambda_{\text{max}}} = 500$ nm.
2.6 Isolation and Acceptor Labeling of the RLC and Exchange into Papain S1

About 5 ml of myosin (80 mg/ml) was dialyzed overnight against 1 L of 0.5 M NaCl, 10 mM Tris, pH 8.5, and ultracentrifuged at 100,000 g (47,000 RPM) in a TLA 100.3 rotor using a Beckman Coulter, Inc. TL-100 Ultracentrifuge for 1 hour at 4 °C. Then, 7.5 parts of myosin supernatant, diluted to 15 mg/ml, was added to 1 part 75 mM 5', 5'-dithiobis-(2-nitrobenzoic acid) or Ellman’s reagent (DTNB), 50 mM EDTA, 200 mM Tris, pH 8.5, before being incubated at room temperature for 10 minutes. Then, cold distilled water was added to myosin at 10:1 and centrifuged at 7,000 RPM for 10 minutes in a Dupont Sorvall® GSA rotor. The supernatant was filtered and concentrated using a 0.45 μM membrane and ultrafilter (molecular weight cut-off, MWCO = 5000 Da), respectively. β-me was added to 1 mM prior to isolation of RLC on a Superdex 75 column, that was equilibrated with 0.1 M NaCl, 2 mM MgCl₂, 10 mM imidazole, pH 7.0. Next, the fluorescent label was prepared: 1.95 mg aminorhodamine B (AmrB, Research Organics, Inc.) was dissolved in 200 μl dimethylsulfoxide (DMSO) and added to 1.42 mg DTPA and incubated for 30 minutes. The product was added to rabbit skeletal RLC, incubated at room temperature for 45 minutes and dialyzed exhaustively in 0.5 M KCl, 5 mM MgCl₂, 10 mM imidazole, pH 7.0. RLC ε<sub>280</sub> = 12,000 M⁻¹cm⁻¹, AmrB ε<sub>550</sub> = 110,000 M⁻¹cm⁻¹, E<sub>m</sub>max = 568 nm. The next step was to exchange the RLC-AmrB with the endogenous RLC bound to myosin. Purified papain S1 was dialyzed against 1 L 50 mM KPr, 20 mM EDTA, 10 mM Kpi, 0.5 mM DTT, pH 7.0 (titrated with KOH), and the same buffer was also used
to equilibrate the gel filtration column (Toyopearl 55F/Superdex 75). The papain
S1 was heated to 30 °C for 15 minutes prior to loading on the gel filtration column
to allow the endogenous RLC to dissociate from the myosin S1 lever arm. After
the S1 devoid of RLC was pooled, the RLC-AmrB was added in 2-fold molar
excess, followed by dialysis against 0.1 M KCl, 10 mM imidazole, 1 mM MgCl2,
pH 7.0. Finally, the unbound RLC-AmrB was removed by gel filtration using the
Toyopearl 55F column. Absorbance spectra show ~50, 7 and 11% efficiency of
labeling (not shown), of TMR, FHS, and AmrB, respectively and an exchange
efficiency of RLC-AmrB with endogenous papain S1 RLC of ~5%.

2.7 Exchange of Chicken TnT-chelate with Endogenous TnT in Rabbit
Skeletal Myofibrils

Rabbit myofibrils stored in 50% glycerol at -20 °C were prepared
according to Shiraishi et al. (1992). Modifications to the protocol included the use
of imidazole instead of MOPS buffer and a DuPont Sorvall® RC-5B centrifuge.
0.7 ml of myofibrils were diluted 50X to 35 ml with 50 mM KCl, 1 mM NaHCO3,
0.5 mM DTT before being centrifuged at 5000 rpm for 10 minutes using an SS-34
rotor at 2 °C. The pellet was gently dispersed using a pipette tip and diluted to
0.01 mg/ml by adding 150 mM KCl, 2 mM MgCl2, 40 mM imidazole, 0.5 mM
ethyleneglycoltetraacetic acid (EGTA), 0.5 mM DTT, pH 6.2. The protein
concentration was measured at 280nm by adding 10 µl of myofibrils to 1000 µl
5.1% SDS, ε_{280} = 0.7 ml·mg^{-1} cm^{-1}. Then 0.5 ml of the chicken TnT-chelate at 0.1
mg/ml was added to 0.5 ml myofibril to a final myofibril concentration of 50 µg/ml, before shaking gently for 60 minutes at 25 °C. As a control, unlabeled chicken TnT was also exchanged. After this, the suspension was centrifuged at 5000 rpm for 10 minutes at 2 °C, the supernatant containing endogenous TnT, TnI and TnC was removed and the pellet was washed with an equivalent volume of 150 mM KCl, 2 mM MgCl₂, 40 mM imidazole, 0.5 mM DTT, pH 6.8. The TnT-chelate-exchanged myofibril pellet was suspended in about 70 µl of 50 mM KCl, 1 mM NaHCO₃. All centrifuged samples were analyzed on a 12% SDS-PAG (not shown). Then, an equimolar amount of Tb³⁺ was added to the chicken TnT-chelate-exchanged myofibril before being tested in the fluorimeter. An emission spectrum using a 337 nm excitation nitrogen laser confirmed the presence of Tb³⁺ and a time trace confirmed the binding of Tb³⁺ to the chelate since the lifetime was measured to be about 1 ms (a faster time would indicate unbound Tb³⁺). An excitation spectrum also confirmed the presence of CS124 with a distinct peak at about 337 nm. Next, the myofibrils were dialyzed in 50 mM KCl, 10 mM imidazole, 1 mM NaHCO₃ for 6 hours before TnI and TnC were added in equimolar amounts with respect to the initial amount of chicken TnT-chelate added and incubated overnight at 4 °C. MgCl₂ was added to 1 mM before the sample was split into two tubes, with one containing CaCl₂ to 0.1 mM and the other with EGTA to 0.5 mM. Only Cy5-ADP was used as the acceptor fluorophore because it is known to bind with high affinity to the ATP binding site on myosin S1. The other acceptor fluorophores used in the myosin S1
experiments were not employed in the myofibril study because they cannot be attached specifically to the target protein due to the presence of all the other muscle proteins in the myofibril. Consequently, the LRET data would be meaningless, therefore only the Cy5-ADP label was used to compare the distance measurements derived from the analogous myosin S1 study.

2.8 Preparative Equipment

Myosin S1 was stored at 4 °C and used within two weeks to ensure its integrity before degradation and all other proteins were flash frozen using liquid nitrogen (-196 °C) and stored at –80 °C. Protein concentrations and absorbance spectra of labels were determined by a diode array spectrophotometer with a bandpass of 1 nm (Hewlett-Packard). The Bio-Rad Laboratories, Inc. Mini-PROTEAN® II system was used with 12 or 15 % SDS-polyacrylamide gels, which confirmed the isolation, purification, and labeling of all proteins. Fluorescent bands in gels were visualized via a UV-lamp and a high performance cooled coupled device (CCD) camera (4910 Series, COHU, Inc). Column chromatography was carried out using a Pharmacia Biotech® Fast Protein Liquid Chromatography (FPLC) System (Controller LCC-501 Plus, Pump P-500), with 254 nm UV detector and conductivity monitor. Centrifugation was performed by a DuPont Sorvall® RC-5B centrifuge (GSA rotors) and a Beckman Coulter, Inc. TL-100 Ultracentrifuge (TLA rotors).
2.9 Sample Preparation and LRET Data Collection

The reconstituted thin filament sample containing actin, Tn (including the chelate-labeled TnT) and Tm was titrated with 5 \( \mu \text{M} \) Tb\(^{3+} \) to saturate binding to the chelate. Once the emission signal of the donor probe (labeled TnT) was maximized, the sample was divided into two tubes, one containing 0.5 mM MgCl\(_2\) and 0.1 mM CaCl\(_2\) and the other with 0.5 mM MgCl\(_2\) and 0.5 mM EGTA. In all, four different acceptor labels were attached independently to myosin S1, so once it was added to the thin filament complex in a 1:1 ratio with respect to the molar concentration of actin monomers, it was possible to determine whether energy transfer or Luminescence Resonance Energy Transfer (LRET) had occurred. This was achieved by observing the difference between the emission spectrum of the donor only versus that of the donor in the presence of acceptor. First, time-resolved fluorescence decay followed by steady-state measurements were obtained using an SLM-Aminco Bowman II luminescence spectrometer. Up to four sequential measurements were made, each for the emission scan and donor fluorescence decay times. These measurements were recorded 1) in the presence of donor only (thin filament complex including TnT-chelate), 2) after addition of acceptor (labeled myosin S1), 3) then after addition of ADP (which causes the myosin S1 to adopt the post-powerstroke state), and 4) where permitting, addition of phosphate analogue (BeF\(_x\) or AlF\(_4\)), which induces myosin S1 to assume the pre-powerstroke state). Essentially, energy transfer between the donor and acceptor probes were measured in up to three myosin S1 states:
rigor (no nucleotide), post- and pre-powerstroke. In each case, fluorescence decay times were measured at the maximum emission wavelength of the donor (545 nm) and acceptor i.e. 670, 567, 520, and 568 nm for Cy5-ADP, TMR, FHS and AmrB, respectively. Not all myosin S1 states were studied for each donor-acceptor pair. For instance, the S1 rigor state using Cy5-ADP was not studied because rigor can only be achieved in the absence of nucleotide. Also, the pre-powerstroke state using S1-TMR was not evaluated because the phosphate analogues are not efficiently incorporated by myosin due to the effect of TMR labeling. This is discussed in more detail in section 5.1. The only acceptor label that was not chemically linked to myosin S1 was Cy5-ADP, so this was added to S1 and myofibrils at 0.5 μM and incubated for 5 minutes prior to recording the LRET measurements. The Two phosphate analogues, BeFx or AlF4 were prepared by mixing 1 mM atomic Be or AlCl3 with 10 mM NaF. Either BeFx or AlF4 was added (in independent experiments) to the contractile complex present with ADP to 0.1 mM and incubated for 15 minutes before data acquisition. The maximum excitation wavelength of Tb3+ was set to 232 nm when the flashlamp was used as the light source or was fixed at 337 nm when the nitrogen laser was used. Being about 1000 times more sensitive in detecting signals than the flashlamp, the laser was necessary for the myofibril-Cy5-ADP and S1-RLC-AmrB measurements, while the other LRET data were collected using the flashlamp. Parameter settings for the SLM-Aminco Bowman II luminescence spectrometer flashlamp were as follows: phosphorescence, >200 μs; delay, 200 μs and gate
width, 4600 µs. Time-trace parameters (fluorescence decay curve) were: 200 µs lower and 4600 µs upper limit, and 200 µs resolution over 500 repetitions. A 16 nm excitation and 16, 8 and 4 nm emission bandpass was used for all labeled myosin S1, myofibril and S1-RLC, respectively. Use of the laser required a 34 ms minimum flash period. All other parameters were default settings and the voltage sensitivity was set to ~60 % (flashlamp) and 30 % (laser).

2.10 LRET Data Analysis: Curve Fitting and Calculation of Distance Values

MacCurveFit version 1.5.5 (Kevin Raner Software), a program that uses least squares to fit curves was used to help determine decay times of the donor in the rigor (no nucleotide), post- and pre-powerstroke state. All decay times at the donor emission wavelength (545 nm) were fit to a double exponential function, $F(x) = a \cdot \frac{1}{b} + c + d \cdot \frac{1}{e}$, except for the donor only curve, which was fit to a single exponential function, $F(x) = a \cdot \frac{1}{b} + c$. Similarly, all decay times at the acceptor emission wavelengths were fit to the single exponential equation, except for the donor only curve that was fit to a value of 0 ($F(x) = c$). These different donor times were used to calculate the efficiency of resonance energy transfer, E, in the tested myosin states (Equation 1). The long-lived apparent lifetime of the acceptor ($\tau_{da}$), in the presence of Tb$_3^{3+}$ is equal to that of the quenched Tb$_3^{3+}$, or lifetime of the donor in presence of acceptor. Thus, E was calculated by comparison of $\tau_{da}$ to the lifetime of the donor in the absence of acceptor, $\tau_d$. 

34
E = 1 – \(\tau_{da} / \tau_d\)  \hspace{1cm} (Equation 1)

Next, J, the spectral overlap integral (Equation 2) was determined where \(\Phi_D(\lambda)\) is the relative emission of the donor at a certain wavelength, \(\varepsilon_A(\lambda)\) is the extinction coefficient of the acceptor at the same wavelength, and \(\Delta\lambda\) is the interval between wavelengths (1 nm).

\[
J = \sum \left[ \Phi_D(\lambda) \varepsilon_A(\lambda) \lambda^4 \Delta\lambda \right] / \sum \left[ \Phi_D(\lambda) \Delta\lambda \right] \hspace{1cm} (Equation 2)
\]

The J value was then used to determine \(R_o\), the critical transfer distance at which energy transfer is 50% (Equation 3), where \(Q_D\) is the donor quantum yield (\(\tau_{da} / \tau_d\)), \(\eta\) is the refractive index of the protein medium (typically 1.4), \(\kappa^2\) is the orientation factor assumed to be 2/3, and J is the overlap integral that depends on the chosen acceptor.

\[
R_o = (8.785 \times 10^{-11} Q_D \eta^{-4} \kappa^2 J)^{1/6} \hspace{1cm} \text{nm} \hspace{1cm} (Equation 3)
\]

Finally, R, the distance separation between the donor and acceptor probe in each tested condition was calculated (Equation 5) after rearranging Equation 4.

\[
E = R_o^6 / (R_o^6 + R^6) \hspace{1cm} (Equation 4)
\]

\[
R = R_o ((1/E)-1)^{1/6} \hspace{1cm} (Equation 5)
\]

2.11 3-D Mapping of C-TnT into Acto-Myosin Atomic Models

All three S1-head labels (Cy5-ADP, TMR and FHS) helped determine distance measurements in the pre- and post-powerstroke state and the average values obtained in the presence/absence of calcium were used to triangulate the
position of C-TnT. The position of C-TnT was visualized using an atomic model with myosin S1 bound to the thin filament by first determining the 3-D co-
ordinates of C-TnT (x, y, and z) using the solver function on the LRET-derived distances. All calculations were performed on Microsoft ® Excel and a molecular viewing software (RasMol) was used to observe all XRC models. The position of C-TnT was observed in three different acto-myosin atomic models designated as Model 1, Model 2 and Model 3 with various interactions and distance measurements noted. All three models were previously generated using crystal structures of actin (Lorenz et al., 1993; Holmes et al., 1993) and skeletal chicken myosin (Rayment et al., 1993a) in the rigor state, however, each utilized a different method of docking myosin S1 to the actin filament. Model 1 (Rayment et al., 1993b) is based on the objective docking of myosin S1 with actin guided by cryo-EM density maps (Milligan et al., 1990). Model 2 is also based on EM maps; however, docking of myosin S1 onto filamentous (F) actin (Holmes et al., 1990) was done computationally (Mendelson and Morris, 1997). The coordinates for this model represent only C<sup>α</sup> atoms, so the distance from C-TnT to the sulfur atom of Cys707 and to the terminal nitrogen atom of Lys553 was calculated from Model 1 using the RasMol program. Finally, Model 3 is based solely on computational docking of myosin S1 (Rayment et al., 1993a) to F-actin (Holmes et al., 1990), which was performed by Root (2002b).
2.12 Conformational Search of the Tb³⁺ Chelate Bound to Chicken Troponin T

Maestro modeling software (Schrödinger, Inc.) was used to construct the Tb³⁺ chelate (DTPA-CS124-NEM) according to Getz et al. (1998) and to mutate the human cardiac TnT (c-TnT) component of the Tn core XRC structure (Takeda et al., 2003) (PDB 1J1E) to the chicken breast isoform (accession NP_990253). Therefore, the human cardiac TnT component served as a 3-D template because the chicken breast TnT crystal structure had not been solved. The chicken breast isoform (287 residues) contains a unique cysteine residue at position 263 to which the Tb³⁺-chelate attaches, thus to achieve an accurate representation of the probe conformation attached near the C-terminal of chicken breast TnT, the appropriate residues were substituted into the existing human cardiac TnT XRC model (Takeda et al., 2003). Also, the flexible loops that do not appear in the original atomic model due to their inability to crystallize were manually modeled into the structure. After the model was modified, the cysteine residue of chicken TnT was assigned position 263 and conformational searches of the chelate bound to this cysteine were performed. This determined the most probable conformation of the chelate with respect to the Tn complex. The procedure for the conformational search was as follows: 1) The OPLS-AA (Optimized Potential for Liquid Simulations – All Atom) forcefield was used with water as the solvent at normal distance and a constant dielectric value of one. 2) A distance constraint (2.5 Angstroms (Å) maximum) was applied between each closest oxygen atom of the chelate and Tb³⁺ atom pair and a force constant of
100 was applied. 3) The substructure was designated as the binding interface comprised of the region between the chelate and TnT residues. Therefore, only the chelate and those residues in immediate proximity to the chelate (binding interface) were allowed to move during the computer simulations. All the surrounding atoms located greater than a 3 Å radius from the substructure were designated as the frozen atoms and thus not included in the calculations. 4) Energy minimization of structures were completed via the Polak-Ribiere Conjugate Gradient energy minimization (PRCG) method at 1000 iterations and carried out to a convergence threshold of 0.05. 5) Conformational searches incorporated the Mixed MCMM/LowMode method (Landau and Binder, 2005) with default automatic setup parameters (e.g. minimum distance, 3 Å, maximum distance, 6 Å) and default settings for the rotation/translation of the Tb\(^{3+}\) atom (e.g. 180° rotation, translation range 1-6 Å). After each round of 1000-step iterations, the lowest energy structure was used as the starting structure of the next iteration until no change in conformation and energy was observed.

2.13 Engineering of α-Tropomyosin Deletion Mutant

This work and the related assays were performed in Dr. J.-P. Jin’s laboratory at Evanston Northwestern Healthcare, Chicago, IL. Because Tm is a highly conserved protein in mammals, a plasmid already available in the lab that encoded chicken skeletal α-tropomyosin (α-Tm) was used instead of waiting for the availability of the rabbit isoform. Deletion of actin binding sites 2 and 3 of α-
Tm (dAc23 Tm) was accomplished using oligonucleotide-directed mutagenesis following a modified protocol described by Hitchcock-DeGregori and An (1996). All complementary DNA (cDNA) was cloned in pAED4 plasmid (Ogut and Jin, 1996) and transformed into E.coli strains JM109 (for mini-expression) and BL21(DE3)pLysS (Ogut and Jin, 1996) for large-scale expression. The nucleotides encoding residues 47-123 were deleted using two pairs of oligonucleotide primers 1) T7 primer (forward) with dAc23R (reverse):
5’-CCTCTTTCCAGAGCCACCAGCTC-3’ and 2) dAc23F (forward):
5’-TGGCTCTGGAAAGAGGAATGAAGG-3’ with pETR (reverse). For nucleotides encoding wild type (WT) Tm, T7 and pETR primers were used (Figure 4).
Figure 4. A double-stranded cDNA map of chicken skeletal α-Tm. Oligonucleotide-directed mutagenesis was used to eliminate two internal actin binding sites of Tm (2 and 3). Oligonucleotide primers T7 and R generated the N-terminal 46 residues, while F and pETR generated the C-terminal 161 residues (124-284) shown by the wavy brackets. This eliminated 77 internal residues (black cross), which correspond to actin binding sites 2 and 3. Primers R (dAc23R) and F (dAc23F) were designed with 5' overhangs bearing sequences corresponding to the opposite strand (hatch and horizontal marks). They are important in annealing the N and C-terminal PCR products to eventually generate the deletion mutant Tm (dAc23 Tm). Some restriction sites (XbaI, NdeI and BamH1) are indicated that reside in the vector DNA (dotted segments). The portion of cDNA corresponding with WT Tm is 852 nucleotides long and represents 284 amino acids, which was amplified using T7 and pETR.
Tm was purified from the heat-stable fraction by anion-exchange chromatography on a DE52 column (eluted using 0-300 mM KCl, 10 mM Tris, pH8) and gel filtration on a G-75 column (eluted using 1 M KCl, 10 mM Imidazole, pH 7.0). When 6 M urea was used to improve the purification protocol, exhaustive dialysis was performed in 1 M KCl, 10 mM Imidazole, pH 7.0. SDS-PAGE and Western blots using CH1, a polyclonal antibody specific for Tm, were used to identify fractions containing the protein.

2.14 Enzyme Linked Immunosorbant Assay (ELISA) of Muscle Proteins

A protein binding assay (Wang and Jin, 1998) was used to investigate the interactions Tm with actin and TnT. First, the type and amount of antibody to use for the detection of Tm was determined by titrating a known Tm specific polyclonal antibody (CH1) and analyzing its binding response to three types of Tm. A microtiter plate was coated with 100 μl/well of each purified Tm (WT, dAc23 and chicken muscle α-Tm at 1 μg/ml) into triplicate wells in 0.1 M KCl, 3 mM MgCl₂, 20 mM Piperazine-1,4-bis(2-ethanesulfonic acid) or (PIPES), pH 7.0 (Buffer A) overnight at 4 °C. The wells were washed once (5 minutes) with Buffer A plus 0.05 % Tween-20 (Buffer T), followed by blocking with 1 % bovine serum albumin (BSA) in Buffer T (Buffer B, 100 μl/well) at room temperature for 1 hour. After three washes with Buffer T (5 minutes each) and blot-drying the wells, serial dilutions of CH1 antibody (10⁻² to 10⁻⁸ and 0) in Buffer B (100 μl/well) were done and incubated for 1 hour (for all other muscle protein binding assays, the binding
partner to be studied was serially diluted, e.g., 3-fold and incubated 2 hours. After three washes (5 minutes each) with Buffer T, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin secondary antibody (Sigma) was added at 100 μl/well and incubated for 45 minutes at room temperature. Finally, after three washes with Buffer T, H2O2-ABTS (2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) substrate was added (100 μl/well) at room temperature and color development was monitored at A405 at a series of time points e.g. 5, 10, 20, 30, 40 and 50 minutes by an automated microplate reader (Bio-Rad Laboratories, Inc. BioRad Benchmark reader). Data from the linear portion of the time course was plotted (typically after 20 minutes) to determine the saturated amount of CH1 to use for all subsequent ELISAs in which Tm binding was to be measured. Note, for all other muscle protein binding assays, prior to adding HRP, the antibody specific for the serially diluted protein was added and incubated for 1 hour. Also, wash times varied depending on the specific binding assay e.g. 7 minutes to reduce the background noise.

A similar protocol was used to measure the binding affinity between the following muscle proteins 1) each coated Tm form (1 μg/ml) and serial dilutions of TnT using CT3 antibody, 2) coated filamentous actin (F-actin) at 30 μg/ml and serial dilutions of each Tm using CH1, 3) coated F-actin pre-incubated with each Tm form for 1 hour and serial dilutions of TnT using CT3. Adjustments in serial dilutions were made to yield an appropriate sigmoidal binding curve.
2.15 F-actin Cosedimentation Assays

F-actin was prepared from rabbit skeletal muscle acetone powder as described by Colowick and Kaplan (1982) and the actin-binding assay was carried out in 0.1 M KCl, 3 mM MgCl₂, 5 mM DTT, 20 mM PIPES, pH 7.0. 100 µl reactions contained the appropriate proteins (actin, Tm, TnT) at stoichiometric amounts e.g. 90, 20 (for non-mutant Tm) and 12 µg, respectively. Following incubation at room temperature for 2 hours and ultracentrifugation for 30 minutes at 100,000 g, the pellet and supernatant were analyzed on SDS-polyacrylamide gels. Densitometric analysis of the protein bands from the gel helped determine the amount of each type present and thus the stoichiometry of binding between actin, Tm and TnT. A range of Tm amounts were incubated that helped determine a saturation binding curve.

2.16 Myosin S1 ATPase Assay

The following buffers were prepared: 1) 10X Mg²⁺ ATPase (20 mM MgCl₂, 0.2 M imidazole, pH 7.0). 2) 10X CaCl₂ (1 mM). 3) 10X EGTA (5 mM in 20 mM imidazole, pH 7.0). 4) 10X imidazole (0.2 M, pH 7.0). 5) 0.25 M H₂SO₄. 6) Fresh 10X ATP (10 mM in 0.2 M imidazole, pH 7.0). 7) Sodium phosphate monobasic (1 mM). Fresh 15 % w/v ammonium molybdate was prepared in distilled water (dH₂O), one part of which was mixed with 4 parts of malachite green stock as was done by Baykov et al. (1988). A sodium phosphate standard curve was
determined using 1 mM sodium phosphate monobasic stock, from which 1 ml of each sodium phosphate standard sample was prepared (0,2,4,8,16,32,64, 128 and 256 μM) each containing 100 μl 10X Mg\textsuperscript{2+} ATPase and 100 μl 10X CaCl\textsubscript{2} or 10X EGTA buffer. 10 μl of each standard along with its 10 μl control sample were delivered to separate microfuge tubes. The data points were obtained as follows: A) 50 μl 0.25 M H\textsubscript{2}SO\textsubscript{4} was added to the control sample. B) 10 μl of malachite green mix was added and incubated for 5 minutes. During the incubation, part A and B were carried out for a phosphate standard sample. C) In the meantime, the absorbance wavelength at 630 nm (A\textsubscript{630}) of the control sample was set as the blank on the spectrophotometer (after its 5 minute incubation time). D) The cuvette was thoroughly rinsed and replaced with a standard sample once the A\textsubscript{630} was ready to be measured. Frequently, dilution of the sample (10 μl reaction mix + 50 μl 0.25 M H\textsubscript{2}SO\textsubscript{4} ) with water was required prior to the addition of 10 μl malachite green mix to keep the A\textsubscript{630} value within the optimal range of the spectrophotometer (<1.5).

2.17  S1 ATPase Data Analysis

The ATPase data were fit to an equation that describes binding kinetics similar to how the Michaelis-Menten equation describes enzyme kinetics. The reversible binding between actin and myosin S1 can be illustrated as follows:

\[
\text{actin-S1} \xrightleftharpoons[k_1]{k_2} \text{actin} + \text{S1}
\]

(Equation 6)
The actin-S1 complex can dissociate into its constituent parts (actin and S1) and can also re-assocate to form the complex. The rate of dissociation and association depends on the dissociation \((k_2)\) and association \((k_1)\) rate constant, respectively (Equation 6) and the equilibrium between the two rates is described by the dissociation equilibrium constant, \(K_d\) (Equation 7):

\[
K_d = \frac{[\text{actin}] [\text{S1}]}{[\text{actin-S1}]} = \frac{k_2}{k_1} \quad \text{[actin]} << K_d \quad \text{(Equation 7)}
\]

In the ATPase assays, since the myosin S1 concentration ([S1]) was varied and the actin monomer concentration fixed at 1 µM, the S1 was designated the ligand and actin as the protein. Also, note that the actin monomer concentration is considerably smaller than the \(K_d\) value because the \(K_d\) corresponds to the [S1] (ligand) at which the binding site on the actin monomer is half occupied. More clearly, it is the [S1] at which the concentration of the actin-S1 complex is equal to the actin concentration with no S1 bound. Continuing from Equation 6, the derivation of the curve-fitting equation is as follows:

\[
[\text{actin-S1}] = \frac{[\text{S1}]B_{\text{max}}}{[\text{S1}] + K_d} \quad \text{(Equation 8)}
\]

The format of Equation 8 is similar to the Michaelis-Menten equation that is typically encountered in enzyme kinetic studies (Equation 9):

\[
[ES] = \frac{[E_T][S]}{K_m + [S]} \quad \text{(Equation 9)}
\]

where, ES represents concentration of enzyme-substrate complex, \(E_T\) is the concentration of total enzyme, S is the substrate concentration and \(K_m\) is the Michaelis-Menten constant corresponding to the substrate concentration at which
the binding site on the enzyme is half occupied. The $K_m$ is analogous to the $K_d$ in Equation 8. Finally, the equation of interest is reached by multiplying both sides by $k_{cat}$ and incorporating the velocity term, $v$ (Equation 10):

$$k_{cat}[\text{actin-S1}] = [S1]B_{\text{max}} \times k_{cat} = \frac{v}{[S1] + K_d}$$  \hspace{1cm} (Equation 10)

where, $k_{cat}$ is the turnover number or maximum number of substrate molecules that can be converted to product by an enzyme per second and $B_{\text{max}}$ is the concentration of $S1$ binding maximally to the actin filament.

2.18 Confocal Microscopy of Labeled Rabbit Skeletal Myofibrils

100 µl of rabbit myofibrils stored in 50 % glycerol at -20 °C were incubated with 1) Adenosine 5’-triphosphate Alexa Fluor 647 2'(or 3)-O-(N-(2-aminoethyl) urethane), hexa(triethylammonium) salt (Molecular Probes ®) and 2) Cy5-ATP, each to 1 µM for 5 minutes at room temperature. Next, the labeled myofibrils (~30 µl) were placed on a glass microscope slide, taped onto a turntable and spun for 10 minutes (720 rpm, 115 V motor) to induce stretching of the myofibrils. An equal volume of oxygen scavenging (OS) solution, as used by Mitchison’s lab (http://mitchison.med.harvard.edu/protocols/flowcell.html#A.%20%20Oxygen %20Scavenging) was added prior to placing a coverslip on the sample.
CHAPTER 3

EXPERIMENTAL STRATEGY

3.1 Fluorescence and Competing Processes

Before discussing the technique of FRET in more detail and how it is used to report nanometer distances, it is first important to understand the phenomenon of fluorescence itself. Some atoms and molecules have the ability to absorb light of a certain wavelength and after a short interval, called the fluorescence lifetime, re-emit light at a relatively longer wavelength, producing fluorescence. Absorption of light energy, for example, by fluorescent probes, occurs between a number of narrowly spaced vibrational and rotational excited states located in different orbitals. As described by Alexander Jablonski in 1953, absorption of light occurs rapidly, in about $10^{-15}$ seconds (s) and elevates the energy of the fluorophore from the ground state ($G_0$) to an excited state. Then, relaxation to the lowest excited singlet state ($S_1$) occurs within $10^{-11}$ s via internal conversion (Herman, 1998). This process occurs as the excited state fluorophore collides with solvent molecules and results in a loss of energy without the emission of light. Still in the lowest excited singlet state then, the fluorophore resides here on the order of nanoseconds ($10^{-9}$ s), after which relaxation to the ground state is accompanied with emission of a photon.
(Lakowicz, 1983). It is this emission that is otherwise known as fluorescence.

However, molecules residing in the lowest excited singlet state can also participate in a relatively scarce event called intersystem crossing, where electrons “cross” over to the triplet state (T1), resulting in a longer lived emission called phosphorescence (Rost, 1991). Also, transitions from the triplet back to the singlet state can occur before proceeding to the ground state, in which case a delayed fluorescence is observed.

A number of energy depleting processes compete with fluorescence which includes internal conversion, phosphorescence and quenching. Since quenching refers to the non-radiative relaxation of excited state electrons to the ground state (without emission of a photon), internal conversion is actually a form of quenching that results due to the collision of an excited fluorophore with another molecule (Herman, 1998). This process is also called dynamic or collisional quenching that can reflect the presence of acceptor molecules which, by diffusion or conformational change, can collide with the donor fluorophore. Another type of quenching, called complex or static quenching occurs when static complexes of donor fluorophore and acceptor (quencher) function to reduce absorption. Here, the numbers of excitable fluorophores are reduced, causing a decrease in the fluorescence intensity without affecting the excited state lifetime (Herman, 1998).

Another quenching phenomenon that is perhaps more significant is photobleaching or fading of the fluorophore, which typically occurs when the sample is being irradiated (Rost, 1991). It is generally thought that
photobleaching is mainly due to photochemical reactions between the fluorophore, oxygen and excitation light (Menter et al., 1978), although it has been observed to occur in non-oxidative conditions (Picciolo and Kaplan, 1984). In the former case, an excited fluorophore in the long-lived triplet state can permit a higher number of reactions to occur with the surrounding components of the cell, or it can induce production of singlet oxygen after collision and energy transfer with molecular oxygen (Menter et al., 1978). The highly reactive singlet oxygen species causes the fluorophore to bleach and also damages biological components depending upon the oxygen concentration, diffusion coefficient and lifetime of the singlet oxygen species (Herman, 1998). This fading phenomenon may also be attributed to a change in the quantum efficiency and photochemical destruction of the fluorophore. For example, evidence has shown a loss of quantum efficiency that is indicative of the fluorophore converting into a non-fluorescent, but absorptive species (Rost and Pearse, 1971). Fluorophores have a defined photochemical life over which a certain number of photons are emitted regardless of whether the excitation light is absorbed in pulses or continuously. Therefore, even at low energy levels photobleaching is not eliminated, but the rate of the process is reduced. Though the mechanism of photobleaching is poorly understood and theoretically, the photochemical lifetime of a fluorophore should not be affected whether a pulsed or continuous excitation light source is absorbed, empirical evidence supports short pulses of light actually reduces the phenomenon (Kaufman et al., 1971; Bergquist, 1973). It is thought perhaps the
short pulses allow interaction with oxygen in closest proximity to the fluorophore, thus limiting the degree of photobleaching. However, when a continuous excitation source is used, multiphoton absorption along with bond photolysis may occur resulting in a photobleached probe (Herman, 1998). Since oxygen favors photobleaching, several methods can be used to reduce the problem depending on the specific application. For example, singlet oxygen quenchers such as histidine, diphenylisobenzofuran or reducing agents like DTT and β-me can be used. Oxygen scavenging systems like glucose oxidase and catalase and deoxygenating solutions provide other alternatives. Also, reducing the exposure time or excitation energy can limit photobleaching, though a compromise must be met with acquiring an adequate signal to noise ratio.

3.2 Principle of Fluorescence Resonance Energy Transfer (FRET) and Förster’s Theory

The process of fluorescence resonance energy transfer occurs when a donor fluorophore in an excited electronic state, transfers its excitation energy to an acceptor probe, which although is not required to be fluorescent, most often is in many applications. Here, the acceptor probe quenches the fluorescence emission of the donor probe and correspondingly causes a decrease in the fluorescence lifetime, which in turn results in increased or sensitized fluorescence emission of the acceptor (Clegg, 1995; Selvin, 2000). This is possible if the emission spectrum of the donor probe overlaps the absorption (or
excitation) spectrum of the acceptor probe and the two are positioned within a minimum radius (Figure 5).
Figure 5. Overlap of the Tb$^{3+}$ donor emission spectrum and acceptor excitation/emission spectra. Tb$^{3+}$ emission is characterized by four narrow peaks between which the excitation/emission spectra of acceptors A) fluorescein (FHS) and B) tetramethylrhodamine (TMR) overlap. Such an overlap couples the donor and acceptor pair to allow FRET to occur, in which case a decrease in the emission intensity of the donor and an increase in the emission intensity of the acceptor occurs (sensitized emission, not shown).
First proposed in the late 1940’s, Theodor Förster stated a theory supporting energy transfer between two probes occurring in a non-radiative manner, i.e. without emission of a photon, via long range dipole-dipole coupling (Förster, 1946; Förster, 1948). Here, the excited fluorophore can be considered as an oscillating dipole that can transfer energy with another dipole possessing the same resonance frequency. Radiative energy transfer on the other hand, depends on the emission and reabsorption of a photon as well as the specimen dimensions, optical properties of the sample, wavefront pathways and the geometry of the container. However, this type of energy transfer mechanism cannot produce significant structural information like the non-radiative process can.

There are other criteria that must be met in order for resonance energy transfer to occur besides the aforementioned overlap of the donor emission and acceptor absorption spectra. As Förster’s theory states, because the efficiency of energy transfer rapidly decreases in proportion to the inverse sixth power of the separation distance between donor and acceptor probes, FRET can only occur over a range of 1-10 nm (Clegg, 1995). The fact that energy transfer is very sensitive over this distance makes this a suitable tool to study interactions between macromolecules e.g. protein-protein interactions, especially because many protein dimensions fall within this range. Another requirement for energy transfer is that the time-span of the donor fluorescence lifetime must be adequate to allow the process to occur. Based on Förster’s theory the rate, $K$, of energy
transfer, is related to the fluorescence lifetime of the donor in the absence of acceptor (Equation 11):

\[ K = \frac{1}{\tau_d}(R_o/R)^6 \]  

(Equation 11)

where \( \tau_d \) is the lifetime of donor fluorescence in the absence of acceptor, \( R_o \) is the Förster distance or critical transfer distance at which energy transfer is 50 % i.e. 50 % of the donor excitation energy is transferred to the acceptor probe and \( R \) is the distance separation between the donor and acceptor probe. \( R_o \) also represents the distance at which the transfer rate (\( K \)) is identical to the rate of donor decay in the absence of acceptor. Revisiting the equations used to calculate distance values between donor and acceptor probes from section 2.10 in more detail, Förster’s theory relates the efficiency (\( E \)) of energy transfer to the lifetime of the donor in the presence and absence of acceptor (Equation 1):

\[ E = 1 - \frac{\tau_{da}}{\tau_d} \]  

(Equation 1)

where \( \tau_{da} \) is the lifetime of donor fluorescence in the presence of acceptor and describes the degree of donor quenching due to the acceptor. \( \tau_{da} \) is also sometimes referred to as the long-lived apparent lifetime of the acceptor in the presence of the donor, but in any case is still equal to that of the quenched donor fluorescence lifetime. The ability to measure these parameters, then, allows one to calculate the distance separating donor-acceptor pairs, but only with the aid of additional Förster equations.
The first equation determines the spectral overlap integral, $J$, which quantifies the extent of overlap between the donor emission spectrum and the acceptor excitation or absorption spectrum (Equation 2):

$$J = \sum \left[ \Phi_D(\lambda) \varepsilon_A(\lambda) \lambda^4 \Delta \lambda \right] / \sum \left[ \Phi_D(\lambda) \Delta \lambda \right] \quad (\text{Equation 2})$$

where $\Phi_D(\lambda)$ is the relative emission intensity of the donor at a specific wavelength, $\varepsilon_A(\lambda)$ is the extinction coefficient of the acceptor at the same wavelength, $\lambda$ is the wavelength in nanometers (nm) and $\Delta \lambda$ is the interval between wavelengths, typically 1 nm. The $J$ value, which depends on the chosen acceptor is then used in the second additional equation to determine the critical transfer distance ($R_o$) in nm (Equation 3):

$$R_o = \left( 8.785 \times 10^{-11} Q_D \eta^{-4} \kappa^2 J \right)^{1/6} \quad (\text{Equation 3})$$

where $Q_D$ is the quantum yield of the donor, $\eta$ is the refractive index of the solvent (typically $= 1.4$ for protein media, (Van Der Meer et al., 1994) and $\kappa^2$ is the orientation factor describing the relative orientation of the donor emission and acceptor absorption dipole moments with respect to each other (typically $= 2/3$).

Finally, Equation 4 shows how the efficiency of energy transfer is proportional to the inverse sixth power of the distance, $R$ separating the donor-acceptor pair and how this can be rearranged to obtain the distance separation value shown in Equation 5:

$$E = \frac{R_o^6}{(R_o^6 + R^6)} \quad (\text{Equation 4})$$

$$R = R_o((1/E)-1)^{1/6} \quad (\text{Equation 5})$$
Equation 4 supports that the efficiency of energy transfer is most sensitive when the R value approaches the critical transfer distance for the donor and acceptor. At distances below $R_o$, the efficiency of energy transfer quickly approaches 100%, while distances above $R_o$ results in a rapid decrease in E to 0% (Figure 6).
Figure 6. The exponential relationship between distance and efficiency of energy transfer. Since efficiency of energy transfer, $E$ rapidly decreases in proportion to the inverse sixth power of the separation distance, $R$ between donor and acceptor, FRET measurements are only accurate when $R$ is within $R_o$ by a factor of about 2 (shaded region). Distances outside this region cannot be determined reliably.
The critical transfer distance that is calculated essentially dictates the range of separation distances that can be determined by FRET for a donor-acceptor pair. Because the efficiency of energy transfer is highly sensitive to changes in distance when \( R \) is close to \( R_0 \), the dimensions of the specific molecular interaction under study is the main factor to be considered when choosing the appropriate donor-acceptor pair. Other important aspects to be considered when selecting FRET probes, depending on whether a steady state or time-resolved measurement is being performed include the quantum yield, fluorescence emission decay lifetimes and chemical stability. Besides probe selection affecting the \( R_0 \) value, other parameters shown in Equation 3 can also impact the calculated value. Since the quantum yield \( (Q_D) \) and spectral overlap \( (J) \) depend on local environmental conditions, the \( R_0 \) values must be determined using the identical experimental conditions as those used to detect energy transfer. Also, because \( Q_D \) and \( J \) values appear as the sixth-root in the \( R_0 \) equation, small variations in these values will not impact the critical transfer distance to an appreciable extent. The parameter most difficult to assess, if not impossible, to determine is the orientation factor, \( \kappa^2 \), which can vary between a value of zero and 4. Zero indicates the donor and acceptor dipoles are oriented perpendicularly to each other, which would therefore result in minimal energy transfer, while a value of 4 indicates both dipoles are parallel and co-linear, resulting in maximal energy transfer (Lakowicz, 1983). However, these extreme values require complete fluorescence polarization of the probes, a highly
improbable scenario, thus favoring intermediate $\kappa^2$ values. Due to the uncertainty of $\kappa^2$, though, a dynamically averaged value of $2/3$ is often assumed, which represents unrestricted random orientation via rotational diffusion of the donor-acceptor dipoles. Thus, critical transfer distances are usually determined by employing this averaged value, in which case a maximum error of 35% is possible because of the sixth-root relationship of $\kappa^2$ to $R_o$.

A variety of methods have been utilized for minimizing the uncertainty associated with the orientation factor, such as measuring the fluorescence anisotropy (mobility) of the donor and acceptor that can determine the range of $\kappa^2$. Also, utilizing fluorophores with low fluorescence polarization or assuming that a range of static orientations can exist and are not altered during the excited-state lifetime of the donor can minimize the uncertainty of the orientation factor. These techniques can potentially improve the calculation error of $R_o$ to about 10% (Lakowicz, 1983), but still in many instances the factor is often considered an impossible value to measure and so is assigned a value of $2/3$. The use of lanthanide atoms as fluorescent donors also significantly reduces the uncertainty of the orientation factor and is explained in more detail in section 3.6 and 3.7.

To summarize, then, any event that alters the distance separation between a donor-acceptor pair will cause a change in the rate of fluorescence resonance energy transfer. This rate is a function of the critical transfer distance, $R_o$, which in turn is influenced by the a) quantum yield of the donor fluorophore ($Q_D$), b) degree of overlap of the donor emission and acceptor absorption spectra.
(J), c) orientation factor describing the relationship between the donor and acceptor dipole moments ($\kappa^2$) and d) distance separating the donor-acceptor pair (R).

3.3 Measuring FRET via Steady-State Methods

A variety of methods can be used to detect and measure the event of FRET. The first is a fluorescence intensity-based detection method that is typically used in steady-state FRET (Selvin, 2002). Here, changes in the relative emission intensity at the two wavelengths corresponding to the donor and acceptor fluorophores are monitored. Although FRET can be characterized by an increase in emission intensity of the acceptor, $I(A)$ and a concomitant decrease in emission intensity of the donor, $I(D)$, often the ratio of the two values, $I(A)/I(D)$ is used to measure the event. Because the value of the ratio is dependent on the distance between the donor-acceptor pair, any perturbation in the system that alters the relative distance between the two probes would produce a change in the fluorescence intensity of the donor and acceptor. Some of the disadvantages though are that the concentration of the donor and acceptor fluorophores must be carefully matched in two different samples, thus being susceptible to titration errors. Also, excessive fluorophore levels can also cause self-quenching to occur and decrease the accuracy of FRET measurements.

To ensure quantitative results in steady-state FRET, it is important to minimize direct excitation of the acceptor fluorophore at the excitation
wavelength of the donor, and also minimize overlap of the donor and acceptor emission at the wavelength where acceptor emission occurs. Such crossover or spectral bleed-through is accounted for by using appropriate filter sets in the spectrophotometer enabling a specific range of wavelengths to pass, thus helping to increase the true signal-to-noise ratio associated with FRET (Herman, 1998). Another problem is presented by filter crosstalk, which occurs over a specific wavelength range when a donor and acceptor emission bandpass filter is placed in series. Here the origin of the emission signal cannot be attributed to either the donor or acceptor because of a certain degree of overlap and is of concern when matching excitation and emission filters for fluorescence sets. Dichromatic mirrors, which function to reflect light below a certain wavelength (usually excitation light) and transmit light above a certain wavelength (usually emission light) help minimize filter crosstalk. Additionally, background noise from the intrinsic fluorescence of a sample (autofluorescence), along with scattered light and signal from instrument components e.g. filters, lenses and cuvettes, must be subtracted from the fluorescence signal generated from extrinsic probes (Yuan and Wang, 2004). Here, the alternative use of longer lifetime lanthanide donor probes (1 µs to 10 ms), coupled with time-resolved fluorimetric techniques eliminates the contribution of the relatively short lifetime of background noise (1 ns to several µs). As Figure 7 shows, exciting these lanthanide probes with a short nanosecond pulse and measuring the fluorescence emission after a µs to ms delay, eliminates background noise and considerably improves the detection
sensitivity. The use of lanthanide probes as alternatives to the conventional FRET probes are discussed in more detail in section 3.6.
Figure 7. Eliminating unwanted fluorescence signals. A delay of about 150 µs between excitation and emission allows short-lived non-specific background fluorescence (autofluorescence) to decay before the long-lived emission of the donor (e.g. lanthanide probe) is measured. The region of data acquisition has been shaded.
3.4 Measuring FRET via Time-Resolved Fluorescence Methods

The second method used to measure FRET that doesn’t require careful control of donor and acceptor fluorophore concentrations is time-resolved fluorescence (Selvin, 2002). This technique is based on measurements of the donor fluorescence lifetime in the presence and absence of acceptor that allows quantitative determination of separation distances between the two probes. Measuring this decay in fluorescence intensity over time, which is not resolvable using the steady state approach, reveals the emission profile of the excited state fluorophore presenting a more detailed description of the donor-acceptor interaction and hence molecular interactions. Fluorescence decay data showing an identical average lifetime value, when measured as steady-state intensity normalized to absorption, may represent very different decay curve profiles compared to those generated via time-resolved methods, thus suggesting differences in the intermolecular processes involved in decay.

Fluorescence decay can be described in a single exponential form (Equation 12):

\[ I(t) = I_0 \left( \frac{1}{e^{t/\tau}} \right) \]

(Equation 12)

where \( I(t) \) is the fluorescence intensity measured at time \( t \), \( I_0 \) is the initial fluorescence emission intensity following the pulse of excitation light, and \( \tau \) is the time the fluorophore spends in the excited state before achieving the ground state (fluorescence lifetime). The \( \tau \) value is also defined as the time required for the intensity to decay to 1/e of its initial value, about 37% of \( I_0 \), and is the
reciprocal of the rate constant for fluorescence decay from the excited to the ground state.

Generally, two techniques can be used to determine the fluorescent lifetimes, the time-domain (pulsed) or frequency-domain (phase-resolved) method. In the time-domain method, the lifetime is measured using the emission signal or by counting the number of photons generated by each excitation pulse (Figure 8).
Figure 8. Time-domain method of measuring fluorescence lifetime. The fluorescence lifetime decays to $1/e$ (or 37% of $I_0$) in a specified time, $\tau$. Observed changes in $\tau$ are used to indicate changes in molecular interactions.
In the frequency-domain approach, lifetimes are determined from the reduced phase shift and modulation depth of the fluorescence emission signal that occurs after exciting the fluorophore with a sinusoidally modulated light source. Though this method is less demanding to perform, it is not as sensitive as the time-domain technique, however it can be used to extract multi-fluorophore lifetimes that can arise due to numerous relaxation pathways available in the environment. The disadvantage though, is that long exposure times can destroy the probes and may not provide sufficient temporal resolution, especially for studying live-cell processes. Also, a strong fluorescence signal is required, anticipating a high concentration of the fluorescing species together with a high fluorescence quantum yield.

3.5 Advantages of Time-Resolved Fluorescence

The main advantage of using time-resolved over steady state methods in FRET measurements is that greater quantitative accuracy is obtained in determining the donor-acceptor separation distance. This is due to the fluorescent lifetimes being independent of the fluorescent intensity, fluorophore concentration and for the most part, their insensitivity to photobleaching. However, fluorescent lifetimes are affected by the fluorophore environment, and even probes displaying a similar spectral pattern may yield different lifetimes due to influences posed by different conditions. Consequently, variations in lifetime can be exploited to provide molecular structural information. The environmental
factors that cause a change in the fluorescence lifetime of the fluorophore include the ionic strength, polarity, temperature and viscosity of the solvent. Also, the oxygen concentration and binding to macromolecules along with interactions with acceptor probes that quench the excited state by FRET affects the $\tau$ value. The fact that lifetime measurements do not depend on the fluorophore concentration, makes this a valuable tool to study molecular interactions.

The advantages of measuring FRET via fluorescence lifetime data are that it is highly sensitive in response to changes in distance between probes, the environment conditions, and is possible to observe energy transfer between donor-acceptor pairs exhibiting similar emission spectra. Unlike in steady state methods, direct fluorescence lifetime measurements can determine FRET without destroying the donor-acceptor probes via light. Also, because FRET reduces the fluorescence lifetime of the donor in the presence of the acceptor, comparison of the lifetime in its absence allows the efficiency of FRET to be easily determined.

3.6 Luminescence Resonance Energy Transfer (LRET) and Lanthanide Probes

There are some limitations associated with conventional FRET: A) the lifetimes of donor probes are very short (several nanoseconds) and multiexponential thus limiting the accuracy of lifetime measurements. B) The relative size and orientation of the organic-based dyes introduce uncertainty in
distance measurements. C) The signal of the sensitized emission can be low because of direct excitation of the acceptor and fluorescence noise from the donor (Selvin and Hearst, 1994). Many of these drawbacks can be potentially eliminated by using a modified technique that employs a luminescent lanthanide donor paired with an appropriate organic-based acceptor. This specific type of energy transfer is appropriately called Luminescence Resonance Energy Transfer (LRET), because lanthanide emission does not arise due to the singlet-to-singlet transition that is characteristic of the fluorescence phenomenon. Instead, emission mainly arises from electric dipole transitions (Xiao and Selvin, 2001), which is the same fundamental mechanism responsible for both FRET and LRET. Therefore, the electric field (E) component of light produced by a lanthanide donor and by an organic donor both decay with distance, R. For distances less than the wavelength of light (λ), E decreases as R⁻³ and for distances much greater than λ, E decreases as R⁻¹. Consequently, this leads to the same distance dependence for resonance energy transfer measurements (R⁻⁶) using either lanthanides or conventional dye probes.

Typically LRET allows for a more accurate distance determination and can measure separation distances in excess of 10 nm. The technique is also insensitive to incomplete probe labeling and generates a greater signal-to-noise ratio compared with using conventional FRET probes. Lanthanide probes include samarium, europium, terbium and dysprosium, but terbium and europium are most commonly used because of their high emission intensity (Sapsford et al.,
2006). Other characteristics of these probes include longer lifetimes (100 µs-10 ms) compared to their conventional counterparts (1-5 ns), sharp emission peaks and clear separation between excitation and emission peaks (large Stokes shift) (Yuan and Wang, 2004). The main benefit of longer lifetimes is the ability, via time-resolved fluorescence methods, to eliminate background signals, thus significantly improving sensitivity to the degree where concentrations as low as $10^{-12}$-$10^{-15}$ M can be detected (Sammes and Yahioglu, 1996). Also, because their lifetime decay is about a million times slower than their FRET counterparts, they are ideal to use in cases where distance changes occur on timescales slower than nanoseconds but faster than milliseconds (Selvin and Hearst, 1994).

Collectively, these properties also allow for temporal and spectral discrimination thus enabling lanthanide probes (e.g. Tb$^{3+}$) to be coupled with a large range of acceptor dyes e.g. fluorescein, tetramethylrhodamine, rhodamine, Cy3 and Cy5. Recall that the orientation factor is often a source of uncertainty in FRET measurements, however the use of lanthanide probes significantly reduces the uncertainty associated with $R_o$ and $R$. Since the lanthanides are spherical, donor emission is typically isotropic i.e. the same intensity is emitted regardless of the orientation of the atom. This is in contrast to the larger, rigid and planar organic dyes used in FRET that display anisotropic emission, which obviously depends on orientation of the donor/acceptor dipoles with respect to each other. The property of isotropic emission then eliminates the requirement in FRET that the donor and acceptor dipole moments must be almost parallel for most efficient
energy transfer to occur, and so eliminates uncertain distance measurements that are associated with the conventional FRET technique. In the case of terbium or europium, where the donor is unpolarized (unlike in FRET) and the acceptor is either perpendicular ($\kappa^2 = 1/3$) or parallel ($\kappa^2 = 2/3$) to the radius vector, the greatest error in $R_o$ would be $\pm 10\%$ if $\kappa^2$ is $2/3$. This value is a fair assumption to make because given the millisecond lifetimes of the lanthanides, there would be ample time for the acceptor probe to rotate, making $\kappa^2$ close to $2/3$. For these reasons then, the accuracy with which distance can be measured by LRET is far superior to FRET (Selvin, 2002).

3.7 Lanthanide Chelates

A lanthanide cannot function in LRET exclusively in ionic form, and so is usually complexed within a chelate ligand that serves several functions (Sapsford et al., 2006). The chelate binds the lanthanide ion tightly, so that high thermodynamic and photochemical stability is achieved (Sapsford et al., 2006). It also functions to shield the ion from the quenching effects of water, provides a means for attachment of an antenna group and a reactive group for linkage to biomolecules. Common reactive groups include isothiocyanates that react with amines (Li and Selvin, 1997), and maleimides, bromoacetamides or pyridyl dithio which all react with thiol groups e.g. on cysteine residues (Chen and Selvin, 1999). Though these reactive groups function to attach the chelate to biomolecules, their interaction with antenna groups can lead to multiple
conformations and thus yield multi-exponential lanthanide decays (Chen and Selvin, 1999). Presence of the antenna, usually an organic chromophore (e.g. in this study carbostyril 124, CS124) is required because of the inherently weak absorption of the lanthanide ion (molar extinction coefficient = 1 M⁻¹cm⁻¹). Essentially, the antenna absorbs excitation light e.g. from a nitrogen laser (337 nm) or a flash lamp (232 nm) and transfers the energy with some probability, $Q_{\text{transfer}}$ to the lanthanide (Figure 9).
Figure 9. The photophysics of chelates. The CS124 antenna (fused ring moiety) enhances the probability of exciting the Tb$^{3+}$ ion via energy transfer ($Q_{\text{Transfer}}$). This leads to photon emission of the lanthanide ($Q_{\text{Ln}}$). The linker group, N-ethylmaleimide, $R$ allows conjugation to macromolecules e.g. proteins via cysteine. The black jagged arrow represents the initial excitation light.
The lanthanide, in turn, emits the signal with some probability, $Q_{Ln}$ (the probability that a photon will be emitted from the lanthanide) as luminescence (Xiao and Selvin, 2001). Thus, the total quantum yield, $Q_{Total}$, the total probability that the lanthanide ion will emit a photon after excitation of the antenna, is (Equation 12):

$$Q_{Total} = Q_{Transfer} \times Q_{Ln}$$

(Equation 12)

Typically, for terbium or europium bound to DTPA-CS124, the range of $Q_{Transfer}$ is 0.4 to 0.75 and $Q_{Ln}$ is 0.25 to 0.53, thus making $Q_{Total}$ between 0.1 and 0.4 (Xiao and Selvin, 2001). For conventional organic dyes though, $Q_{Transfer}$ is 1 because there is no antenna group present, therefore $Q_{Total} = Q_{Ln}$. Since $Q_{Total}$ only affects the total brightness of the sample, only the $Q_{Ln}$ is proportional to the efficiency of energy transfer because it determines the strength of the donor electric field (Xiao and Selvin, 2001). Commercially available chelates including LANCE (Perkin-Elmer) and Cryptate (CIS-Bio, Packard) have been used in high-throughput screening assays, while polyaminocarboxylate chelates like DTPA-CS124 have been applied in conformational studies where subpicomolar detection limits have been obtained (Selvin, 2002). In the present study, the properties of the lanthanide Tb$^{3+}$ bound to DTPA-CS124 are exploited to measure distance changes between myosin and the C-terminal region of TnT upon myosin binding to the regulated actin filament. Energy transfer is characterized by both a decrease in the donor’s fluorescence emission intensity.
and lifetime of the excited state (reduced quantum yield), as well as an increase in the acceptor’s emission intensity.

3.8 Summary on Measuring FRET/LRET

As previously mentioned, there are several ways to measure FRET, and while some methods can be applied to LRET as well, others are exclusive to FRET. The methods common to both FRET and LRET include measuring E by: 1) a decrease in the donor emission intensity in presence of acceptor, 2) a decrease in the donor fluorescence lifetime, or 3) an increase in acceptor emission (sensitized emission). Though an increase in donor fluorescence intensity after photodestruction of the acceptor is a potential way to measure LRET, it is more often used in FRET (Jovin and Arndt-Jovin, 1989; Selvin, 2002). Only in FRET can an increase in the photostability of the donor in the presence of acceptor be used to measure E. Here, energy transfer to the acceptor causes a decrease in the donor lifetime, thus reducing the rate of donor photobleaching.

3.9 Conformational Searching of the Tb$^{3+}$ Chelate

A protein or chemical structure is predicted on the basis of a two step process that first scores the structure and then carries out a conformational search. The scoring function can be divided into three general classes: physics-based, knowledge-based or a mixture of the two (Lazardis and Karplus, 2000). In
the present study, a well established physics-based scoring function is used, termed the OPLS-AA forcefield. This forcefield has been extensively tested to detect native protein conformations from searching almost 49,000 decoy structures, and in comparison with six other empirical scoring functions consistently ranked the native structure higher than the decoys (Park and Levitt, 1996).

In our case, the fact that a relatively large chemical moiety, NEM-DTPA-CS124 is required to coordinate the donor Tb$^{3+}$ atom and position it adjacent to the TnT molecule of the Tn complex raises questions about the conformation of the chelate. Consider the extreme scenarios where the chelate is either fully extended or compact, where the distance measured from the point of chelate attachment (Cys263 TnT) to the Tb$^{3+}$ atom would be significantly different. In the extended structure, the Tb$^{3+}$ atom would be located further from the surface of TnT and thus would not represent the most accurate location of the C-terminal region of TnT (Cys263). Conversely, if the Tb$^{3+}$ atom was located close to the surface of TnT, then it would be a more accurate marker for the position of C-TnT. It is therefore important to determine a likely conformation of the chelate that indicates the degree of certainty for the location of TnT. The XRC structure of Tn that was used in the conformational search and atomic models was determined at a resolution of 3.3 Å. Thus, if a distance value higher than the resolution of the XRC structure is observed (< 3.3 Å) between Cys263 and the Tb$^{3+}$ atom, it would mean there is a higher degree of variability with respect to the
true location of Tn. However, if a distance value lower than the resolution of the XRC structure is observed (> 3.3 Å), then this would correlate with a highly accurate localization of C-TnT.

Conformational searching of small structures are often accomplished by computational methods that calculate the energy of the structure as a function of the degrees of freedom. More specifically, the molecular mechanics and energies are generally associated with the sum of the components including harmonic bond stretching, bond angle bending, torsional energies and non-bonded interactions of the molecule(s) of interest (Mackerell Jr., 2004). These parameters along with electrostatic terms describing the effect of partial charges of the atoms as well as solvent/solute interactions comprise the forcefields used to search for all the lowest energy, thus most energetically favored structures. The range of energies exhibited by a structure due to changes in the aforementioned parameters leads to a “turbulent” energy profile consisting of peaks (energy maxima), troughs (energy minima) and shoulders (saddle points). Thus, equations describing the total energy are combined with a variety of algorithms to search, ideally for the lowest energy structure in the entire conformational energy profile. This so-called global minimum can be rather difficult to locate because of the presence of multiple minima that represent local minima. The outcome of the search would also depend on the type and rigor of the forcefields (Klepeis and Floudas, 1999; Maranas and Floudas, 1994).
The ideal conformational search would be to systematically search the entire conformational landscape i.e. sample the energy over the entire range of each degree of freedom at specific intervals. However, the sheer quantity of conformations that are generated cannot be analyzed in a reasonable time frame. For example, if a two amino acid peptide was subject to a complete conformational search at 30° increments over a range of 360° and assuming each amino acid, n, exhibits a total of three rotatable bonds, the total number of conformations would be about 2.99 x10^6.

\[(360/30)^3n\]  
(Equation 13)

As Equation 13 suggests, the number of structures can be reduced by limiting the range over which the bonds are rotated (e.g. from 360° to 180°) as well as increasing the rotational increment of the bond (e.g. from 30° to 60°), in which case 729 structures would be generated ((180/60)^6). The number of rotatable bonds can also be decreased to reduce the value of the exponent along with the number of amino acids that are analyzed. Evidently, having fewer structures can significantly reduce the computational time making the process more practical.

Once the conformations are generated, they are energy minimized and each unique structure is saved along with the respective energy values. Finally, the fraction of a specific conformer can be calculated by an equation that uses the Boltzmann value, which is a weight factor related to the percent contribution of a certain conformation to the global properties of the protein/molecule under study (Wilson and Cui, 2004). Some of the methods used to generate these low
energy conformations include the Monte Carlo (MC), Molecular Dynamics (MD) and Stochastic Dynamics (SD) algorithms. The MC method randomly and not deterministically changes a few degrees of freedom at a time, which produces a wide array of conformational states, while allowing for many thermal fluctuations and searching higher energy structures. Here, structures are typically selected by the Metropolis algorithm where the energy of a conformer is accepted if the calculated transition probability value is greater than a random number between 0 and 1.

The MD method is based on the fact that molecules exhibit dynamic structural changes. These molecular motions are modeled using forcefields where Newton’s equations of motion are applied to describe the force and movement of atoms in a protein as a function of time. These simulations are termed deterministic, in the sense that the motion of atoms are determined over time from starting co-ordinates, velocities and forces (Landau and Binder, 2005). Though the scope of this discussion is not to explain the technical aspects of the algorithms, it is important to address some critical requirements of the technique. For instance, to eliminate significant errors in the energy calculation, small time steps, typically between 1/100-1/20 the duration of the fastest oscillation are specified. This timescale translates to about 0.1-1 femtosecond (fs) (http://mcdermott.chem.columbia.edu/ biophys_2002/search/backgroundII.html). The consequence of employing relatively longer time steps, for example, greater than 10 fs, without re-evaluation of the forces and velocities leads to the system
gaining more energy and eventually attaining a high energy, unstable state. However, even though the use of much shorter time steps provides a more accurate structural evaluation, proteins approaching about 15,000 atoms with MD are commonly limited to 300-400 picoseconds (ps) simulation time. MD is thus more appropriate for analyzing properties that depend on high frequency vibrations, but not for protein domain shifts, or rotations that occur on much slower timescales (http://mcdermott.chem.columbia.edu/biophys_2002/search/backgroundII.html).

A third method that can be used to generate low-energy conformers is similar to MD, but instead utilizes a non-explicit solvent model. This method consequently does not take all degrees of freedom into account like MD does and therefore results in a shorter simulation time. The SD method involves simulation of random forces and provides heat equilibration. The collisions of a solvent with a protein/molecule producing stochastic rather than deterministic dynamics can be approximated by the Langevin Dynamics (LD) or Brownian Dynamics (BD) method (Haile, 1997). In the former case, several parameters take into account the systematic force, the frictional effect of the solvent on the protein/molecule and the collisional effect of the solvent (random force). The energy of the protein/solvent will not drift, but it may fluctuate about a value, however in MD, the energy of the system must remain constant. When the collisional frequency (picosecond$^{-1}$) value is relatively large, the kinetic energy of the protein is rapidly attenuated by solvent collisions, and so the dominant type of
dynamics is described as Diffusive or Brownian. Conversely, when the collisional frequency value is relatively small, the system follows the previously described LD behavior (http://mcdermott.chem.columbia.edu/biophys_2002/search/backgroundII.html).

Though the SD method is not used in the present study to search the conformation of the Tb$^{3+}$ chelate, it’s one example of many conformational sampling methods that could be employed. Instead, the MC and MD conformational search algorithms are used to gain insight into the location of Tb$^{3+}$ with respect to the point of chelate attachment to chicken TnT. One of the main factors to consider when running a simulation is the duration of the computational time. Therefore, it is common to implement distance and other parameter constraints/limits that for example, freeze domains and allow other segments to move making the conformational search more time efficient and practical.
CHAPTER 4

RESULTS

4.1 Labeling of Myosin S1 and RLC with Acceptor Fluorophores

The contractile protein components, myosin, actin, Tn and Tm were isolated and reconstituted as described in the Materials and Methods section. The chemical labeling of α-chymotryptic S1 using tetramethylrhodamine-5-iodoacetamide dihydroiodide (TMR) and fluorescein-5(6)-carboxamidocaoroic N-hydroxysuccinimide ester (FHS), and labeling of the RLC of papain S1 with aminorhodamine B (AmrB) is shown in Figure 10.
Figure 10. A 15% SDS-PAG of purified and labeled myosin S1. From left to right: coomassie blue stained rabbit myofibril standard, α-chymotrypsin-digested S1, fluorescent labeled S1 with FHS, TMR and AmrB and their respective coomassie blue stained samples.
Note that α-chymotrypsin digested S1 contains only the ELC while papain digested S1 contains both light chains (latter not shown in Figure 10). In order to establish the side of the actin filament to which myosin S1 binds, papain S1 was used for LRET measurements between AmrB-labeled RLC and the C-TnT region. Absorbance spectra show ~50, 7 and 11 % efficiency of labeling (not shown), of TMR, FHS, and AmrB, respectively and an exchange efficiency of RLC-AmrB with endogenous papain S1 RLC of ~5 %. The specific amino acid that reacts with TMR (Cys707) and FHS (Lys553) and the one close to where Cy5-ADP binds (Trp131) is highlighted on the XRC structure of myosin in Figure 11 (Rayment et al., 1993a). The three labels that bind to the head domain of myosin S1 are located in or very close to locations that are likely to undergo conformational changes when binding to the actin filament. Trp131 resides in the ATPase site, where ATP/ADP can bind and influence the kinetic state of the myosin head, while TMR and FHS are located close to the actin binding site and lever arm, respectively.
Figure 11. Atomic model of labeled myosin S1. Myosin S1 heavy chain residues that react with or are located near acceptor fluorophores, Cy5-ATP, TMR, and FHS are shown as space-filled structures located at Trp131, Cys707, and Lys553, respectively. AmrB labels lysine(s) at the N-terminal of the RLC.
4.2 LRET in the Reconstituted Thin Filament and Myofibrils: Emission and Lifetime Decay Curves

In the reconstituted thin filament system, the distance change between the donor and acceptor-labeled S1 was monitored upon the addition of ATP and phosphate analogue in the presence and absence of calcium. Cy5-ATP is hydrolyzed by myosin to Cy5-ADP, thus indicating that myosin S1 assumes the post-powerstroke state, and that subsequent addition of phosphate analogue induces the pre-powerstroke state. Figure 12 shows an increase in the emission intensity of the appropriate acceptor labeled S1 and thus correlates with LRET between the donor (D) and acceptor (A). This also indicates that the donor and acceptor probes are within 1-10 nm for energy transfer to occur resulting in sensitized emission of the acceptor. Conditions tested include the control, i.e., in the presence of donor only, followed by addition of acceptor (A), ADP and then phosphate analogue (PA), either BeF$_x$ or AlF$_4$. 
Figure 12. Emission spectra of Tb\textsuperscript{3+}-labeled chicken TnT. The signal was measured in the reconstituted thin filament and in the presence of S1 labeled with A) Cy5-ATP, B) TMR, C) FHS, D) RLC-AmrB, E) Same as A), but in myofibrils (Myo). Each trace has been normalized at 545 nm (Em\textsubscript{max} of Tb\textsuperscript{3+}).
As previously mentioned, a more accurate method of showing the occurrence of LRET is by measuring the fluorescence lifetime decay of the Tb\(^{3+}\) signal and comparing it in various conditions. Here, a relatively gentle slope indicates a slow decay time because there is not a probe present that can accept the energy from the donor so the rate at which the energy decays is significantly slower. However, a relatively steeper slope indicates a faster decay time of the donor as observed upon the addition of acceptor, thus supporting energy transfer between the two probes. For instance, Figure 13 shows fluorescence decay curves in conditions similar to those described in Figure 12. However, measurements were obtained at the donor (D) \(\text{Em}_\lambda = 545\) nm and the specific emission wavelength of the acceptor (A). Ultimately, these lifetime decay curves were converted into distance measurements as described in section 3.2.
Figure 13. Fluorescence lifetime decay of Tb$^{3+}$-labeled chicken TnT. Myosin S1 labeled with A) Cy5-ATP, B) TMR, C) FHS, D) RLC-AmrB and E) Same as A), but in myofibrils (Myo).
4.2.1 LRET-derived Distances Between Donor and Acceptor Probes:

A) S1-Cy5-ATP

The fluorescence lifetime decay signals for all four S1 acceptor probes A) Cy5-ATP, B) FHS, C) TMR, D) AmrB and also Cy5-ATP-labeled myofibrils, were used to determine real distance values. Table 1 shows up to three myosin states: rigor, post- and pre-powerstroke, which were induced prior to the distance measurements in the presence (Y, yes) and absence (N, no) of calcium (Ca). Note, when using Cy5-ATP as the acceptor, the rigor state (no nucleotide state) was obviously not induced. Also, since TMR labeling of S1 is thought to hinder entry of phosphate analogues (PA), BeF$_x$ or AlF$_4$ was not used to induce the pre-powerstroke state. These conditions have been appropriately termed not applicable (NA). Recall that the $R_0$ value shows the distance at which E is 50%.
Table 1. LRET-derived distances in myosin S1 and myofibrils. The efficiency (E) of energy transfer between each donor (D) and acceptor (A) pair along with the distance separation (R) in nanometers (nm) is summarized for A) Myosin S1 and B) Myofibrils. Each value represents an average of three experiments.

### A) Myosin S1

<table>
<thead>
<tr>
<th>A Site</th>
<th>Ca PA</th>
<th>Ro</th>
<th>E</th>
<th>R</th>
<th>E</th>
<th>R</th>
<th>E</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy5-ATP</td>
<td>Y AlF$_4$</td>
<td>4.84 ±0.09</td>
<td>0.48 ±0.05</td>
<td>4.90 ±0.10</td>
<td>0.43 ±0.06</td>
<td>5.09 ±0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BeFx</td>
<td>4.88 ±0.06</td>
<td>0.53 ±0.07</td>
<td>4.78 ±0.17</td>
<td>0.51 ±0.07</td>
<td>4.85 ±0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.11 ±0.03</td>
<td>0.60 ±0.06</td>
<td>4.79 ±0.25</td>
<td>0.55 ±0.05</td>
<td>4.93 ±0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.08 ±0.05</td>
<td>0.61 ±0.06</td>
<td>4.73 ±0.16</td>
<td>0.56 ±0.05</td>
<td>4.90 ±0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FHS Lys553</td>
<td>Y AlF$_4$</td>
<td>4.02 ±0.02</td>
<td>0.72 ±0.04</td>
<td>3.44 ±0.11</td>
<td>0.76 ±0.05</td>
<td>3.33 ±0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BeFx</td>
<td>4.04 ±0.02</td>
<td>0.75 ±0.07</td>
<td>3.36 ±0.21</td>
<td>0.73 ±0.06</td>
<td>3.43 ±0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.01 ±0.05</td>
<td>0.75 ±0.07</td>
<td>3.33 ±0.11</td>
<td>0.76 ±0.03</td>
<td>3.30 ±0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.02 ±0.04</td>
<td>0.73 ±0.07</td>
<td>3.40 ±0.08</td>
<td>0.74 ±0.01</td>
<td>3.38 ±0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMR Cys707</td>
<td>Y NA</td>
<td>5.26 ±0.03</td>
<td>0.72 ±0.03</td>
<td>4.49 ±0.07</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N NA</td>
<td>5.24 ±0.02</td>
<td>0.67 ±0.01</td>
<td>4.65 ±0.06</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AmrB RLC</td>
<td>Y BeFx</td>
<td>5.20 ±0.01</td>
<td>0.02 ±0.01</td>
<td>9.97 ±0.36</td>
<td>0.02 ±0.01</td>
<td>9.86 ±0.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.11 ±0.03</td>
<td>0.02 ±0.01</td>
<td>9.97 ±0.36</td>
<td>0.02 ±0.01</td>
<td>9.86 ±0.80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### B) Myofibrils

<table>
<thead>
<tr>
<th>A Site</th>
<th>Ca PA</th>
<th>Ro</th>
<th>E</th>
<th>R</th>
<th>E</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy5-ATP</td>
<td>Y AlF$_4$</td>
<td>5.40 ±0.22</td>
<td>0.41 ±0.03</td>
<td>5.74 ±0.32</td>
<td>0.47 ±0.10</td>
<td>5.50 ±0.31</td>
</tr>
<tr>
<td></td>
<td>BeFx</td>
<td>5.52 ±0.13</td>
<td>0.34 ±0.04</td>
<td>6.17 ±0.31</td>
<td>0.35 ±0.07</td>
<td>6.15 ±0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.81 ±0.13</td>
<td>0.59 ±0.03</td>
<td>5.48 ±0.08</td>
<td>0.60 ±0.02</td>
<td>5.44 ±0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.74 ±0.16</td>
<td>0.44 ±0.04</td>
<td>5.97 ±0.10</td>
<td>0.50 ±0.05</td>
<td>5.76 ±0.31</td>
</tr>
<tr>
<td>#1 Cy5-ATP</td>
<td>Y AlF$_4$</td>
<td>5.65 ±0.08</td>
<td>0.62 ±0.07</td>
<td>5.20 ±0.22</td>
<td>0.63 ±0.03</td>
<td>5.15 ±0.02</td>
</tr>
<tr>
<td></td>
<td>BeFx</td>
<td>5.41 ±0.05</td>
<td>0.50 ±0.01</td>
<td>5.41 ±0.08</td>
<td>0.53 ±0.05</td>
<td>5.29 ±0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.73 ±0.16</td>
<td>0.63 ±0.01</td>
<td>5.23 ±0.11</td>
<td>0.64 ±0.02</td>
<td>5.19 ±0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.64 ±0.12</td>
<td>0.56 ±0.06</td>
<td>5.42 ±0.22</td>
<td>0.61 ±0.03</td>
<td>5.25 ±0.20</td>
</tr>
</tbody>
</table>

---

91
First, in the case of S1-Cy5-ATP, the results show that calcium does not cause a significant effect on the efficiency of resonance energy transfer in either the pre- (induced by phosphate analogue) or post- (ADP) powerstroke state, or even during the transition between the two states. This suggests the binding of calcium to the N-domain of TnC does not cause a substantial distance change between the C-terminal of TnT and the myosin ATPase site. This does not however, discount the possibility of a conformational change, since such an event can occur while maintaining the same distance between the two probes. Therefore in any case where two probes are used for LRET, one cannot assume there is no conformational change when no significant distance change is observed between various experimental conditions. Table 1A also shows comparable efficiency of energy transfer both in the presence and absence of calcium that indicates the pre-powerstroke state of myosin S1 forms irrespective of calcium levels. The distance values observed when using the acceptor Cy5-ATP probe range from about 4.5 to 5.3 nm.

4.2.2 B) S1-TMR

Second, for S1-TMR, results indicate the distance between probes in the rigor state is maintained (~4.3 nm) irrespective of calcium levels. Activated Tn (presence of calcium) also appears to maintain the distance separation between the labeled Cys707 and C-TnT during the transition from the rigor to the post-powerstroke state. However, a small but significant increase in distance (0.2-0.64
nm) is observed in the absence of calcium during the rigor-to-post-powerstroke state transition, along with 0.04-0.3 nm increase in distance in the absence of calcium observed in the post-powerstroke state (Table 1A).

### 4.2.3 C) S1-FHS

Third, for S1-FHS, the distance between the labeled Lys553 and C-TnT does not change significantly during the transition from rigor to the post/pre-powerstroke states or even between the post/pre-powerstroke states, either in the presence or absence of calcium. In accordance with Lys553 located close to the actin binding site, these results suggest the residue may reside at a fulcrum about which the myosin S1 head pivots during the powerstroke phase, thus yielding a consistent distance value in all three myosin states. The approximate distance between probes is 3.4 nm regardless of calcium levels, which is ~1 nm less in the analogous S1-TMR data, supporting then that Lys553 is located relatively closer to C-TnT. Again, low calcium levels do not appear to prevent formation of the pre-powerstroke state (Table 1A).

### 4.2.4 D) S1-RLC-AmrB

Fourth, a small but significant change is observed in the distance between S1-RLC-AmrB and C-TnT during the transition from rigor (~12 nm) to the pre/post powerstroke state (~10 nm). This may be indicative of rotation, bending
or simply high mobility of the RLC region upon addition of ADP/phosphate analogue (Table 1A). Also, these relatively large distances can be explained by the fact that very little energy transfer was observed suggesting the probes were positioned barely within the upper limit of the LRET technique. However, this data was vital in eliminating one of the two possible sites of the location of C-TnT with respect to the actin filament since the other potential site was located well outside the operating range of LRET.

4.2.5 E) Myofibril-Cy5-ATP

Finally, as observed in S1-Cy5-ATP studies, LRET measurements in myofibrils labeled with Cy5-ATP support that calcium does not cause a significant effect on the efficiency of resonance energy transfer in either the pre- (phosphate analogue) or post- (ADP) powerstroke state, or even during the transition between the two states. Of course as mentioned earlier, no detectable distance change can still mean a change in conformation. Interestingly, the distances measured in each myosin state are up to ~1 nm larger in the myofibril than those observed in S1-Cy5-ATP experiments. This appears to be most likely due to the influence of other contractile/structural muscle proteins native in myofibrils. For example, the binding geometry of myosin attaching to actin, which is influenced by the geometry of protruding myosin heads from the thick filament, may result in a slightly different binding geometry than free myosin S1 exhibits. Table 1B shows distance values for two different sets of myofibrils (#1 and #2) and in the
two tested myosin states (pre- and post-powerstroke) the distances were calculated to be between the mid 5 nm to low 6 nm range.

4.3 Myosin Binds Preferentially near Troponin

The fraction of donor-labeled Tn molecules (Tb$^{3+}$-labeled TnT) located close to acceptor-labeled myosin S1 on the actin filament was calculated based on a derivative of Equation 12 where the coefficient values (amplitude) of the quenched and unquenched signal of Tb$^{3+}$ correspond to the presence and absence of LRET, respectively. The results are summarized in Figure 14, where for S1-Cy5-ADP (post-powerstroke) and after the addition of AlF$_4$ (pre-powerstroke), calcium appears to have no significant effect on the fraction of Tn present near a bound myosin on the actin filament (~65-75%). However, in the pre-powerstroke state induced by BeFx, there is a small but significant decrease in the fraction of Tn present near myosin S1 going from high to low calcium levels (76% +/- 2 versus 62% +/- 7, respectively). S1-FHS binding shows ~80-90% of Tn located near a bound myosin S1 independent of calcium levels either in the pre- or post-powerstroke state. Although in the absence of nucleotide (rigor), ~60% of Tn is located near S1-FHS in the presence of calcium compared to ~75% in the absence of calcium. Evidently, the removal of calcium causes the amplitude of the quenched component to increase, thus suggesting a conformational change and an increase in the number of donor-acceptor pairs located in close enough proximity for LRET to occur. It is possible the observed
lower fraction of Tn located near a bound S1-FHS in the rigor state, is due to the modified Lys553 in the actin binding site of S1, which is also known to cause a decrease in the binding affinity for the actin filament (Bertrand et al., 1995).

Finally, S1-TMR binding suggests ~90% of Tn is located close to this S1 irrespective of calcium both in the rigor and post-powerstroke state. Although the graph for myofibril-Cy5-ATP is not shown, there is no significant difference between the fractions of Tn molecules located close to labeled myosin heads bound to the filament in either the pre- or post-powerstroke state (~30-35%). Clearly then, studies using myosin S1 show a higher proportion of Tn located close to the acceptor-labeled myosin S1 (~60-70%) than in myofibrils. Contrary to all myosin S1 data, the amplitude of the quenched component is smaller than the unquenched component suggesting that myosin binding to actin close to Tn is not as favorable in myofibrils versus that observed in S1 in vitro studies. This observation could also be due to a lower fraction of donor-labeled TnT being able to bind to the actin filament during the exchange of endogenous TnT. Also, the fact that donor quenching is observed in the presence of labeled myosin S1 supports that Tn is close enough to myosin for resonance energy transfer to occur.
Figure 14. Tn fraction located close to acceptor-labeled myosin S1. The ratio between the amplitude value of the quenched component of Tb$^{3+}$-labeled Tn (in the presence of acceptor-labeled myosin S1) and the sum of the amplitudes of the quenched and unquenched component, indicates a relatively high fraction of Tb$^{3+}$-labeled Tn is located near a bound acceptor-labeled myosin S1. This also suggests myosin appears to bind preferentially near Tn, even in the presence of transition state analogues, which induce the pre-powerstroke state.
3-D Mapping of C-TnT in Three Acto-Myosin Atomic Models

The LRET-derived measurements were used to visualize the spatial relationship between the C-TnT and S1 bound to the actin filament. Specifically, three previously derived atomic models show myosin S1 in the rigor state bound to the actin filament, while the distance values used to reveal the location of C-TnT are average values observed in the post-powerstroke state (Table 1). Since there was no significant difference in the distance values in the absence or presence of calcium, it was arbitrarily chosen to use the measurements in the latter condition to map the position of C-TnT. Figure 15 shows three atomic models designated Model 1, Model 2 and Model 3, showing the proposed location of C-TnT (white sphere) with respect to the acto-myosin complex. The Tn complex does not “fit” in the proposed location due to steric constraints presented in the current S1 (rigor) state. Model 1 was generated by the objective docking of S1 with actin using EM maps (Milligan et al., 1990). Model 2 was determined by Mendelson and Morris (1997) by the computational docking of S1 with Holmes et al. (1990) F-actin using EM maps. Finally, Model 3 was derived by conformational search-based docking of S1 with F-actin (Root, 2002b).
Figure 15. Location of C-TnT in three acto-myosin atomic models. A) Model 1, B) Model 2 and C) Model 3, show C-TnT (white sphere) located between myosin and actin. D), E) and F) illustrate a close-up of the respective Model, showing the close association of myosin loop 3 (purple) with C-TnT. Cy5-ATP (cyan), TMR (red) and FHS (yellow). N and C correspond to the actin terminals.
The atomic models were observed using the RasMol program and the LRET-derived distances measured from the Tb\(^{3+}\)-labeled TnT to the three individual myosin S1 labeling sites: Trp131, Cys707, and Lys553 were calculated as 4.84, 4.49, and 3.4 nm, respectively. Because the orientation of the acceptor probes are unknown, the distances were measured to the Trp131 C\(\alpha\) atom, Cys707 S atom and Lys553 terminal N atom. These distance values were then substituted into the atomic models that yielded two possible solutions to the exact position of Tb\(^{3+}\)-labeled TnT. To help determine which of the two would be the most probable location for C-TnT, the RLC of myosin S1 was labeled in the N-terminal region with AmrB. Because very weak energy transfer occurred (1-2 %), it suggested the distance between C-TnT and S1-RLC-AmrB was just close enough to account for the observed energy transfer. Thus, the likely location of C-TnT was found to be nearest the actin filament as observed in Figure 15. Visualization in the three atomic models shows C-TnT located near loop 3 (Pro570-Ala575) of myosin S1. Also, note that because triangulation of this position is calculated with respect to myosin S1, C-TnT relative to these sites is identical in all three atomic models. Therefore the position of C-TnT with respect to the actin filament will appear different in each model due to the different methods used to dock myosin S1 to the actin filament.

Table 2 summarizes the distances (nm) observed between C-TnT and various sites in the three atomic models: Model 1 (objective docking using EM maps), Model 2 (computational docking using EM maps) and Model 3 (purely...
computational-based docking). For example, the distance between C-TnT and loop 3 (purple) of myosin S1 (green) was about 0.7-0.9 nm, suggesting the possibility of direct contact between these two regions. Also, distances between C-TnT and the actin monomer that binds myosin S1 are shown with respect to the N- and C-terminal and actin helix Ser350-Ile357 (light blue).
Table 2. Comparison of distances from each acto-myosin atomic model. Summary of distances (nm) measured between Tb$^{3+}$-labeled TnT and various sites of the actin monomer binding directly to myosin S1 (light blue) or the adjacent monomer (dark blue) in three atomic models of acto-myosin. Colors in the table correspond to those regions/labels indicated in the atomic models in Figure 11 and 15. LRET-derived distances (gray row) were translated into each atomic model and underlined numbers correspond to the shortest distance.  

<table>
<thead>
<tr>
<th>S1 Label</th>
<th>Atom</th>
<th>Site</th>
<th>LRET</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy5-ATP</td>
<td>C$^\alpha$</td>
<td>ATP binding (Trp39)</td>
<td>4.49</td>
<td>4.49</td>
<td>4.49</td>
<td>4.49</td>
</tr>
<tr>
<td>TMR</td>
<td>S (C$^\alpha$)</td>
<td>SH1 (Cys707)</td>
<td>4.49</td>
<td>4.49</td>
<td>4.49</td>
<td>4.49</td>
</tr>
<tr>
<td>FHS</td>
<td>N (C$^\alpha$)</td>
<td>Actin binding (Lys553)</td>
<td>3.40</td>
<td>3.40</td>
<td>3.40 (2.90)</td>
<td>3.40</td>
</tr>
<tr>
<td>Protein/Motif</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin monomer that binds S1</td>
<td>C$^\alpha$</td>
<td>Actin N-term. (Glu2)</td>
<td>-</td>
<td>3.70 (4.32)</td>
<td>4.35 (1.55)</td>
<td>2.63 (4.42)</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>Actin C-term. (Arg372)</td>
<td>-</td>
<td>1.56 (4.12)</td>
<td>4.30 (3.45)</td>
<td>1.38 (4.68)</td>
</tr>
<tr>
<td>Actin helix</td>
<td>C$^\alpha$</td>
<td>Ser350</td>
<td>-</td>
<td>2.30 (4.89)</td>
<td>3.27 (2.65)</td>
<td>1.58 (5.14)</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>Thr351</td>
<td>-</td>
<td>1.92 (4.97)</td>
<td>3.17 (2.88)</td>
<td>1.21 (5.26)</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>Phe352</td>
<td>-</td>
<td>1.95 (4.62)</td>
<td>3.51 (2.67)</td>
<td>1.24 (4.93)</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>Gln353</td>
<td>-</td>
<td>1.87 (4.52)</td>
<td>3.62 (2.49)</td>
<td>1.18 (4.83)</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>Gln354</td>
<td>-</td>
<td>1.50 (4.68)</td>
<td>3.50 (2.80)</td>
<td>0.81 (5.04)</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>Met355</td>
<td>-</td>
<td>1.64 (4.42)</td>
<td>3.73 (2.80)</td>
<td>1.02 (4.80)</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>Trp356</td>
<td>-</td>
<td>1.91 (4.16)</td>
<td>4.05 (2.45)</td>
<td>1.28 (4.51)</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>Ile357</td>
<td>-</td>
<td>1.74 (4.15)</td>
<td>4.31 (2.53)</td>
<td>1.21 (4.54)</td>
</tr>
<tr>
<td>Actin monomer adjacent to the right of the S1 binding monomer</td>
<td>C$^\alpha$</td>
<td>Arg939</td>
<td>-</td>
<td>1.50</td>
<td>3.45</td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>His40</td>
<td>-</td>
<td>1.51</td>
<td>3.48</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>Arg95</td>
<td>-</td>
<td>3.22</td>
<td>0.78</td>
<td>3.20</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>Val96</td>
<td>-</td>
<td>3.24</td>
<td>0.71</td>
<td>3.29</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>Ala97</td>
<td>-</td>
<td>3.05</td>
<td>0.80</td>
<td>3.14</td>
</tr>
<tr>
<td>S1 (loop 3)</td>
<td>C$^\alpha$</td>
<td>Pro570</td>
<td>-</td>
<td>1.02</td>
<td>0.87</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>Ala571</td>
<td>-</td>
<td>0.91</td>
<td>0.74</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>C$^\alpha$</td>
<td>Ala575</td>
<td>-</td>
<td>1.27</td>
<td>1.52</td>
<td>1.27</td>
</tr>
</tbody>
</table>

*a* Model 2 (Mendelson and Morris, 1997) PDB file 1ALM contains only C$^\alpha$ coordinates, thus actual measurements to Cys707 sulfur atom (S) and Lys553 terminal nitrogen atom (N), in brackets, were calculated from Model 1 using RasMol.

---

102
Here, Model 1 and 3 showed the C-terminal of actin (Arg372) to be relatively close (~1.6 and 1.4 nm, respectively) to C-TnT, which again implied a possible interaction between the two regions. Distances between C-TnT and the actin monomer adjacent to the S1-binding monomer are shown in dark blue. Model 2, for example, showed the N-terminal to be about 1.5 nm away, while Model 1 and 3 showed the same location about 4.4 nm away. The interaction between myosin S1 loop 3 and C-TnT is intriguing because the loop has previously been suggested to interact electrostatically with actin helix residues 99-100. Figure 16 shows a close-up view of the loop potentially being able to bridge the gap and make contact with residues of the actin helix, possibly altering the position of C-TnT. Interestingly, conserved residues in these regions of vertebrate skeletal myosins include positively charged Lys572 and Lys574 and on actin, negatively charged Glu99 and Glu100, thus forming the basis for the implied electrostatic interaction. Note residues 572-574 are not mapped in the XRC structure due to their highly flexible nature.
**Figure 16.** Potential contact between myosin S1, TnT and actin. Interactions between myosin loop 3 (purple), C-TnT (white sphere) and actin helix Tyr91-Glu100 (red), particularly of the actin monomer adjacent to the one that binds S1 are possible. Specifically, Lys572/574 previously stated to interact with Glu99/100 (E99/100) is separated by ~3 nm. N and C are the actin terminals.
4.5 \( \text{Tb}^{3+} \) is Held in Close Proximity to Troponin T

To gain insight into the most probable conformation of the \( \text{Tb}^{3+} \) chelate that is attached to chicken TnT, but not necessarily the most accurate, conformational searches were performed computationally until the lowest energy structure was obtained. Indeed, Figure 17A shows the \( \text{Tb}^{3+} \) chelate may assume a relatively compact conformation that is positioned close to the surface of TnT and the \( \text{Tb}^{3+} \) atom is about 1.5 nm from the TnT sulfur atom of Cys263. This distance is about five times less than the resolution of the Tn XRC structure (0.33 nm of the 52 kDa Tn core), thus making the sulfur atom atom a viable site to measure distances. The complex as shown is about 7.5 nm wide by 10 nm high. A closer view of the lowest energy chelate (Figure 17B) shows \( \text{Tb}^{3+} \) coordinated by the closest three carbonyl oxygen atoms from DTPA. Also, the point of chelate attachment to TnT is shown in yellow via NEM. Carbostyril-124 (CS124) serves as an antenna to allow for more efficient \( \text{Tb}^{3+} \) excitation. Figure 17C indicates the residues from TnC (A110-G112 and G148-R149) and TnT (L259-C263) that were allowed to freely move during conformational searches due to their close proximity to the \( \text{Tb}^{3+} \) chelate. For example, L259, R262 and C263 were measured be within 0.5 nm.
Figure 17. Lowest energy structure of the Tb$^{3+}$ chelate. A) A ribbon diagram shows the conformation of Tb$^{3+}$-DTPA-CS124-NEM attached to Cys263 of TnT (yellow) complexed with TnI (orange) and TnC (blue) (Takeda et al., 2003). B) Close-up view of the chelate. Oxygen (red) and nitrogen (blue) atoms. C) Side profile of Tn backbone showing some TnC and TnT residues that were included in the chelate conformational search. Specific amino acids/numbers are shown.
4.6 Binding Assays of Tropomyosin, Troponin and F-actin

Having positioned the C-TnT in several atomic models it was necessary to determine the effect of Tn on myosin S1 binding to the actin thin filament. Here, a method was required to increase the number of Tn complexes binding to the actin filament per unit distance, in order to increase the number of observable myosin S1 molecules binding in close proximity to Tn. Consequently, a shortened construct of Tm was generated (dAc23 Tm) and tested to validate its use to increase the periodicity of Tn. In order to measure the degree of binding between F-actin and each type of Tm i.e. WT Tm, dAc23 Tm (both bacterially expressed) and chicken cardiac Tm, an ELISA binding assay was carried out using CH1, an antibody specific for Tm (Figure 18). It was observed that the binding affinity was comparable between all three types of Tm and CH1, confirming that this antibody can be used to monitor the binding to the actin filament. Also, this titration curve helped determine the saturating amount of CH1 (1:3000) that was then used in subsequent binding assays, which ensured the same binding response regardless of the type of Tm being used.
Figure 18. CH1 antibody titration with Tm. WT Tm, chicken (Chk) cardiac Tm and dAc23 Tm were coated on a microtiter plate and each generated a similar binding curve, supporting that CH1 binds with similar affinity to each Tm. A saturating amount of CH1 (1:3000) was used for all relevant binding assays that followed. A 10-fold serial dilution of CH1 was carried out beginning at 1:100. The inset shows the absolute binding of CH1 to Tm is comparable between all three Tm types.
Next, the binding affinity between cardiac TnT (cTnT) and each type of Tm was tested and not surprisingly, the chicken cardiac Tm exhibited the highest affinity for cTnT (~10-fold higher), while both the WT and dAc23 Tm forms showed similar binding characteristics to cTnT (Figure 19). The higher binding affinity of chicken cardiac Tm to cTnT, characterized by a rightward curve shift, appears to be due to acetylation of the N-terminal Tm region. This post-translational modification, as mentioned in previous studies, appears to enhance the degree of head-to-tail polymerization of Tm and therefore favors a higher degree of cTnT binding. Since acetylation does not occur in the prokaryotic system (thus is not present in the bacterially expressed WT and dAc23 Tm), the resulting perturbed Tm polymerization causes a marked decrease in cTnT binding. Thus, a much higher concentration of WT and dAc23 Tm is required to achieve a similar response compared to when chicken cardiac Tm is used.
Figure 19. Cardiac TnT binding to Tm. WT Tm, chicken (Chk) cardiac Tm and dAc23 Tm were coated on a microtiter plate followed by 4-fold serial dilutions of cardiac TnT (cTnT) beginning at 0.5 μM. Chicken cardiac Tm has the highest affinity for cTnT, while both WT and dAc23 Tm shows similar binding affinities. The inset shows the absolute binding of cTnT with chicken cardiac Tm is the highest.
Next, it was imperative to test the binding affinity of each Tm to F-actin, essentially to observe the effect of deleting actin binding modules 2 and 3. Interestingly, the binding affinity of each Tm to F-actin was similar, especially at lower Tm dilutions (higher Tm concentration), although at much higher Tm dilutions (lower Tm concentration), chicken cardiac Tm exhibited slightly lower binding affinity to actin (Figure 20).
Figure 20. Tm binding to F-actin. Actin (7-mer) was coated on a microtiter plate at 60 µg/ml followed by serial 3-fold dilutions of each Tm. The binding affinity between F-actin and each Tm (WT, Chk, dAc23) doesn’t appear to differ significantly at higher amounts of Tm.
In conclusion, while the inset in Figure 20 shows the absolute binding of dAc23 Tm is significantly less than the WT and chicken cardiac counterpart (fewer mutant molecules bind to the actin filament yielding a lower signal), it is still possible to use dAc23 Tm to increase the density of Tn complexes along the actin filament. Finally, the binding affinity of Tm-bound F-actin and cTnT was observed (Figure 21). In this case, chicken cardiac Tm complexed with F-actin binds cTnT with highest affinity, followed by the mutant form and then the WT Tm.
Figure 21. cTnT binding to F-actin-Tm. 30 µg/ml of F-actin (7-mer) was incubated for 1 hour at room temperature with saturating amounts of each Tm type (2 µM, determined from Figure 20) prior to coating the microtiter plate. 4-fold serial dilutions of cTnT were carried out. Interestingly, cTnT shows the least binding affinity to the WT Tm-actin complex while the mutant Tm appears to increase the cTnT binding affinity up to 4-fold.
As noted earlier, cTnT binds with higher affinity to chicken cardiac Tm most likely due to its ability to polymerize with higher efficiency and therefore form a viable head-tail junction for cTnT to bind. While the affinity is less for F-actin-dAc23 Tm to bind cTnT, the mere fact that it is possible supports that the mutant form can be used to increase the density of Tn molecules along the actin filament. F-actin-WT Tm exhibits weaker binding to cTnT suggesting that the mutant form is altered in such a way as to increase cTnT binding affinity. Note, despite beginning the cTnT serial dilution at 2 μM, the x-axis starts with 0.5 μM cTnT (the next dilution point) because at 2 μM the observed behavior was indicative of competitive binding between cTnT over binding sites on Tm.

4.7 Cosedimentation Assay of dAc23 Tropomyosin

Having determined that the shortened dAc23 Tm binds to actin and can facilitate binding of the Tn complex, via TnT binding our next goal was to determine the stoichiometry of Tm binding to the actin filament. Incubation of Tm dimer:actin at molar ratios of 5:7 and 10:7 confirmed the binding of both the WT and dAc23 Tm to F-actin (Figure 22).
Figure 22. Cosedimentation of F-actin with WT and dAc23 Tm. Tm dimer:actin was added in A) 5:7 and B) 10:7 molar ratio, respectively and incubated at room temperature for 6 hours. BSA was used as an internal control. Samples were divided into the pellet (p) and supernatant (s) fraction by ultracentrifugation at 120,000g for 30 minutes before being analyzed by 12% SDS-PAGE. Saturation of Tm on actin (A) allows both wild type (WT) and mutant (dAc23) forms to bind to actin as observed in the pellet samples.
Since both Tm forms do not form an appreciable pellet in the control samples, their pronounced presence in the pellet fraction along with F-actin supports the interaction and binding of the two thin filament proteins. Though an incubation of Tm dimer: actin at 20:7 molar ratio was also tested, the corresponding gel picture is not shown because the results from the 10:7 incubation were very similar. This was concluded from measuring the density of the gel bands (amount of protein present) in the pellet and supernatant fraction, the results of which are presented as a saturation plot (Figure 23).
Figure 23. Saturating F-actin with each Tm form. Densitometric analysis of the gel bands from the cosedimentation experiment (Figure 22) show WT Tm binds F-actin close to the expected 1:7 molar ratio (Tm dimer:actin), this value is reflected by the $B_{\text{max}}$ value, 1.25. The dAc23 Tm, however shows a 1.75:7 ratio ($B_{\text{max}} = 1.95$) consistent with it binding in a head-to-tail fashion along the actin filament, provided the incubation conditions are at least 10:7, Tm dimer:actin. Doubling the Tm dimer ratio to 20 did not increase the bound molar ratio of Tm dimer to F-actin, thus confirming that the saturation point had been reached. As a control, cosedimentation of chicken (Chk) cardiac Tm with F-actin revealed the expected 1:7 molar ratio, Tm dimer:actin (data point shown, but not fitted to an equation).
The saturation plot shows that when increasing amounts of Tm dimer were incubated with a fixed amount of actin, eventually the amount of WT and dAc23 Tm binding to F-actin reached a maximum. At this point, the actin filament is considered saturated due to all Tm binding sites on the filament being occupied by Tm. Chicken cardiac Tm was not tested at the same saturating conditions because it showed a Tm dimer:actin stoichiometry of 1:7 at much lower incubation ratios. Instead, it was necessary to determine the molar ratio of dAc23 Tm required to achieve complete binding to the actin filament. It has long been established that vertebrate skeletal Tm possesses 7 actin binding modules and therefore binds 7 actin monomers. Consequently, it is not surprising that the WT and chicken cardiac Tm also show the same molar binding ratio of 1:7, Tm dimer:actin. Note though, because of the absence of N-terminal acetylation on the bacterially expressed WT Tm, at least 5 times more Tm dimer is required to saturate the actin filament compared with the chicken cardiac form. Now consider the dAc23 Tm, which has two missing actin binding modules leaving only 5 to interact with 7 actin monomers, therefore it is expected to have a molar ratio of at least 1:5 (or 1.4:7), Tm dimer:actin. However, Figure 23 supports a molar ratio of 1.75:7 (or 1:4) suggesting that missing actin binding modules 2 and 3 affect the binding of modules either side of the deletion or possibly other modules away from the deletion. In this case a 2.35:7 (or 1:3) ratio would also be possible, but not likely since a ratio of 1.75:7 is observed. Therefore, to achieve a saturated
actin filament using dAc23 Tm in a reconstituted thin filament, the incubation conditions should include at least 10:7, Tm dimer:actin.

4.8 Myosin S1 ATPase Assay

The confirmed binding and function of dAc23 Tm on the actin filament allowed us to test a hypothesis that was first formed upon mapping the location of C-TnT (Figure 15). To observe the effect of Tn on myosin S1 binding to actin, a series of ATPase assays were conducted at varying concentrations of myosin S1. First, a phosphate standard curve was generated to help correlate measured absorbance values with phosphate release from myosin S1 (Figure 24).
Figure 24. ATPase phosphate standard curve. Addition of malachite green to varying concentrations of sodium phosphate yielded a linear $A_{630}$ response.
Subsequent myosin S1 ATPase experiments were then carried out ensuring the $A_{630}$ values were within the range of the standard curve. This helped determine the degree of phosphate release from myosin S1. Analysis of the actin-activated myosin S1 ATPase activity revealed that in the presence of calcium, WT Tm bound to actin yielded a greater saturating myosin S1 ATPase activity (Figure 25) than when the mutant form, dAc23 Tm was bound (Figure 26).
Figure 25. S1 ATPase activity with WT Tm reconstituted with actin and Tn. The amount of phosphate release (y-axis) by S1 was significantly higher in the presence of calcium, suggesting that the regulatory components were bound and functional on the actin filament. The dotted lines represent fitted data, while the colored lines are the background-corrected S1-ATPase measurements. Calculated parameters in the presence of calcium are: $B_{max}k_{cat} = 8.39 \mu\text{M} \cdot \text{S}^{-1}$, $K_d$ is 3.67 $\mu$M and root mean square (rms) value is 1.27. The corresponding values in the absence of calcium are 7.33 $\mu$M·S$^{-1}$, 20 $\mu$M, and 0.88, respectively.
Figure 26. S1 ATPase activity with dAc23 Tm reconstituted with actin and Tn. As in Figure 25, the amount of phosphate release was higher in the presence of calcium, suggesting that the mutant Tm and other regulatory components were bound and functional on the actin filament. However, a lower absolute value compared with when the WT Tm was bound to actin, supports dAc23 Tm serves to increase the number of Tn complexes bound along the actin filament. Fitted data is shown by the dotted lines, while the colored lines are the background-corrected S1-ATPase measurements. Calculated parameters in the presence of calcium are: \( B_{max}k_{cat} = 6.19 \, \mu\text{M}\cdot\text{S}^{-1} \), \( K_d \) is 1.31 \( \mu\text{M} \) and \( \text{rms} \) is 0.51. The corresponding values in the absence of calcium are 6.42 \( \mu\text{M}\cdot\text{S}^{-1} \), 17 \( \mu\text{M} \), and 0.22, respectively.
These findings support that the shortened Tm and consequent increase in Tn density results in Tn inhibiting the weak to strong myosin S1 binding transition, or the completion of the powerstroke. As expected, the binding affinity of myosin S1 to actin is much higher in the presence of calcium than in its absence. In Figure 25, where WT Tm was bound to actin, the $K_d$ of myosin S1 binding was 3.67 µM and 20 µM, in the presence and absence of calcium, respectively. Analogously, when dAc23 Tm was bound to actin, the $K_d$ of myosin S1 binding was 1.31 µM and 17 µM (Figure 25), clearly indicating that myosin S1 binds with a higher affinity in the presence of dAc23 Tm.

The ATPase data is also consistent with the deletion of two actin binding modules because the ratio of the calculated $B_{max} k_{cat}$ parameter (dAc23 Tm:WT Tm) in the presence of calcium is 5:7. However, in low calcium conditions this ratio is observed to be 6:7 or sometimes 3:7, suggesting the removal of either one or four actin binding modules, respectively. Originally, these results were thought to be due to an experimental error, but repetition suggests some other unknown element is causing the odd ratio. Typically in the absence of calcium, Tm is thought to sterically block myosin binding sites and prevent myosin S1 from binding productively to the thin filament. Therefore, one would assume to see minimal ATPase activity in low calcium conditions, but Figure 25 and 26 clearly show ATPase activity even in this condition. Such activity is due to a fraction of oxidized myosin S1 molecules (dead heads that always bind like rigor regardless of the presence of ATP). These dead heads will activate the thin
filament even in the absence of calcium and possibly allow more heads to bind actin and undergo the powerstroke, though as the graphs suggest, to a lesser degree than in the presence of calcium. Completely removing these dead heads from the myosin S1 preparation is very difficult, therefore it's important the sample is used within a week and treated with a reducing agent such as DTT to minimize the number of oxidized myosin S1 heads. Even so, as the myosin S1 concentration is increased a small fraction of dead heads will accumulate on the filament resulting in its activation.

4.9 Fluorophore Labeling of Rabbit Myofibrils

Despite prior knowledge of the acceptor label (Cy5-ATP) binding with high affinity to the myosin ATP site, it was important to confirm that the label did not bind to any other sarcomeric component since the LRET measurements using this acceptor were about 1 nm larger in situ (myofibrils) versus in vitro. Therefore, a label known to bind to the myosin ATP site, Alexa 647-ATP was used as the control and confocal images were compared with Cy5-ATP labeled myofibrils. Indeed, Figure 27 supports that both labels bind to the thick filament (A-band), more specifically where the myosin heads are located.
Figure 27. Localization of Cy5-ATP and Alexa 647-ATP in myofibrils. A) Cy5-ATP and B) Alexa 647-ATP both bind to the myosin heads of the thick filament in the A-band indicated by the fluorescent white bands. The distance measured between and including a pair of white bands represents the length of the A-band (small white line) while the white arrows point to the Z-lines, and thus define the sarcomere. In A), the average length of the A-band and sarcomere is 1.6 µm +/- 0.08 and 2.23 µm +/- 0.07, respectively. In B), the analogous measurements are 2.05 µm +/- 0.03 and 3.03 µm +/- 0.04.
5.1 The Effect of Labeling Myosin S1

Energy transfer between the Tb$^{3+}$ donor and each acceptor probe (Cy5-ATP, TMR, FHS and AmrB) is observed at the emission wavelength of the acceptor by an increase in signal upon the addition of acceptor to the sample containing donor. Structural changes that accommodate for the binding of ATP at the catalytic site of myosin and hydrolysis to ADP and inorganic phosphate (Pi) are thought to be transmitted to the actin binding cleft and the converter region (Rayment et al., 1993a), thus allowing movement of myosin heads along the actin filament. Consequently, the ATPase site represents a valuable landmark to assess positional changes with respect to other contractile elements e.g. in our case, the C-terminal region of TnT. The basis of choosing myosin S1 Trp131 to represent the ATPase site is due to its close proximity to the fluorophore moiety as observed in atomic models. Though the use of Cy5-ATP did not modify myosin S1 amino acids, we discuss how labeling with the other three acceptor probes could affect the function of myosin.

TMR attaches to myosin S1 at Cys707 (otherwise called the sulfhydryl 1, SH1 site), which resides near the myosin head/neck junction (converter region),
and is the most reactive labeling site on myosin (Root et al., 1991). While the consequences of complete myosin S1-Cys707 modification include strong inhibition of K⁺ (EDTA) ATPase activity and a decrease in acto-S1 ATPase activity, partial modification of this group shows the acto-S1 ATPase activity to decrease proportionally according to the level of SH1 labeling. However, acto-myosin ATPase activity remains high even at 50-60 % labeling of myosin heads on SH1 (Root et al., 1991). Therefore, because TMR binds with high specificity to SH1 and since ~50 % labeling efficiency was calculated by absorbance spectra, it suggests a sufficient level of acto-S1 ATPase activity was maintained. Note though, because different effects of labeling SH1 on myosin have been observed, the true form of the cross-bridge cycle is open to debate and so data with spectroscopic probes on this site must be interpreted carefully. Interestingly, Greene and Eisenberg (1980) have shown similar S1 binding results between iodoacetamide-labeled SH1 S1 and unmodified S1 in actin binding assays. In the present study, the use of TMR only permits the induction of the rigor state and the post-powerstroke state (addition of ADP). Because the location of TMR bound at Cys707 close to the converter domain is thought to hinder the entry of phosphate analogues, formation of the pre-powerstroke state was not studied.

Though a significant change is not observed in the position of Cys707 relative to C-TnT during the rigor-to-post-powerstroke transition in the presence of calcium, it does not rule out the possibility of a conformational change. This is because a similar distance can still be maintained even after an apparent structural
alteration (Table 1). However, in the absence of calcium there is a small, but significant increase in distance (0.2-0.64 nm) that supports binding of calcium to TnC inhibits a change in the position of Cys707 relative to C-TnT during this rigor-to-post-powerstroke transition. In any case, it was possible to measure the distance to help pinpoint the location of the donor probe attached to C-TnT.

FHS, which reacts with Lys553 of myosin S1, is located in the helix-turn-helix motif (residue 516-558) and is also an important site to monitor structural changes that occur upon myosin binding to actin. This site is near the primary actin-binding site thought to contribute to the tight, hydrophobic interaction of myosin S1 with subdomain 1 of actin (Rayment et al., 1993a). Cosedimentation experiments reveal no disruption of the rigor acto-S1 interaction when Lys553 is labeled and this does not drastically affect the biological activity of myosin S1 (Bertrand et al., 1995). Distance measurements from all three myosin states: rigor, pre- and post-powerstroke are possible due to no known ill-effects from using this particular label.

Although only three myosin S1-head sites (mentioned above) are required to triangulate the position of C-TnT, labeling of the RLC was necessary to eliminate one of two possible solutions to the position of C-TnT. AmrB attaches to the RLC of papain S1, although it is unclear which of the RLC N-terminal lysine(s) is (are) labeled. However, this region is extremely mobile and does not appear in crystal structures, so even knowledge of the specific labeling site is not likely to increase the precision of the modeling (Figure 11). Despite achieving a
very small degree of resonance energy transfer (1-2 %), sufficient data was
gathered to eliminate one of the possible positions of C-TnT. The cause of weak
energy transfer is likely due to the relatively large separation distance between
probes assuming, or even exceeding the upper limit of the LRET technique.

To support the LRET measurements derived from myosin S1-Cy5-ATP
and reconstituted thin filament proteins, Tb\(^{3+}\)-labeled chicken TnT was
exchanged into rabbit skeletal myofibrils. This represents the native state of thick
and thin filaments in the presence of all the other structural and contractile
elements and serves as a reference to compare the analogous myosin S1 data
to. Only Cy5-ATP was added directly to myofibrils because of its high affinity and
specificity to the myosin ATPase site. Similarly, TMR and FHS were not used in
the myofibrillar system to measure distances to C-TnT, because non-specific
labeling to other muscle proteins would invalidate the results. Collectively, the
presence of the full complement of muscle proteins will likely affect the behavior
of myosin interacting with actin. In myofibrils, one may expect myosin to be more
restrained due to the fixed location of the thick filament backbone and thus have
fewer degrees of freedom to approach and bind to the actin filament. These
effects however, are redundant in myosin S1 \textit{in vitro} studies and so myosin is not
restricted in its orientation and location prior to actin binding. Also, the
association of myosin with the thick filament backbone naturally imposes load on
the myosin molecule, which can be disregarded in myosin S1 experiments.
Hence, slightly different behavior of myosin is expected in these two systems.
Note the experiments done on myofibrils are contracted slightly and may cause discrepancies in results compared to myosin S1 data (Table 1).

Our data supports low calcium appears to allow the formation of the pre-powerstroke state due to the occurrence of LRET. It is likely that myosin can bind to the actin filament at low calcium levels, which is also supported by Maytum et al. (1999), whom propose actin filaments are ~67 % blocked, thus leaving ~33 % of the filament potentially available for binding myosin. Also, cryo-EM maps have indicated the N-terminal of Tm to bind the inner domain of the actin filament at low calcium levels, thus unmasking myosin binding sites and allowing myosin to interact with the thin filament (Narita et al., 2001).

5.2 Atomic Models suggest Interaction between Myosin Loop 3 and Troponin T

Our LRET studies indicate a potential contact point between myosin loop 3 and the C-terminal region of TnT, therefore suggesting that this region may play a regulatory role in striated muscle contraction. Model 1 (Figure 15) indicates myosin loop 3 to be the closest structure in proximity to C-TnT than any other myosin S1 or actin region, thus suggesting interaction between the two components. The shortest distance measured to loop 3 Ala571 is about 0.9 nm. This does not however, rule out the possibility of e.g. actin interacting with loop 3, but the mere fact that C-TnT is located closest to loop 3 strongly suggests interaction between these two regions. Indeed, though a relatively longer
distance to C-TnT is observed, for example from the C-terminal Arg372 of the myosin S1-binding actin monomer (~1.6 nm), or from the N-terminal Glu2 (~3.3 nm), the interaction of myosin loop 3 with actin is also thought to occur. For instance, disruption of loop 3 showed a decrease in the sliding velocity of actin filaments (Blotnick et al., 1995) and cross-linking between myosin loop 3 and actin was re-established after the skeletal myosin loop 3 sequence was substituted into Dictyostelium myosin (Van Dijk et al., 1999). Interestingly, the latter study was done because the myosin loop 3 and actin interaction has only been observed in skeletal muscle myosin and not in smooth muscle myosin, or even genetic constructs of Dictyostelium myosin (Van Dijk et al., 1999).

Model 2 (Figure 15) shows C-TnT in closest proximity to Val96 (~0.7 nm) of the actin monomer adjacent to the myosin S1-binding actin monomer. Previous studies have shown this region to interact with the myosin hypertrophic cardiomyopathy (HCM) loop, thus suggesting an interaction with C-TnT (Holmes et al., 2004). Loop 3 Ala571 is also ~0.7 nm away from C-TnT and is about 0.2 nm closer than the corresponding pair in Model 1. Both the N- and C-terminals of the myosin S1-binding actin monomer are located relatively far from C-TnT (~4.3 nm). Consequently, the N- and C-terminals of the actin monomer adjacent to the myosin S1-binding monomer are located closer to C-TnT (1.5 nm and 3.5 nm, respectively). Analogous distances in Model 1 show Glu2 and Arg372 to be located about 4.3 and 4.2 nm, respectively. Model 1 and 3 show the closest association to Gln354 of the helix in actin that binds myosin S1 (~1.5 and 0.8 nm,
respectively). Some regions of this helix are thought to take part in the "strong
binding" of myosin to actin and these results suggest C-TnT plays a role in that.
Model 3, interestingly shows identical distances between C-TnT and loop 3 as
that observed in Model 1. Distances measured to loop 3 Pro570, Ala571, and
Ala575 are about 1, 0.9 and 1.3 nm, respectively. The corresponding values
according to Model 2 are approximately 0.9, 0.7 and 1.5 nm. In Model 3, the
distance between C-TnT and the N-(2.6 nm) / C-(1.4 nm) terminals of the myosin
S1-binding actin monomer are the shortest observed out of all three models,
although it is comparable to that observed in Model 1. The N- and C- terminal of
the actin monomer adjacent to the S1-binding monomer is about 4.4 and 4.7 nm
away from C-TnT, respectively (also comparable to distances in Model 1). In
Model 1 and Model 3, C-TnT is placed identically with respect to myosin loop 3
and similarly with respect to residues Arg39, His40 and Arg95, Val96 and Ala97
of the actin monomer adjacent to the myosin S1-binding monomer (Figure 15
and Table 2). These sites may be the most probable to interact with the C-TnT
region since they are closest in proximity and it may be electrostatic in nature
given the positively charged residues in this actin segment.

All three atomic models represent myosin bound to actin in rigor and
because it may be possible for loop 3 to extend across the gap and make contact
with actin residues Tyr91-Glu100 (Holmes et al., 1990). The position of C-TnT
derived by LRET measurements in the post-powerstroke state, located between
loop 3 of myosin and the adjacent actin helix, Tyr91-Glu100 suggests the
possibility of steric hindrance (Figure 16). This is discussed further in the next section.

5.3 Troponin may Sterically Hinder Completion of the Myosin Powerstroke

It has been shown before that each myosin head interacts with two actin monomers forming primary and secondary binding sites (Holmes et al., 2004; Rayment et al., 1993b). Binding of myosin to actin may consist of a multiphase process, first involving the formation of a weak ionic interaction, then a stronger stereospecific interaction. The weak ionic interaction is thought to be responsible for the ionic strength-dependent "weak binding" of myosin to actin, which is thought to be mediated between the Tyr626-Glu647 segment of myosin and Asp1-Glu4 and Asp24-Asp25 of actin (Brenner et al., 1982). Similarly, myosin "strong binding" to actin is thought to occur via stereospecific contacts between Pro529-Lys553 of myosin and actin Ile341-Gln354 and Ala144-Thr148. However, chemical modifications such as those mentioned in section 5.1 affect myosin S1-ATPase activity, and consequently affect the binding of myosin to actin. For example, depending on the degree of labeling of Cys707 an inhibition of ATPase activity is observed, while labeling of Lys553 causes a decrease in activity (Root et al., 1991; Root, 2002a). This is likely to hinder the extent of strong binding to actin, however we assume that it does not have an effect on the observed position of C-TnT. Note, all three atomic models show C-TnT located near helix 350-358 of the actin monomer that binds myosin S1, and that this includes part of
the actin region (residues 341-354) involved in strong binding to myosin (Table 2). This suggests that myosin Pro529-Lys553, previously proposed to bind strongly to this part of actin may be inhibited from doing so due to the steric hindrance posed by the C-terminal region of TnT. More specifically, as the atomic models suggest (Figure 15), the swinging motion of the myosin lever arm (powerstroke) or the transition from the weak to strong binding at this specific location on the actin filament appears to be inhibited. The steric effects presented by Tn on myosin binding to actin in this region must be accommodated for and this may be done through establishing unique contacts between the individual components of myosin, actin and Tn. Now this accommodation would only be expected to occur with respect to those myosin molecules binding adjacent to the Tn complex, while those myosins binding away from Tn, free from steric hindrance, would likely adopt the conventional weak and strong binding contacts and thus be able to produce force.

To gain more information on whether the motion of the myosin S1 lever arm, upon ATP hydrolysis, is hindered by Tn, the use of a shorter Tm (dAc23) served to increase the number of Tn complexes in the reconstituted thin filament. Here, the absorbance signal generated from myosin S1 ATPase assays (hydrolysis of ATP to ADP and Pi) would be expected to decrease as a result of the increased number of Tn complexes potentially inhibiting the full motion of the myosin S1 lever arm and subsequent hydrolysis of ATP. Consequently, conversion of the measured change in absorbance to the rate of inorganic
phosphate release i.e. release of Pi from the myosin S1 catalytic site was done using the ATPase standard curved shown in Figure 24. The design of the ATPase assays includes performing the experiment using a fixed low thin filament concentration with increasing amounts of myosin S1. This ensures that the measured signal is proportional to myosin S1 binding. Figure 25 and 26 shows the myosin S1 actin-activated ATPase activity in the presence of WT and dAc23 Tm, respectively. Clearly, there is a reduction in ATPase activity even in the presence of calcium when the shorter Tm is bound to the actin filament, this indeed supports that Tn is inhibiting the activity of myosin S1. This inhibition can be explained by a decrease in phosphate release when myosin S1 binds adjacent to Tn and the lever arm is not able to complete the powerstroke presumably due to the physical presence of Tn. The advantage of using the shorter Tm amplifies the decreased ATPase activity of the myosin S1 molecules binding adjacent to Tn, since the mutant Tm effectively increases the number of Tn complexes bound per unit length of actin. This behavior is also apparent in another LRET-based study done in our laboratory where the myosin lever arm could not assume a post-powerstroke orientation when binding close to Tn (Coffee and Root, 2007).

There are also other lines of evidence that support our findings with respect to the interactions between specific contractile components as well as factors that may impact the behavior of the myosin lever arm. For instance, an interesting study by Milligan et al. (1990) showed that myosin S1 possessing the
longer ELC isoform (A1) binds actin with a higher affinity than myosin S1 possessing A2, the shorter ELC isoform, and may stabilize actin-actin contacts promoting actin polymerization in vitro. ELCA1 has an additional 40 residues at the N-terminal which binds to actin residues 360-363 at the C-terminal (Sutoh, 1982; Trayer et al., 1987). This region is close to actin helix 350-358 which is the closest segment to C-TnT (Figure 15 and Table 2). Thus, in our experiments, the fraction of myosin S1 possessing ELCA1 may not be able to interact with actin residues 360-363 due to the perturbation posed by TnT, and so prevent the enhanced binding of this S1-ELCA1 isoform from being realized. How this may affect the swinging of the lever arm is a matter of debate and the implications of the two different ELC isoforms binding close to Tn remain to be seen.

Our LRET data agree with a previous study (Labbé et al., 1995) that show two actin α-helices, 79-92 and 338-368 that bind myosin in the presence of ATP. Table 2 supports loop 3 potentially interacts with both of these actin helices (or residues very close to them) and as before the apparent obstruction on the myosin lever arm imposed by C-TnT indicates that the lever is inhibited from completing the powerstroke. Additional evidence eliminates specific regions of actin involved in myosin binding (Korman and Tobacman, 1999; Korman et al., 2000), while some supports the importance of the aforementioned helices via mutational and FRET studies (Razzaq et al., 1999; Moens and dos Remedios, 1997; Johara et al., 1993; Chen and Lehrer, 2004). For example, mutational studies by Johara et al. (1993) showed a five-fold decrease in in vitro motility.
assays suggesting an important role for the interaction between loop 3 and residues close to actin helix 79-92. The interaction appears to involve positively charged residues on myosin (Lys572 and Lys574) and negatively charged residues on actin (Glu99 and Glu100), the importance of which is noted by their conservation in vertebrate skeletal myosins (Rayment et al., 1993b).

Interestingly, FRET measurements have shown when myosin S1 is induced to assume the rigor state it binds near Tn on the actin filament. However, when the post-powerstroke state is induced, binding is not favored (Coffee and Root, 2007). This suggests the myosin S1 rigor state is accommodated via structural changes potentially occurring in myosin, actin, Tn and Tm, while analogous changes resulting from the induced myosin S1 powerstroke state appear to inhibit the interaction with actin. Without such changes, the Tn complex does not fit into the postulated location observed in the atomic models (Figure 15). Even previous studies have shown atomic models of myosin S1 docked onto F-actin interpenetrating the contact region implying that myosin S1 and actin must undergo structural changes to accommodate for the rigor complex (Schröder et al., 1993).

Several studies have also attempted to shed light on the position of Tn using electron microscopy. For example, Lehman et al. (2001) support the interaction of the globular head (N-terminal) domain of Tn with Tm and the extreme periphery of subdomain 1 and 2 of the same actin monomer in the absence of calcium. The association with actin, however, is abolished upon the
addition of calcium, which then allows myosin heads to bind to the actin filament. Our data supports that the C-terminal region of Tn does not shift appreciably upon the addition of calcium and is consistent with Lehman’s data since the so-called stalk region of Tn, which is believed to include the C-terminal region of TnT, does not shift significantly. Note that our LRET studies are carried out in the presence of myosin S1, which may restrict the motion of C-TnT even when myosin binds weakly and strongly to actin. Our LRET data also supports previous findings that myosin S1 binds to thin filaments even in the absence of calcium (Chalovich and Eisenberg, 1982), hence supporting the formation of the pre-powerstroke state or a weakly bound state as described by Greene and Eisenberg (1980). Besides effects on TnT, other studies support that in the absence of calcium, TnI forms a C-clamp configuration around the N- and C-terminal region of actin, and together with Tm, inhibits muscle contraction (Narita et al., 2001; Johara et al., 1993). It is conceivable, though, that Tn alone sterically hinders myosin S1 from completing the powerstroke as discussed earlier.

So far our results imply myosin S1 behaves differently, depending on where it binds with respect to Tn on the actin filament. For instance, myosin S1 binding away from Tn can easily proceed through the motions of the powerstroke via swinging of the lever arm, while the powerstroke of the myosin S1 binding adjacent to Tn appears to be inhibited due to the physical presence of Tn. Next, we discuss the likely structural basis for this differential behavior of myosin S1
and the favored target zones that result to permit productive binding along the actin filament, as well as the functional role this may play in muscle cells.

5.4 Tropomyosin and Troponin Determines Myosin Binding Target Zones along the Actin Filament

N-terminal acetylation is required for striated Tm to bind in a head-to-tail fashion with high affinity to the actin filament, but in the presence of Tn, unacetylated Tm binds appreciably and regulates the acto-myosin ATPase activity (Heald and Hitchcock-DeGregori, 1988; Urbancikova and Hitchcock-DeGregori, 1994). Bacterially expressed dAc23 Tm and WT Tm is unacetylated, so a saturating amount of Tm along with Tn ensured that the regulatory component was able to bind to the actin filament. The notion of Tm serving to space out Tn complexes along the actin filament and to define where myosin S1 binds productively along the thin filament is supported by protein binding, cosedimentation and ATPase assays. First, the ELISAs confirm that dAc23 Tm binds actin and TnT much like the WT form does (Figure 19-21) and the cosedimentation assay demonstrates a molar ratio of 1.75 dAc23 Tm dimer bound per seven actin or one dimer to every four actin monomers (Figure 22 and 23). Based on the fact that Tm actin binding modules 2 and 3 have been deleted, one would expect the resulting Tm molecule having five actin binding modules to bind the same number of actin monomers. However, a slight discrepancy is observed between this theoretical value and the one observed in the
cosedimentation assay, implying that the deletion affects the structure of Tm to the extent that the stoichiometry of actin binding modules to actin is slightly more than 1:1. It is quite feasible that the regions adjacent to the Tm deletion can be structurally altered and impact how Tm binds to actin, it can also potentially have long range effects on the structure of Tm, ultimately influencing its stoichiometry with actin. Although it is not known how the structure of Tm may be altered, evidence strongly suggests it is not a rigid coiled-coil, but rather is a highly dynamic structure that is prone to fluctuations. For example, Nitanai et al. (2007), showed via XRC studies that Tm is comprised of multiple domains that suggest the coiled-coil is not smooth and homogenous, but rather undulating and highly flexible. Also, the conformational flexibility of Tm, as shown by Nuclear Magnetic Resonance and Circular Dichroism methods, appears to be very important for the ability of the Tm C-terminal to form complexes with Tn (Greenfield et al., 2002). In addition, the structure of Tm is relatively susceptible to changes depending on the ionic strength and other experimental conditions used (Hitchcock-DeGregori and An, 1996). In any case, the cosedimentation data suggest the short Tm is binding head-to-tail and thus able to increase the number of Tn molecules binding along the thin filament (Figure 22 and 23). Tm mutants of different lengths have also been previously observed to saturate the thin filament as indicated by the same Tm:actin mass ratio, thus implying that Tm aligns in a head-to-tail manner (Hitchcock-DeGregori and An, 1996). Consequently, this eliminates the possibility of the actin filament structure being responsible for Tn
binding every seven actins, instead it is the Tm length that governs the periodicity of Tn along the thin filament.

Several models depict how this shorter Tm affects the alignment of Tn compared with WT Tm or native Tm. First, Figure 28A shows head-to-tail binding of WT Tm is believed to cause the regulatory complex to align across from one another on each actin protofilament (Chen and Lehrer, 2004). If the Tn complexes were instead staggered along the actin filament then the arrangement may look like that modeled in Figure 28B. If the short Tm (dAc23 Tm) binds to the original location as the WT Tm on the actin filament, then despite Tn being aligned, Tm would not bind head-to-tail and so would form gaps along the filament (Figure 28C). Such an arrangement would likely decouple allosteric mechanisms associated with the full length Tm that cooperatively unmask myosin S1 binding sites on the thin filament. The proposed model in Figure 28D shows how the shortened Tm may bind to the actin filament and also fits the ATPase assay results that support Tn hindering myosin S1 function. As supported by the co-sedimentation assays (Figure 22 and 23), head-to-tail binding of the short Tm increases the number of Tn complexes, which correspondingly reduces the length of the myosin S1 binding target zone.
Figure 28. Effect of Tm on the alignment of Tn along the actin filament. Head-to-tail binding of WT can cause Tn to A) align or B) stagger with respect to Tn on the other actin protofilament. C) dAc23 Tm binding in the same location as WT Tm cannot bind head-to-tail. D) Head-to-tail dAc23 Tm binding would increase the number of Tn complexes reducing the myosin S1 binding target zone.
5.5 Myosin Binding adjacent to Troponin may Function to Attenuate the Overlap of Thick and Thin Filaments in Muscle Contraction

It was determined that myosin binding in immediate proximity to the Tn complex (Figure 15) is inhibited from completing the powerstroke, implying that it would not contribute to force generation when muscle contraction is triggered. Though it is not clear what physiological purpose these myosins serve, the data implies they may act as mechanical sensors, perhaps operating in a communication pathway with the more well-documented structural proteins like titin. As suggested by Coffee and Root (2007), this type of myosin S1 behavior could imply a safety mechanism to attenuate the degree of thick and thin filament overlap. Consequently, this would ensure excessive and potentially damaging overlap does not occur and that elasticity is maintained for the filaments to revert to their original relaxed position. For optimal force generation to occur during contraction, it is important the sarcomere length is maintained and even hyperextension is protected against (Wang et al., 1991, 1993). Recall that the relaxed sarcomere is typically about 2.3 µm along the long axis of the muscle fiber and one of the most important structural/mechanical elements that helps maintain this length is the titin molecule.

Titin is the largest protein discovered to date at 3.7 megadaltons (MDa) and comprising about 38,000 amino acids (Luft, 2006), it functions as a molecular spring providing elasticity and passive tension, while also keeping thick filaments centered between the Z-lines (Trinick, 1994; Trinick and Tskhovrebova,
This elastic property is attributed to three distinct structural domains, the tandem immunoglobulin (Ig) repeats, the PEVK region (rich in proline, glutamate, valine, and lysine) and the N2B/A region, an extensible region that differs in length and passive stiffness (Helmes et al., 1999; Lu and Schulten, 1999; Lu et al., 1998; Krammer et al., 1999; Marszalek et al., 1999). An estimated six titin molecules associate with the myosin thick filament (Carzola et al., 2000) and span half the sarcomere (greater than 1 µm), with its N- and C-terminal located at the Z- and M-line, respectively (Furst et al., 1988). With the aid of other supporting structural proteins that help sense the degree of muscle contraction/extension (Luft, 2006), titin exerts force similar to a compressed spring relaxing back to its original length and enhances the rate of relaxation of the contractile elements to their original position, thus priming the filaments for another contraction cycle. Conversely, titin also generates tension when the sarcomere is stretched, thus maintaining thin and thick filament overlap. For example, hyperextension of the sarcomere to 3.7 µm would cause loss of filament overlap to where force production would be eliminated. Similarly, hypercontraction of the sarcomere to 1.3 µm would not allow force to be generated because the ends of the thick filament would collide with the Z-lines and thus reduce the number of viable cross-bridges able to produce force (Gordon et al., 1966a, 1966b). It could be possible then, that myosin binding adjacent to Tn plays a pseudo-regulatory role in muscle contraction by transmitting the contractile status of the muscle fiber to the main mechanical
proteins that maintain the structural integrity of the sarcomere e.g. titin. It is also conceivable that Tn, considering its close proximity to myosin participates in a possible communication pathway, even with Tm, to constantly provide input to titin and other mechano-sensory proteins on the structural condition of the muscle.

5.6 Justification of using dAc23 Tropomyosin and Effect of removing other Actin Binding Modules

An interesting observation from the ATPase assay is the observation of myosin S1 binding with higher affinity to the actin filament complexed with dAc23 Tm versus that complexed with WT Tm (Figure 25 and 26). This behavior may be explained by evaluating the K_d values of the binding assays. Recall that the K_d is the myosin S1 concentration at which the binding sites on the actin filament are half occupied. Now because dAc23 Tm increases the periodicity of Tn complexes along the thin filament, it reduces the number of actin monomers available for myosin S1 binding. Therefore, this effectively reduces the myosin binding target zone along the actin filament (compared with the WT) and so it potentially becomes easier to occupy half the binding sites for a given filament length using the same amount of myosin S1, hence explaining the lower K_d value. Also, several studies strongly indicate the conformation of the actin filament changes along with Tm-Tn and myosin S1 when the thin filament state changes between the blocked to the open state (Lu et al., 2003; Lu et al., 2006; Singh and
Hitchcock-DeGregori, 2007; Tobacman, 1996; Hill et al., 1992; Lehman et al., 1994; Lorenz et al., 1995, Cassel and Tobacman, 1996). These structural changes are likely to impact the binding affinities of each of the proteins, thus making it difficult to assess which protein(s) cause an alteration in $K_d$ of which other protein(s).

Although the dAc23 Tm binding affinity to acto-myosin S1 and \textit{in vitro} sliding speed has been observed to decrease (Lu et al., 2003) along with a decrease in force and stiffness (Lu et al., 2006), it has long been established that the removal of actin binding modules 2 and 3 of Tm preserves Ca$^{2+}$ regulation of \textit{in vitro} motility and actin activated myosin S1 ATPase activity (Hitchcock-DeGregori and Varnell, 1990; and Hitchcock-DeGregori and An, 1996). This validates the sole purpose of using the shortened Tm in our experiments to increase the density/periodicity of Tn complexes that bind to the actin filament, ultimately to determine the ATPase behavior of myosin S1 binding adjacent to the Tn complex. Also, the shortened Tm possesses actin binding modules 1 and 5, which are thought to provide the optimal degree of affinity to the thin filament, and allow Tm to shift dynamically between the blocked and fully activated actin filament state (Singh and Hitchcock-DeGregori, 2007; Hitchcock-DeGregori et al., 2001; Hitchcock-DeGregori et al., 2002; Landis et al., 1997, 1999; Sakuma et al., 2006).

The effect of removing other Tm actin binding modules has been demonstrated, for example by Landis et al. (1997,1999) and Sakuma et al.
(2006), where missing modules 3-5 appear to reduce the ability of myosin to shift the thin filament to the open, fully activated state. Curiously, a research group that used a Tm with only four acting binding modules present (dAc234) observed a decrease in myosin S1 ATPase activity, with weaker myosin S1-ADP binding, but with increased cooperativity than when WT Tm was bound (Rosol et al., 2000). However, this group’s hypothesis of an increased periodicity of Tn on actin hindering myosin S1 binding to the thin filament could not be supported. This was due to an assay performed in the absence of Tn that showed a strong inhibition in motility (Landis et al., 1997; Rosol et al., 2000) and therefore suggested Tn was not responsible for the observed decrease in myosin S1 ATPase activity. Despite these findings, the presence of Tn actually increases Tm’s ability to bind actin (Hill et al., 1992), which in turn is likely to alter its structure and behavior upon myosin S1-binding. Therefore, the assay doesn’t seem to provide unequivocal evidence that Tn does not hinder myosin S1 ATPase activity.

With respect to the affinity of Tm binding to actin, several lines of evidence show that deleting internal actin binding modules from Tm does not significantly weaken actin-Tm interactions in the absence of myosin S1 (Hitchcock-DeGregori and Varnell, 1990; Landis et al., 1997; Hitchcock-DeGregori and An, 1996), however, a strong binding enhancement is observed in the presence of myosin S1 and is therefore more influenced by the deletions (Landis et al., 1997, 1999; Singh and Hitchcock-DeGregori, 2007). For example, a dAc234 Tm binds to
acto-myosin S1 about a hundred times more weakly than the control Tm (Landis et al., 1997). Collectively then, the deletion of specific actin binding modules affects Tm’s ability to bind actin and the switching of the actin filament to the active state (Rosol et al., 2000).

5.7 Relationship between Tropomyosin Actin Binding Sites and Actin Monomers

Analysis of the striated Tm amino acid sequence has shown the presence of seven quasi-equivalent repeats thought to correspond to the number of actin monomers spanned by a molecule of Tm. (McLachlan and Stewart, 1976; Hitchcock-DeGregori and Varnell, 1990). The hypothesis of periodic actin binding sites on Tm was tested by comparing the effects of deleting one-half, two-thirds and one full actin binding module on actin binding and regulatory function (Hitchcock-DeGregori and Varnell, 1990). Additionally, whether or not the Tm repeats 1-7 contribute equivalently to the overall actin affinity was tested by deleting actin binding modules 2 and 3 (Hitchcock-DeGregori and An, 1996). Collectively, in order for Tm to bind cooperatively, with high affinity to the actin filament, and inhibit acto-myosin S1 ATPase activity, it was determined that 1) The deleted Tm amino acid segments must be in multiples of 7 to retain the heptapeptide repeat essential for coiled-coil formation, which is also supported by Crick (1953) and McLachlan and Stewart (1975). 2) An integral number of actin binding repeats are more important than the quantity. 3) The internal actin
binding modules function primarily as weakly interacting spacers that align the ends of Tm to the actin filament.

As defined by McLachlan and Stewart (1976) and based on the finding that striated Tm spans seven actin monomers, one actin binding module in Tm is approximately 39 1/3 residues long. Therefore, the number of actin monomers that would be involved in binding a molecule of Tm of a given length can be calculated (Equation 19):

\[ N = \frac{A}{39\, \frac{1}{3}} \]  

(Equation 19)

where N is the number of actin monomers and A is the number of amino acids in Tm. Equation 19 does not take into account that Tm is highly flexible, but rather assumes it is a true coiled-coil structure. It does, however, consider slight variations in the number of Tm residues per actin monomer that result due to: 1) The interaction between the two α-helices in the coiled-coil of Tm where the side chains of residues that protrude from one α-helix fit into the spaces between the side chains in the other α-helix. 2) The bending of the coiled-coil around the actin filament (Stewart, 2001). Therefore, 39 1/3 residues represents an average value that associates with each actin monomer of the thin filament.

5.8 Cy5-ATP Localizes with the Myosin Thick Filament

Recall that three fluorescent acceptor probes bound to myosin S1 (Cy5-ATP, FHS and TMR) each paired with the same Tb³⁺ donor probe, in independent experiments were used to triangulate the location of the C-terminal
region of TnT in the reconstituted thin filament system via LRET. In the ideal scenario, one would use the same probes to determine the location of C-TnT in situ i.e. in myofibrils. However, trying to specifically label the target sites, especially using FHS and TMR would be impossible since they would react with other accessible lysine and cysteine residues, respectively. Cy5-ATP, though, does not form a covalent link with an amino acid and instead binds with high affinity to the nucleotide binding pocket of the myosin molecule. Therefore, this is the only probe that can be used to compare a distance value measured in the reconstituted thin filament system versus one that contains the full complement of contractile proteins and elements (myofibrils). The purpose of comparing the distance measured in the reconstituted thin filament and in myofibrils would help validate the measurements that were obtained in vitro and thus make the findings physiologically significant – at least in the rabbit model.

Previous fluorescence confocal images of rabbit myofibrils labeled with Cy5-ATP support the fluorophore binds to the myosin thick filament or A-band. One would expect this since the ATP moiety binds in the nucleotide cleft of the myosin head, presumably with the Cy5 probe protruding into the milieu away from the myosin head. However, the LRET data shows the distance between Tb$^{3+}$ and Cy5-ATP to be ~1 nm larger in myofibrils compared with in the reconstituted system, perhaps suggesting that Cy5-ATP binds elsewhere and thus causing the discrepancy (Table 1A, B). Indeed, this raises the question whether Cy5-ATP can bind to other targets and if so, which targets are they?
Consequently, to accurately observe the true location of the label with respect to the specific sarcomeric regions e.g. I-, A-, and Z-band, myofibrils labeled with Cy5-ATP were stretched prior to being viewed in the confocal microscope. Stretching the fibers essentially decreases the overlap between thin and thick filaments, thus making it easier to establish the exact location of the label. It should be noted though, that addition of Cy5-ATP causes the fibers to contract slightly, therefore it’s imperative the fibers are stretched immediately after adding the label.

Taking a brightfield differential interference contrast (DIC) image of the labeled myofibril along with the confocal fluorescence image helps analyze which features of the sarcomere the fluorophore interacts with. Typically, in a brightfield image, one would encounter a relatively wide dark zone that corresponds to the bipolar thick filaments, with a subsection of that zone interacting with thin filaments appearing even darker. The center of the bipolar filament mass is the M-line, either side of which exists a so-called “bare-zone” that is devoid of myosin heads. This zone is less dense and consequently gives rise to a lighter appearance on the image. Positioned further out from the poles of the thick filaments are two, thin dark bands called the Z-lines that perpendicularly bisect the filaments. This also marks the boundaries of one contractile unit – the sarcomere (Figure 1).

In accordance with the brightfield images then, the fluorescence images show that both Cy5-ATP (Figure 27A) and the control Alexa 647-ATP (Figure
bind predominantly to the A-band (thick filament). The Cy5-ATP-labeled myofibril shows an average sarcomere length of 2.23 µm, which is close to the typical resting value of 2.3 µm (Wang and Wright, 1988). However, the length of the sarcomere in the Alexa 647-ATP-labeled myofibril was measured to be 3 µm, suggesting it was more stretched than the former fiber. Also indicated are the A-bands i.e. the thick filament, which were measured at 1.6 µm (the typical length, Figure 27A) and 2.1 µm (Figure 27B) for the Cy5-ATP- and Alexa 647-ATP-labeled myofibrils, respectively. The slightly larger value of 2.1 µm could be attributed to the duration of the stain and the degree of permeability of the skinned fibers i.e. a relatively long incubation and fast infiltration time is more likely to yield thicker bands and result in a larger A-band measurement. In any case, we show here that both Cy5-ATP and the control Alexa probe are specific for the myosin thick filament and that they do not bind appreciably to other zones of the sarcomere. Consequently, the high affinity binding Cy5-ATP is appropriate for measuring distances between the myosin ATP binding site and the Tb$^{3+}$ donor.

5.9 Implications of Myosin Binding near Troponin

Taking all the data thus far including: 1) LRET measurements between C-TnT and various sites on myosin S1, 2) the mapping of C-TnT’s position in acto-myosin atomic models, 3) the myosin S1 ATPase activity in the presence of an increased number of Tn molecules bound to actin, Figure 29 shows a model
illustrating the effect of myosin binding close to the Tn complex. Here, a scheme similar to Figure 2 shows the behavior of myosin binding in the target zone (myosin A) and myosin binding close to Tn (myosin B) during the ATPase cycle.
Figure 29. Myosin binding close to Tn. Myosin molecules, A and B, bound with ADP and Pi approach actin (1) and bind weakly to the filament (2). Myosin A can undergo the powerstroke and assume the rigor conformation (3), the motion of the lever arm is shown by the dotted arrow. However, myosin B cannot complete the powerstroke due to the steric hindrance posed by Tn (crossed out dotted arrow). Therefore, Pi is not released and so this myosin does not contribute to force production. Since the interaction of myosin B is still weak, it can dissociate from the actin filament, while myosin A in the rigor state dissociates once ATP binds (4).
Since myosin binding close to Tn is inhibited from completing the powerstroke, the weak association with actin would favor the myosin to cycle between the detached and weak binding state (state 1 and 2, Figure 29), thus by-passing the post-powerstroke state and rigor condition. Note though, that myosin rigor binding close to Tn has been previously observed in in vitro studies (Resetar et al., 2002), suggesting that this structure induces a structural change in at least Tn and potentially actin and Tm, in order to form this state. Still, as the ATPase assays suggest, productive binding (transition from weak to strong myosin binding) in the presence of ATP and calcium appears to be inhibited. The physiological implications of myosin binding close to Tn are not clear, however, it is conceivable that it may function as a mechanical sensor and drag along the actin filament to dampen the effect of the productive myosins binding within the target zones between the Tn complexes. As previously discussed in section 5.5, potential interaction with Tn and Tm suggest it could also play a regulatory role in sensing and controlling the extent of contraction, while maintaining the sarcomere length within optimal limits via the help of other structural proteins, such as titin.

5.10 Potential Future Projects

While these experiments have bolstered previous findings and shed light on new notions, such as myosin binding actin to “target zones” on the actin filament, other aspects to be addressed have emerged. 1) When myosin S1 rigor
binding occurs where does the Tn move along the actin filament, with respect to a) the filament itself and b) myosin? 2) Can the Tn complex block rigor binding due to steric influences imposed by Tn or perhaps by some other mechanism? A feasible answer to the latter question is that Tn can block the weak binding of S1 to the actin monomers in close proximity to the regulatory complex. This is consistent with the hypothesis of there being a target zone on the filament for myosin binding, but the ATPase data does not tell us how many actin monomers are affected by each Tn. 3) What are the relative amounts of myosin binding near Tn versus those binding in the target zones and how does this affect acto-myosin function and regulation? 4) If myosin binding adjacent to Tn acts as a sensor, how does it communicate with other regulatory/structural elements and how significant is this? 5) A more refined atomic model, especially one that includes Tm is required by gathering more FRET/LRET data between other locations that would allow for a more detailed understanding on the regulation mechanism of muscle contraction.

5.11 Conclusion

Overall, this data helps establish a plausible model that accounts for the structural changes occurring in skeletal muscle contraction when myosin binds adjacent to the Tn complex in the reconstituted thin filament. The acto-myosin XRC models coupled with LRET-derived results provide an atomic resolution view of the location of C-TnT. Now, sufficient evidence while supporting previous
findings also provides insight into the exact amino acid residues involved in specific molecular interactions between myosin loop 3, C-TnT and important actin helices, Tyr91-Glu100 and Ile341-Gln354. The location of C-TnT may perturb the secondary binding interaction (responsible for myosin strong binding) thought to occur between loop 3 and actin residues Ala341-Gln354 and Ala144-Thr148 in the post-powerstroke state, implying a unique interaction between myosin and Tn. Calcium does not cause a statistically significant effect on the position of C-TnT with respect to myosin S1, thus Tn appears to sterically hinder myosin S1 binding and inhibits the completion of the powerstroke as suggested by the acto-myosin models. An engineered Tm, shortened by two actin binding modules still binds in a head-to-tail fashion like the native form, therefore eliminating the possibility that the actin filament structure pre-determines the binding location of Tm on actin. The effect of a shorter Tm increases the density of Tn complexes on the actin filament, which inhibits actin-activated S1-ATPase activity even in the presence of calcium. This supports that Tn and Tm determine where myosin binds along the actin filament, to so called “target zones”, which could play a pseudo-regulatory role in dictating where productive binding and force generation is most effective.
REFERENCES


