Characterization of Short ADP Ribosylated Oligomers Developed by
Modification in F-actin Structure

by

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ABSTRACT

CHARACTERIZATION OF SHORT ADP RIBOSYLATED OLIGOMERS DEVELOPED BY MODIFICATION IN F-ACTIN STRUCTURE

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University of Guelph, 2014

Advisor: Dr. John F. Dawson

Researchers have been trying to determine the mechanism of the crossbridge cycle, containing actomyosin complex, formed by the interaction of F-actin and myosin. But, because actin forms polymers of varying lengths, it has been difficult to crystallize this complex. My research is aimed at developing a homogeneous actomyosin complex with a short actin oligomer that possesses one myosin-binding site. ADPr-oligomers are produced by chemical crosslinking of F-actin and inhibiting polymerization by ADP-ribosylation. Specifically, I determined the behavior of an ADPr-trimer and dimer with myosin and its effect on actomyosin activity. Individual myosin ATPase activities of ADPr-trimer/dimer were similar to basal myosin activity. However, in the presence of long ADPr-oligomers (tetramer, pentamer, etc.) or F-actin, ADPr-dimer/trimer show binding and activity with myosin thick filaments. Thus, I was unable to produce a short actomyosin complex through these efforts and present a model for interaction of short actin oligomers with each other in the presence of long actin filaments and myosin.
Acknowledgments

I would like to thank Dr. John Dawson for believing in me and handing me this project. He has been a great source of inspiration and encouragement throughout my research, be it troubleshooting or interpreting experiments. I am glad to be a part of his team and hope to take a lot from the experience.

I would also like to thank my advisors Dr. Rod Merrill and Dr. Matthew Kimber for being on my committee and encouraging me to perform better throughout my time in the department. Their experience and expertise in the field has definitely been very helpful for interpreting data for my project.

I would like to thank the entire second floor of the MCB department for being a great team; specially Dr. Danielle Visschedyk for her guidance with Photox toxin and endless encouragement to keep up during the difficult times of research. In addition, I would like to thank Maria Anillo and Tijana Matovic for being best friends and teaching me life lessons through small things. Also Dawson lab members, Amila Omeragic, Mackenzie Charter, Matiyo Ojehomon, Ethan Shore, Chelsea Coumoundouros, Kris Mendoza, Michael Brodzikowski and Haidun Liu for being supportive and helpful. Lastly my thankfulness reaches out to Peihua Liu, Thomas Keeling, Jaspreet Kaur, Rob Taylor, Husam Al Sarraf, Danoo Vitsupkorn, Evan Mallette, Agota Ferenc and Dan Krska for being a part of this wonderful experience.
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<th>Description</th>
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<tbody>
<tr>
<td>10X poly</td>
<td>10X F-buffer</td>
</tr>
<tr>
<td>ABP</td>
<td>Actin binding proteins</td>
</tr>
<tr>
<td>ADPr-Oligomers</td>
<td>ADP ribosylated oligomers</td>
</tr>
<tr>
<td>βME</td>
<td>Beta mercaptoethanol</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>ECP32</td>
<td>Actin specific protease</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
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<tr>
<td>F-buffer</td>
<td>Buffer for the formation of F-actin</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence Resonance Energy Transfer</td>
</tr>
<tr>
<td>G-actin</td>
<td>Globular actin</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LD</td>
<td>Lower dimer</td>
</tr>
<tr>
<td>pPBM</td>
<td><em>para</em> phenylenebismaleimide</td>
</tr>
<tr>
<td>Pyrene</td>
<td>N-(-1)-pyrene iodoacetamide</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total internal reflection fluorescence</td>
</tr>
<tr>
<td>TMR</td>
<td>Tetramethylrhodamine</td>
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Statement of Work

A previous graduate student in the Dawson lab, Dr. Alex Perieteanu, had performed preliminary ATPase experiments with ADPr-oligomers trimer and dimer. Cloning of the \textit{photox} gene in Rosetta cells was done by Dr. Danielle Visschedyk from the Merrill lab and stored as glycerol stocks in -80°C. The progress of purification gel from actin oligomer isolation was jointly performed by the author and an undergraduate student Amila Omeragic. All other work was performed by the author.
Chapter 1 - Introduction

The Biological Requirement for Directed Movement

Movement is one of the most essential properties of living cells. It is controlled by molecular motor proteins in the cells of living organisms. Molecular motors play roles in crucial functions in the living cell, e.g., cell division, migration, cytokinesis, and immunity. These motors occur in a wide range of organisms from amoeba to the blue whale (Bustamante et al., 2004).

The cell cytoskeleton is mainly composed of actin. Actin is a molecular motor protein that helps in shaping the cell and directing its movements, and in processes like cell migration that are dictated by polymerization (Sheterline et al., 1998). Myosin, another molecular motor protein, coordinates with actin to form the actomyosin complex and enables essential properties like contraction of muscles during movement. The presence of these two proteins suggests a strong general connection between their mechanisms of action (Tyska and Warshaw, 2002). The significance of their function is demonstrated by the conserved nature of these two proteins among a wide range of organisms (Huxley, 2000). This significance encourages us to study the atomic interactions associated with actin and myosin.

It is important to map the connections between molecular motor proteins like actin and myosin. Understanding the atomic details of the fundamental machinery in biological movement has been the goal of researchers ever since the discovery of the proteins involved over 70 years ago. Diseases like cardiomyopathy are a result of changes in the amino acid sequences of molecular motor proteins, but the details connecting the changes and disease development are still unclear (Elliott et al., 2008). Understanding the
interactions of the contractile machinery has been obstructed by actin’s ability to form filaments of varying length (Kabsch et al., 1990). After years of study, scientists have still been unsuccessful in creating a protein complex which is stable enough to determine a crystal structure of F-actin. The Dawson lab has developed a novel method to modify actin proteins to solve this problem. These methods alter actin to make it non-polymerizable without changing its intrinsic structural properties. I used these methods to produce short oligomers of actin to characterize their interactions with myosin. With our adapted processes of cross-linking and ADP-ribosylating short actin oligomers, we hope to achieve a practical complex for a crystal structure of the actomyosin.

1.1 Actin

1.1.1 Globular Actin

Actin, a 42 kDa protein, is present in all eukaryotic organisms with high sequence conservation. In mammals, actin occurs in three isoforms: alpha(α), beta(β) and gamma(γ). Alpha-actin is found in skeletal, cardiac and smooth muscles whereas beta and gamma isoforms are present in both muscle and non-muscle cells. In mammals, actin occurs in two major forms as a folded protein: monomeric globular (G-actin) or polymeric filaments (F-actin) (Dominguez and Holmes, 2011).

The mature monomeric form of actin has been crystallized and consists of 375 amino acid residues (pI 5.23) (Figure 1). G-actin is segregated into two parts: a large and small domain. These two domains contain two subdomains each, numbered 1 through 4 as shown in Figure 1. Subdomains 1 and 2 (SD1/SD2) constitute the small domain and subdomains 3 and 4 (SD3/SD4) represent the large domain. Between SD2 and SD4 lies a
**Figure 1: Globular Actin.** The figure above shows the ribbon diagram of Globular actin with ADP nucleotide shown as a stick figure. A Ca$^{2+}$ ion is attached as a yellow sphere in the nucleotide-binding cleft close to ADP. The subdomains are labelled as 1, 2, 3 and 4. Image generated using the PyMol program (PDB 2HF3).
divalent cation, Ca$^{2+}$ or Mg$^{2+}$, with a tightly-bound ATP or ADP. These divalent cations have a strong affinity for the phosphate groups of nucleotides and are responsible for the stability of actin (G- or F-) (Domínguez and Holmes, 2011).

The spatial area of nucleotide-binding is called the nucleotide-binding cleft. This region is different from the hydrophobic cleft (residues 137 – 146 and 334 – 337) which serves as a region for interacting with actin-binding proteins (ABPs) or toxins specific to actin (e.g., macrolides) (Kudryashov and Reisler, 2013). The N- and C- termini are both located in SD1. The SD2 is considered the most flexible region in the actin structure because it contains the D-loop (residues 40-51) which mediates most interactions with DNase – I (Kabsch et al., 1990) as a result of either the extension or unwinding (Kudryashov and Reisler, 2013).

Other important regions in actin are the Hydrophobic plug/H-plug (residues 264 – 271), the WH2 binding loop (residues 165 – 172) and V-stretch (residues 227-237) (Domínguez and Holmes, 2011; Kudryashov and Reisler, 2013). Among these, the H-plug between SD3 and SD4 of one subunit interacts with SD1 and SD2 of another subunit in F-actin. All the G-actin subdomains are important sites for the binding of various ABPs (Kabsch et al., 1990).

1.1.2 Filamentous actin

Polymerization of actin follows a series of steps in which actin monomers self-associate to form a two-stranded, right-handed helical structure called F-actin (Domínguez and Holmes, 2011; Vavylonis et al., 2005). F-actin is polar and contains two specific ends, a barbed end and a pointed end. The process of polymerization is divided
into three parts: nucleation, elongation, and steady state (Figure 2A). In the nucleation phase, a trimer (three subunits) or a tetramer (four subunits) forms the seed of the polymer. More monomers bind to both ends of the seed which leads to elongation. The last phase is steady state during which the polymer length is maintained by the continuous addition of monomers to the barbed end and subunit removal from the pointed end (Vavylonis et al., 2005). Usually, ATP-bound actin associates with the barbed end and ADP-bound actin dissociates from the pointed end. The entire mechanism of association and dissociation is called treadmilling. During treadmilling, conformational change in SD2 leads to the release of an inorganic phosphate from ATP (Holmes, 2009). All of these intrinsic nucleotide changes are associated with subdomain alterations that modify connections between subunits. ATP was found to be a factor that signals the difference between new and old actin subunits for ABPs (Kudryashov and Reisler, 2013).

Monomers associate and dissociate at both ends, but association occurs at a much higher rate at the barbed end and dissociation is faster at the pointed end. Recent FRET and TIRF microscopy studies have shown that more than two states of actin monomer structures are formed in the reaction of polymerization (Morimatsu et al., 2012), suggesting an intermediate monomeric state called “fg” which prepares the monomer for polymerization and binding to another actin. An “fg” state supports results seen by Rich (1976), who found that proteolytic digestion of actin decreased in a solution exposed to KCl where polymeric actin forms. F-actin is not cleaved by proteolysis. The study shows how proteolysis is still seen in partially formed F-actin stages which signify the presence of an intermediate G-actin form. The studies mentioned above show the importance
Figure 2: F-actin Tradmilling and Twisting. A) Treadmilling. The formation of F-actin takes place by the addition of actin with ATP on the Barbed end (+) and removal of actin with ADP from the Pointed end (-). Addition and removal of actin monomers takes place at both ends but the frequency of addition on the Barbed end is higher and the Pointed end is lower while dissociation frequency is higher on the Pointed end and lower on the Barbed end. B) Intermolecular Twisting. The crossover region of F-actin ranges 36-37 nm in length for each helical pitch (taken from Oda et al. 2009 with permission). C) F-actin Intramolecular Twisting. Actin monomers become planar in shape after twisting its major domain (SD1 and SD2) by 20° (taken from Dominguez et al. 2011 with permission).
of different conformational states and the dependence or alterations in these conformations based on associated nucleotides.

1.1.3 Filament Twisting

F-actin is the filamentous form of actin and appears as a two-stranded, right-handed helical structure with a helical pitch length of about 36-37 nm (Figure 2B) (Oda et al., 2009). F-actin undergoes two kinds of twisting in its conformation when it is transformed from G-actin. The first kind of twisting is an intramolecular twisting as a free G-actin monomer changes structure in F-actin. X-ray fibre diffraction studies carried out on F-actin uncovered a significant change in the structural orientation of the monomers when changing from G-actin to F-actin (Oda et al., 2009). F-actin subunits adopt a flattened structure compared to monomeric G-actin with a 20° reorientation of the major domains into one plane (Figure 2C). There are alterations in all the four subdomains of every subunit. The D-loop extends toward the upper subunit proline-rich regions (residues 108-112) in F-actin to form a longitudinal interaction allowing the catalytic reaction of ATP hydrolysis to occur (Murakami et al., 2010). To illustrate how the twisting of domains affects the helical structure, Oda et al. (2010) compared the actin-formin (ABP) complex non-helical structure to F-actin, and found that twisting generates an interaction between alternate subunits not seen in F-actin without helical rotation. This twist also places Glu-137 close to the β and γ phosphates of ATP, allowing F-actin to hydrolyze the nucleotide.

Holmes (2010) illustrated the importance of the intermolecular twisting of the F-actin helix in generating a strong bond with myosin and guiding the process of mechanical contraction in cells. F-actin-binding proteins have the ability to modify the twist of F-actin (Egelman 2003). A major change in the helical twist of F-actin is seen with the
binding of proteins like actin depolymerizing factor (ADF) and coflin (McGough et al., 1997). Regions of crossover after filament twisting (as observed in 2 dimensions) were shorter when F-actin was exposed to coflin and the diameter of the helix was 20-30% larger than in normal actin filaments. McGough’s experiments calculating the crossover length along actin filaments with half the filament naked and half covered with coflin, revealed that the shortening of crossover lengths occurred only in regions covered with coflin and was not propagated throughout the filament.

Tsaturyan (2005) showed an increase in length of the actin filament in the rigor actomyosin state. Myosin is assumed to be inducing this F-actin inter-subunit stretch as measured from the radial position of an actin Cys-374 fluorescent probe. The helical twist of F-actin elongates and the helical angle decreases when myosin pulls on actin. These studies suggest that long-range F-actin inter-subunit connections may play a role in the ability of myosin to bind and release from F-actin, but this idea has not been tested directly (Uyeda et al., 2011). Other F-actin studies have shown that the interaction between F-actin and ABPs like coflin is also dependent on filament tension. If the filament is stressed it will not interact with such ABPs (Hayakawa et al., 2011; McCullough et al., 2008). Thus, each G-or F-actin conformation is predisposed to bind a particular type of ABP, which could explain how actin manages multiple protein-protein interactions. My research will help us understand if long range F-actin interactions are required for normal actomyosin activity.
1.2 Actin Modifications

Following from studies related to F-actin-like conformations, the Dawson lab has adapted some methodologies to obtain F-actin-like structures from monomers.

1.2.1 Crosslinking Reactions

The ability of actin subunits in F-actin to be chemically-crosslinked by treatment with \( p-N,N'-\text{phenylenebismaleimide (PBM)} \) was first characterized by Knight and Offer (1978). This crosslinking reaction is specific for Cys-374 of one actin subunit and Lys-191 of another subunit, as the amino acids are only 1.2-1.4 nm apart on the two longitudinal strands of F-actin (Elzinga and Phelan, 1984) (Figure 3). This reaction needs an alkaline pH 9.0 to render the amino acids reactive. Decreases in pH lead to lower efficiency of crosslinking; usually 0.5 crosslinks per subunit. No strain has been observed on the F-actin structure due to this chemical crosslinking. This technique is used by the Dawson Lab to obtain different lengths of actin oligomers derived from F-actin.

Deciphering the structure and function of short actin oligomers could lead to a better understanding of the initial formation of F-actin and its kinetics. Millonig et al. (1988) found differences between two kinds of dimers seen after PBM crosslinking reactions where “upper dimers” (UD) lead to polymerization of actin while “lower dimers” (LD) did not participate in the polymerization. These results indicated different conformations between these two states, possibly longitudinal (along strand) and lateral (between strand) subunits. Dawson et al. (2003) used the same oligomers to determine the conformation of monomers in a polymeric form. A PBM-crosslinked trimer was bound by gelsolin segment-1 to determine the conformational differences in its subunits. This experiment showed how F-actin conformation is different from F-actin subunit structure with
Figure 3: Crosslinking of Actin Subunits. $p$-N,N’-phenylenebismaleimide creates a connection between Cys-374 in SD1 of one actin subunit to Lys-191 in SD3 of another actin subunit to form lateral crosslinks. The reaction of the chemical with cysteine takes place first and this reaction renders the other side of the chemical reactive to lysine as they are close to each other in an F-actin formation.
gelsolin and how gelsolin severing of F-actin is processed. When gelsolin attaches to one subunit, it stretches the PBM-crosslinked trimer longitudinally by placing itself between two subunits. This changes the twist in actin making it impossible for the structure to mimic F-actin (Dawson et al., 2003). We strive to use naked actin oligomers for our study to avoid the influence of other proteins and mimic the original F-actin structure. In addition to using polymerization-inhibiting proteins as mentioned above, restriction of polymerization can be done by methods as described in Section 2.2.

1.2.2 Control of Polymerization

One of the many approaches to study the atomic structures of F-actin includes controlling actin polymerization. This control can be performed by at least three different methods. Covalent post-translational modifications like ECP32 cleavage between Gly42 and Val43 (only under Ca-ATP conditions) or TMR (tetramethylrhodamine)-modified G-actin have proven to be helpful for atomic studies on monomers (Klenchin et al., 2006; Kudryashov and Reisler, 2003). Other posttranslational modifications like AP-actin, developed by point mutations in SD4 (A204E and P243K) and studied in a baculovirus expression system have performed equally well in rendering actin non-polymerized (Joel et al., 2004; Rould et al., 2006). However, mutational studies that are aimed at controlling polymerization may result in the protein losing its usual properties and function. Recently, methods to control actin polymerization without the allosteric changes have been developed through the process of post-translational modification.

Actin is a highly conserved structure throughout eukaryotes; it is commonly targeted by bacterial toxins which affect polymerization (Patel and Galan, 2005). One of the first
bacterial toxins found to inhibit actin function was Botulinium C2 toxin which ADP-ribosylates actin (Aktories et al., 1986). Photox, a mono-ADPribosyltransferase toxin derived from Photorhabdus luminescens, modifies actin monomers to render them incapable of polymerization. This alteration is specific for Arg-177 of actin (Visschedyk et al., 2010). Arg-177 is located close to the ATP binding site on G-actin. When Arg-177 is ADP-ribosylated, the ability of ATP to hydrolyze and monomer to polymerize is lost (Margarit et al., 2006) (Figure 4A). This hindrance is thought to inhibit across-strand interactions in F-actin because of involvement of intersubunit interactions associated with Arg 177 (Visschedyk et al., 2010) (Figure 4B). Although, several other bacterial toxins are known to affect actin polymerization (Aktories et al., 2012), the Dawson lab uses Photox, to inhibit polymerization for structural studies of actin. A 1:1 ADP-ribose to actin subunit was found by mass spectrophotometric data from Perieteanu et al. 2010. Also, actin dimer and trimer were observed to be associating with the barbed end of F-actin inspite of ribosylation in a Dual colored microscopy assay.

1.3 Myosin

1.3.1 Full length Myosin

The myosin superfamily consists of at least 31 classes of myosin according to phylogenetic and genome analysis of these superfamilies (Sebe Pedros et al., 2014).

Conventional myosin from muscle is called myosin-II. Myosin-II is a 500 kDa molecular motor protein with 1,940 amino acids. Three important regions in its structure are the head, neck, and tail. A heavy chain extends in one direction to form the head and
Figure 4: ADP Ribosylation of Actin Subunits. A) Reaction of Arginine with NAD
NAD reacts with Arg-177 of actin and gives ADP ribose and Nicotinamide. B) Hindrance from ribosylation Three actin subunits (grey and green ribbon) in F-actin like state are seen. Addition of ADP-ribose (red stick model) to Arg leads to hindrance among the subunit before reacted actin (n-1) and another subunit after the reacted subunit (n+1) (grey ribbon structures). This is the proposed mechanism of how ADP-ribosylated subunits cannot be polymerized (taken from Aktories et al. 2011 with permission).
an alpha-helical tail exists on the other end. Light chains are attached to the head region of the heavy chain. These helices of the heavy chain form a coiled coil, resulting in a 2-headed complex (Sellers, 2000).

Diversity of amino acids in the tail region of myosin is responsible for the variety of myosin functions in cells, e.g., contraction, vesicle transportation and cytokinesis. In myosin-II, the tail region combines to form thick filaments. These filaments are the core of sarcomeres that bind actin in muscles (Oliver et al., 1999). Self-associated myosin-II can be purified from muscle tissue using a variety of ionic concentration changes (Sellers, 2000).

In unconventional myosin that carries cargo along actin filaments, the tail region serves as an adaptor between the head regions, binds to actin on one side and cargo binding domains at the opposite end of the tail (Sellers, 2000).

### 1.3.2 Myosin S1

The head region is the most dynamic region of myosin, possessing ATPase activity and the binding sites for actin and nucleotides. The head of myosin is responsible for the contraction and movement of actin filaments. The combination of myosin head and neck is called subfragment-1 (S1). The head region is the most conserved of all three parts. The neck region has a long alpha-helix stabilized by the essential and regulatory light chains in myosin-II. The light chains bind Ca\(^{+2}\) ions and are regulated by phosphorylation. They in turn regulate the myosin ATPase activity and control the assembly of myosin into thick filaments. The function of the neck region includes
movement during contraction of the actin-myosin complex and regulation of some myosin proteins (Baker and Voth, 2013; Houdusse et al., 2000).

Myosin S1 is an elliptically-shaped protein fragment, composed of part of the heavy chain (~95 kDa) and light chains of 35 kDa (Figure 5). An upper 50 kDa domain and actin-binding domain form the head of S1. A nucleotide-binding domain lies between the N-terminus (SH3-like domain, residues 30-75) and the upper domain. The N-terminal domain lies between the upper 50 kDa domain and the converter domain (Preller and Manstein, 2013). A flexible lever arm region lies in the end of the converter domain that helps with the movement of the head. Three different links connect these domains: switch I (Ile 461–Asn 470), relay (Asn 489-Asp 515) and SH1 helix (Cys 693–Phe 707) (Koppole et al., 2007; Preller and Holmes, 2013). Studies have shown changes in these domains during interactions with actin and the ATPase activity of myosin S1 is half the activity of that of full-length myosin (Baker and Voth, 2013; Reggiani et al., 2000). However, S1 is sufficient for binding and providing actin-activated ATPase activity.

1.4 Actin and Myosin

1.4.1 Actin and Myosin Complex

A muscle consists of multiple myocytes which are assembled into myofibrils. Myofibrils are tubular cells containing sarcomeres as their basic structural unit which are responsible for contraction and relaxation. Actin and myosin generate force to make the sarcomere contract through what is called the crossbridge cycle (Figure 6A) (Hwang and Lang, 2009).

In a relaxed muscle, myosin motor protein is bound to ADP and a phosphate
Figure 5: Structure of Myosin. Myosin S1 is composed of Upper, Lower 50 kDa domains, the N-terminal and Converter Domain acting as major motor units. Links that guide motor movements between these domains are Relay Helix, SH1 Helix and Switch II (Adapted from Coureux et al. 2004).
Figure 6: Sarcomeres and the Crossbridge Cycle. A) Sarcomeres in muscles. According to the crossbridge cycle theory, with an increase in Ca$^{2+}$ in cells, F-actin is pulled over myosin filaments, an event that decreases the space between F-actin and results in contraction of muscles. B) Cross-bridge Cycle. Myosin S1 with ADP and P$_i$ attaches to F-actin (1). The release of P$_i$ leads to pulling of actin over myosin, followed by the release of ADP from myosin (2). This tight binding state is called rigor. ATP then attaches to myosin and detaches from F-actin (3).
group. When a nerve impulse is received at the neuromuscular junction, it triggers the release of Ca$^{+2}$ ions into the sarcomere. This release allows troponin and tropomyosin regulatory proteins to expose the myosin binding site on F-actin (Behrmann et al., 2012). When myosin binds to actin, ADP and P$_i$ are released from myosin, resulting in a conformational change in the neck region of myosin. The actin filament is then pulled in what is referred to as the power stroke (Hwang and Lang, 2009). Myosin is then released from F-actin with the binding of an ATP molecule. The last step in the crossbridge cycle is hydrolysis of ATP to ADP and Pi, with the myosin head returning to its initial pre-power stroke conformation (Figure 6B). The net result of a power stroke is the conversion of chemical energy to mechanical energy and 1 ATP molecule is spent per cycle (Hwang and Lang, 2009).

In the absence of ATP, actin and myosin bind tightly to form the actomyosin “rigor” complex. This complex is attained after a weak interaction between positively-charged residues of actin and negatively-charged residues of myosin (residues 100-200). These weak interactions then result in tight binding after a couple reactions between the hydrophobic loop of myosin and the actin-binding site (Geeves and Holmes, 2005). The mechanism for this conformational change in the rigor state is not known due to the overall lack of actomyosin structural studies and lack of consistency of these few studies. However, it is known that interaction of myosin with actin results in a decrease in the affinity of myosin for nucleotides (Coureux et al., 2004). This rigor actomyosin complex can demonstrate the atomic interactions of actin and myosin, as well as give us insight into conformational changes during the crossbridge cycle. The affinity of myosin for F-actin is dependent on the type of nucleotide bound to myosin, which can change up to
10,000-fold based on myosin’s conformation during a particular stage of crossbridge cycle (Preller and Holmes, 2013). This affinity is also related to conformational change in G-actin after polymerizing into filaments. Therefore, maintenance of conformational changes is important for binding of actin to myosin.

1.4.2 Actomyosin Crystal Structures

Previously, actomyosin complex models have been proposed using the crystal structure of myosin-V. When Holmes et al. (2004) looked at the cleft region of myosin S1 in X-ray crystal structures with no actin, they proposed how this region closes in a non-nucleotide-binding state and opens in the presence of a nucleotide. Studies performed on the closed cleft state showed energy change resulting from torsional strain. However, this state could not explain its tight binding to F-actin during the rigor state (Rayment et al., 1993).

Later in 2010, Lorenz et al. created an electron microscopic model of the actomyosin complex at 13 Å resolution. This model focused on the contacts between one myosin S1 head and two subunits of F-actin.

Oztug Durer et al. (2012) attempted to provide a better connectivity map of actomyosin with the help of radiolytic oxidative protein footprinting of F-actin in the absence and presence of myosin S1. Oxidative modification of the side chain residues is carried out without any change in protein structure. Myosin S1 is then bound to F-actin and the experiment is repeated again. Residues in F-actin that interact with myosin are protected and are not detected during the second trial. These experiments demonstrated that residues 29-39, 119-147, 148-77, 239-254 and 336-359 on actin were involved with
strong interaction on myosin binding sites. These results were supported by residues previously determined for major interactions except 239-254.

Cryo-electron microscopy at 8 Å resolution performed by Behrmann et al. (2012) gave a higher resolution picture of the amino acid interactions between the actin and myosin domains. Here, myoE was used from *Dictyostelium discoideum*. A 16° rotation in myoE is detected as the nucleotide-binding cleft changes after interaction with F-actin. Salt-bridge interactions were seen between the negatively-charged amino terminus of actin (D1, E2, D3, E4) and positively-charged loop 2 and helix HW (K556, K557, R558, R567) of myoE. Also, negatively-charged SD1 (D24, D25) in actin interacts with the myosin CM loop (R323, K331, R332) (Figure 7).

Cryo EM studies on proteasome like *Thermoplasma acidophilum* have provided deeper insights on other techniques for structural studies. They obtained a 3.3 Å resolution structure by increasing the number of frames per second and recording videos to obtain non-blurry impressions (Doerr, 2014; Li et al., 2013). These biochemical studies provide some insight into new advances in technology that are alternates to X-ray crystallography that can be used to not only observe the interactions between these proteins but to decipher details regarding the crossbridge cycle mechanism.

1.5 Limitations of Current Crystal Structures

To determine atomic details of protein-protein interactions, we require crystal structures that show patterns of the protein arrangement. Scientists have determined better resolution 3D helical reconstructions of F-actin, and subsequently better models of
Figure 7: Actomyosin Interface Interactions. Taken from Behrmann et al., 2012 with permission, this figure shows the connections between actin 0 and -2 (relative to position), tropomyosin and myosin in an actomyosin complex. Here two monomers actin 0 and actin -2 are interacting with myosin, actin -1 is on the further side not interacting with myosin but interacting with actin 0 and -2. Asterisks represent the amino acids on actin -2. The color of residues corresponds to their charge at pH 7.4 (red for negative and blue for positive). This information has not been confirmed yet due to lack of actomyosin crystal structures.
myosin S1 head with different nucleotides bound to it, with or without F-actin (Baker and Voth, 2013; Coureux et al., 2004; Tsaturyan et al., 2005). But, the structure of actomyosin, the force-generating complex, is still of low resolution.

The problem lies with actin: it has the ability to form polymers of varying length under crystallization conditions. This is a challenge because a homogenous preparation is required for crystallization. Therefore, a way to control actin polymerization and isolate F-actin of specific length for structural studies has to be applied to overcome these barriers.

To understand this concept, I will apply our methods adapted in the Dawson lab to isolate short F-actin segments (oligomers) as substrates for myosin binding.

1.6 Short Actin Oligomer Studies

My aim is to determine the interactions of the actomyosin protein complex by producing short, homogeneous actomyosin complexes. Extensive tests have been performed previously to study the behavior of n-meric actin proteins, to understand the mechanism or structures of actin-binding proteins with actin. Long-range inter-subunit interactions might be important for activity of the actomyosin complex as pointed by Oosawa (1975), leading us to the question whether small actin oligomers will behave the same as F-actin.

Studies performed with monomeric actin and myosin revealed that the myosin S1 head interacts with two actin subunits at once. Interaction between actin and myosin proteins was observed through pyrene stopped-flow experiments and displayed isomerization (interchange in the two forms from reversible bimolecular reaction) of GS
(one monomer with S1) complex with G2S (two monomers with S1) due to reversible reactions. The G2S and GS dissociation rates were compared with F-actin and G2S values were closer to F-actin. Thus, they considered G2S as a better model to study interactions with myosin (Blanchoin et al., 1995).

Kim et al. (2002) chemically-crosslinked F-actin and studied its interactions with myosin. In vitro motility and stopped-flow experiments were carried out with dimers from a different kind of crosslinking, where longitudinal (intrastrand) links were produced between Gln-41 of a lower subunit and Cys-374 of an upper subunit. This crosslinking resulted in inhibition of the motion and force generation when combined with myosin. It is assumed that this inhibition is due to the stretch in the regular inter-subunit distance caused by the crosslinking. The importance of the subdomain 2/C-terminus between subunits for interaction with myosin was demonstrated by their experiments, but movement and force generation were inhibited.

Mockrin and Korn (1981) mentioned the potential presence of two kinds of dimers resulting from pPBM crosslinking. These dimers are different in their attachment in crosslinking i.e., they are either crosslinking with an intra-strand subunit (longitudinal dimer) or an inter-strand subunit (lateral dimer). In contrast, Steinmetz et al. (1997) showed how one of the two dimers with pPBM crosslinking resulted from fast cysteine reactions between two monomers, but pPBM did not create longitudinal interactions.

Previously, the Dawson lab successfully created long-pitch actin dimers that were unable to polymerize but still retained important protein-protein interactions (Morrison et al., 2010). Four types of long-pitch dimers were assessed: a mutated dimer, dimer bound to DNase-I, ADP-ribosylated dimer, and an unmodified dimer. All the dimers nucleated
actin polymerization, but with different kinetic values. Interactions with myosin were also observed, but dimer polymers may have been induced by the interaction.

Perieteanu et al. (2010) characterized purified ADP-ribosylated, PBM-crosslinked dimers and trimers. There was no observed polymerization in these modified dimers and trimers, which proved fruitful for using them as members of complexes. The conformation of trimer was more open or spread out as opposed to the compact F-actin structure. This showed how the three subunits were different from F-actin proceeding to polymerization. Both ADPr-dimer and trimer bound DNase-I, but dimer bound to DNase-I with a better affinity than the trimer. This observation suggests that the presence of both lateral and longitudinal interactions gives the ADPr-trimer a twisted conformation that partially inhibited DNase-I binding. Importantly, the removal of the ADP-ribose from the Arg-177 showed that the dimers and the trimers still retained the properties of the original protein and polymerized.

The idea for my research is rooted from an unpublished experiment performed by Dr. Perieteanu from the Dawson Lab. Dr. Perieteanu derived some preliminary myosin ATPase data using ADPr-dimer and trimer. The ADPr-dimer does not contain a putative myosin binding site, and should therefore result in no increase in myosin ATPase activity. Conversely, ADPr-trimer possesses a putative myosin binding site that might activate myosin ATPase activity. However, myosin activity actually decreased in the presence of ADPr-trimer. This led to questions about the conformational change of actin during contraction: Does the trimer developed by our lab bind to myosin tightly and inhibit its activity?
The main advantage of using ADPr-trimer is the presence of both long-pitch (intrastrand) and short-pitch (interstrand) F-actin interactions, providing an F-actin like environment for myosin binding. In this regard, we may finally reveal conformational changes in F-actin that are critical to contraction and have only been suggested in the past. On a practical note, the PBM-crosslinking strategy uses only wild-type actin protein which is readily available in gram quantities from muscle tissue.

Having stated the relevance of my research in the Dawson Lab, I will hypothesize my research and describe the methods and procedures that I propose to use during my study. I hypothesize that short actin oligomers like dimer and trimer formed by crosslinking and ribosylation will interact with myosin S1, proving to be good models for crystallization studies. To achieve my goals I planned on specific aims:

**Aim 1: To determine the interaction and effect of short ADPr-oligomers with myosin using myosin ATPase activity assays.**

The ATPase activity of myosin induced by F-actin could be affected depending on the interaction of ADPr-oligomers with myosin. I want to observe the behavior of ADPr-oligomers on ATPase activity of myosin in presence or absence of F-actin. If any of its components are interacting with myosin, a change in normal activity of F-actin should be seen.

**Aim 2: To determine the interactions between ADPr-oligomers and full-length myosin using co-sedimentation assays.**

Co-sedimentation of ADPr-oligomers with full-length polymerized myosin should result in oligomers bound to myosin in the pellet and leave any oligomer not interacting in the supernatant.
Chapter 2 – Materials and Methods

2.1 Standard Experimental Methods

2.1.1 Protein Concentration Measurements

The protein concentration of α-skeletal actin was measured using the Bradford colorimetric assay (Bradford, 1976) with known actin concentrations as standards. The concentration of actin obtained is taken as subunit concentration. During the dimer and trimer calculation, the subunit concentration obtained by Bradford was divided by two and three respectively to measure the concentration per complex of trimer or dimer. This method of calculation was done because we believe one complex of trimer and dimer contained one myosin binding site by itself.

Myosin S1 protein concentration was measured using a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA) at absorbance of 280 nm with the 7.5 cm⁻¹ as extinction coefficient (Siemankowski et al., 1985). Full-length myosin concentration was measured at 280 nm on a Beckman Coulter DU 800 Spectrophotometer (Mississauga, ON) with an extinction coefficient of 0.57 M⁻¹ cm⁻¹ (Ouyang et al., 1995). In the ATPase assay, concentrations were measured in mg/ml of full-length myosin due to viscosity of myosin and all the assays had the same myosin concentrations. When performing the co-sedimentation assay, the concentration of full-length myosin was back calculated using online tools (Practical Molecular Biology http://molbiol.edu.ru/eng/scripts/01_04.html). Also, manual calculations were done to confirm the concentration using 500 kDa as the molecular mass of myosin. Therefore, 10 μM of myosin was back calculated to 5 mg/ml which was diluted from 40 mg/ml of
myosin stocks and used in the assay. All chemical reagents were purchased from Fisher Scientific (Missisauga, ON) or Sigma-Aldrich (St. Louis, MO) unless otherwise mentioned.

2.1.2 Polyacrylamide Gel Electrophoresis

8% SDS polyacrylamide resolving gels and 5% stacking gels were used for better visualization of higher molecular mass proteins including actin trimers and tetramers. Laemmli buffer (2X) was mixed at a 1:1 ratio with the sample and loaded on the gel. Running buffer was set up in a Bio-Rad Mini PROTEAN tetra cell system and gels were run at 180 V for 40 min. Solutions were prepared and followed according to Laemmli et al. 1970.

2.1.3 Native Polyacrylamide Gel Electrophoresis

Native-PAGE gels contained 8% polyacrylamide resolving gel (0.04 mM CaCl$_2$) and 5% stacking gel (0.02 mM CaCl$_2$). Loading buffer was mixed with sample at a 3:1 sample:loading buffer ratio. Running buffer (1X) was placed in the same system as above and the gel was run at 85 V and 4°C for approximately 2 hours.
2.1.4 Silver Staining

Silver staining was performed for better visualization of faint protein bands in Native-PAGE gels following the protocol from the manufacturer (Thermo Scientific Pierce Product #24612).

2.2 Protein Purification

2.2.1 Actin Purification

Extraction of actin protein was done with lean turkey breast muscles (pectoralis and supraceracoideus) as turkey contains more protein per ounch than chicken (Royston, 2009). Breast muscles are low on blood vessels and therefore the majority of actin proteins present in them are alpha actin. The sequence identity between turkey and chicken, human and rabbit is 97% (Bertola et al., 2008). Purity in muscle is high enough to be able to isolate crystal structures (Kabsch et al., 1990).

Lean turkey meat was used to prepare acetone powder. First, the extraction with 0.1 M KCl and 0.15 M potassium phosphate (pH 6.0) was done with manual stirring for 10 min. This extraction was spun at 4000 rpm for 5 min. Subsequently, extraction was carried out with 0.05 M sodium bicarbonate, 1 mM EDTA (pH 7.0), and water followed by spinning after each extraction. The remaining meat was extracted with a total of 4 L of acetone, stirring the meat manually with 1 L acetone each time for 10 min, and filtering out the acetone with cheesecloth. The extracted meat was spread on a tray with aluminum foil and left to dry in the fumehood overnight. Actin was extracted from acetone powder
of lean turkey meat. 45 gm of acetone powder was stirred with 900 ml of G-buffer twice. The liquid obtained after filtering through the cheesecloth was mixed together. This supernatant was spun at 19,000 rpm for 30 min to clarify the supernatant and the actin protein was then polymerized at room temperature for one hour. This solution obtained was raised to a concentration of 0.6 M KCl and stirred for 15 min. Centrifugation was carried out at 42,000 rpm for 1 hour in Beckman Coulter Ti 45 rotor in Beckman Coulter ultracentrifuge optima 90L. Pellets obtained from centrifugation were homogenized and dialyzed against G-buffer with three buffer changes. Protein was taken out of dialysis and spun at 95,000 rpm for 15 min in a TLA 110 rotor on a table-top Beckman Coulter Optima 100 MAX-E centrifuge. Supernatant was separated; final protein concentration was measured and flash frozen in N₂(l) and stored at -80°C.

2.2.2 Purification of Actin Oligomers

To obtain actin oligomers, the pH of the solution was adjusted to 9.0 with 10 mM sodium tetraborate and p-N,N’-phenylenebismalaemide (pPBM) (Aldrich Chemical Company P2, 398-9) was added to F-actin after addition of 0.6 M KCl described in the section above. A 1.5X molar excess of pPBM compared to actin concentration was added and stirred for 30 min at room temperature. The reaction was stopped by adding a 160X molar excess of βME relative to pPBM. This solution was spun at 40,000 rpm for 1 hour in an ultracentrifuge, and pellets were homogenized and dialyzed against G-buffer with 5 buffer exchanges in the next two days. The procedure was modified by an undergraduate student (Amila Omeragic) to be more time efficient (Figure 8). The centrifugation and
dialysis of polymerized actin was skipped in lieu of immediately crosslinking actin after treatment with high salt concentrations. This modification decreased the processing time for protein purification by 2 days, saving time and increasing the yield of actin.

2.2.3 Ribosylation of Actin Subunits

Ribosylation of Arg-177 of actin subunits rendered it unable to polymerize. The pH of solution is raised to 8.0 by adding 10 mM of Tris, pH 8.0. One to 100 nM of photox toxin was added to the already crosslinked actin solution. The reaction was completed with the addition of a 5X molar excess of β-NAD (Sigma Aldrich N1511) compared to actin concentration and incubated for 15 min. The reaction was carried out overnight at 4°C. The “slurry” (combination of all crosslinked actin oligomers) obtained was spun to remove any non-ribosylated subunits with the ability to polymerize. The supernatant was concentrated to approximately 50 ml (Amicon Ultra-15 Centrifugal Filter Unit MWCO 10 kDa) to load onto a Superdex 200, 26/60 Amersham Biosciences Fast Performance Liquid Chromatography gel filtration column (GE Healthcare, Piscataway, NJ) to isolate the different oligomers of actin according to size. Fractions collected were analyzed using 8% SDS-PAGE gels. Relevant fractions were concentrated and either used within three days or flash frozen and stored at -80°C.
Figure 8: Modified ADPr-Oligomer Purification Protocol. Comparison between the original ADPr-oligomer protocol and the modified protocol. The modification saved us two days of wait time or labour.
2.2.4 Photox Purification from *E. coli*

Glycerol stocks of Photox toxin grown in Rosetta competent cells were kindly provided by Dr. Rod Merrill. Photox purification was performed following Perieteanu *et al.* 2010. 5 ml of LB + Kan inoculated overnight with the glycerol stock was transferred to a 50 ml LB + Kan and incubated for 1 hour at 37°C. This was then transferred to 2 L of 2X YT+ Kan solution that grew till OD 600 ~ 0.6. Then, IPTG to a final concentration of 1 mM was added to the culture to grow for 3 hours. After 3 hours, the culture was spun at 5,200 rpm for 5 min in Avanti J25 I centrifuge with a JLA 10.5 Beckman Coulter rotor. Pellets that contained the cells were broken with 30 ml cold lysis buffer (20 mM Tris, 50 mM NaCl, pH 7.5). The resuspended pellet was French pressed twice and centrifuged at 8,600 rpm for 25 min and obtained pellet was resuspended in Wash buffer (50 mM Tris pH 7.5, 2 mM EDTA, 100 mM NaCl, 0.05 % deoxycholate and 0.5 mg/ml lysozyme). This resuspension was centrifuged at 7,400 rpm for 20 min and pellet was resuspended in 15 ml denaturing buffer (500 mM NaCl, 50 mM Tris pH 7.5, 3 M GnHCl). Rigorous vortexing and pipetting was used to dissolve the pellet and the solution obtained was again centrifuged for 40 min at 10,000 rpm speed. A chelating Sepharose fast flow column (GE Healthcare 17-0575-01) was pre-equilibrated with Zn$^{2+}$, and the supernatant obtained after centrifugation was loaded on the column twice. The fractions were collected using gravity by washing the column with increasing step concentrations of imidazole (5 mM, 20 mM, 40 mM, and 100 mM).

Fractions containing Photox were pooled and dialyzed against binding buffer (20 mM Tris, pH 7.5, 50 mM NaCl). Photox was concentrated to 0.5 mg/ml with an Amicon concentrator, and the concentration was calculated using the DU 800 Beckman Coulter.
Spectrophotometer where concentration in mg/ml = (Abs 280/0.67) * Dilution Factor (Visschedyk et al., 2010). The activity of Photox was tested using a CARY Eclipse Fluorescence Spectrophotometer (Varian, Missisauga, ON) at an excitation wavelength of 305 nm and an emission wavelength of 405 nm by adding a 5 to 10 fold molar excess of ε-NAD⁺ (Sigma Aldrich N2630) relative to the concentration of G-actin. The fluorescence is increased upon ribosylation due to internal quenching of ε-NAD caused by chemically removed nicotinamide (Pergolizzi et al., 2011).

2.2.5 Myosin and S1 Purification

Full-length myosin was purified from rabbit muscles as described by Margossian and Lowey (1982). The ground muscle mince from rabbit was extracted with solution A (0.3 M KCl, 0.15 M K₂PO₄, pH 6.5, 0.02 M EDTA, 5 mM MgCl₂, 1 mM ATP). This mixture was diluted with water twice, 4-fold and again to a 10-fold dilution of solution A at 4°C. The diluted myosin mix was allowed to sit for at least 3 hours to obtain thick myosin settled at the bottom of the flask. The clear buffer on top was carefully siphoned off, and the settled myosin was centrifuged at 7,000 rpm for 15 min in a JLA 10.5 rotor using Avanti J25 I centrifuge. Resuspension of the pellet was carried out with 220 ml of solution B (1 M KCl, 25 mM EDTA and 60 mM K₂PO₄). The resuspension was dialyzed against 6 L of solution C (0.6 M KCl, 25 mM K₂PO₄, 10 mM EDTA and 1 mM DTT) overnight. The next day, an equal volume of water was added to the dialyzed solution and centrifuged at 10,000 rpm for 10 min. Isolation of myosin thick filaments was performed by centrifuging the supernatant for 1 hour at 19,000 rpm using a JLA 20.5 rotor. The supernatant obtained was diluted 8-fold to let the myosin precipitate again overnight.
Precipitated myosin was centrifuged at 10,000 rpm for 10 min in a JLA 10.5 rotor. The pellet was resuspended with solution E (3 M KCl and 10 mM K$_2$PO$_4$) and dialyzed overnight in solution D (0.6 M KCl and 50 mM K$_2$PO$_4$). Myosin was finally separated from lipids after centrifugation for 2 hours at the speed of 19,000 rpm. The supernatant containing full-length myosin was dialyzed against 50% glycerol v/v and stored in -80°C after flash freezing with N$_2$(l).

The myosin head S1 was isolated through papain digestion of full-length myosin and used the next day. Myosin full-length was kept at room temperature for an hour and papain to a final concentration of 0.03 mg/ml was added to it. This reaction was run for 7 min after which it was stopped by the addition of 1 mM iodoacetic acid final concentration. The stopped reaction solution was centrifuged at 70,000 rpm for 15 min in a TLA 110 Beckman Coulter rotor using an Optima tabletop ultracentrifuge. Supernatant and pellet fractions were run on a gel to confirm purification. The S1 activity was confirmed by binding F-actin with S1, centrifuging at 95,000 rpm for 15 min in Beckman Coulter TLA 110 rotor, and then separating the supernatant and pellet. Active S1 bound to F-actin and was in the pellet fraction.

2.3 Myosin ATPase Assay

2.3.1 F-actin vs Slurry

The myosin ATPase activity assay is a colorimetric assay based on the production of a blue product between released inorganic phosphate (P$_i$) and molybdate. The protocol employed a modification of Trybus (2000), adapting the chemistry to a 96-well format.
The 2X stock solutions of actin and myosin were prepared in 1X AB buffer (10 mM imidazole, pH 7, 75 mM KCl, 1 mM MgCl2, 1 mM EGTA, 1 mM DTT, 1 mM NaN3). Different concentrations of actin ranging from 0 – 100 μM were prepared. G-actin was polymerized using 10X AB buffer in a 9:1 ratio, and equimolar (relative to G-actin concentration) gelsolin (24.6 μM stock concentration) was added to control filament length. This F-actin was incubated at room temperature for half an hour before using it in the assay. Different concentrations of actin and slurry stocks from 0 – 100 μM containing 100 mM Mg/ATP mix were prepared with actin added before the slurry. The final concentration of myosin was 0.125 mg/ml. Actin and myosin were mixed in a 1:1 ratio of volumes in 200 μl reaction volumes in a 96-well plate. Each reaction was performed in triplicate. At 10 min intervals for 40 min, 45 μl of each reaction was added to 50 μl of stop solution (60 mM EDTA, pH 6.5, 6.6% SDS) in another 96-well plate. Then 200 μl of colour development solution (75% H2O (v/v), 25% stock of 2% ammonium molybdate in 4N H2SO4 (v/v), and 0.5% ferrous sulphate (w/v)) was added to the stopped reactions and incubated for 20 min. The sample absorbance was read at 750 nm on a plate reader (Thermomax Molecular Devices Microplate reader, Minnesota, USA). Calculations for K_M and V_max and scatter/bar graphs were created with Sigmaplot 12.5 using dynamic curve fitting. The standard error of the mean of the three replicates was used to represent error bars.
2.3.2 F-actin vs ADPr-oligomers

Myosin ATPase reactions with 1X AB buffer as a negative control were performed using ADPr-dimers, trimers, and pure full-length myosin. 5 μM of each oligomer was added to 0.125 mg/ml (250 nM) of myosin and the values in μM of inorganic phosphate released by each component over a period of 40 min were measured. In all the assays where F-actin is mixed with ADPr-oligomers, the already incubated F-actin and ADPr-oligomers were mixed together and then added to myosin.

2.3.3 ADPr-oligomers added to F-actin

ADPr-oligomers were added to F-actin in 1:8 ratio of ADPr-oligomer to F-actin (5 μM) in per complex ratios. ADPr-oligomers were also added to 3 μM of F-actin in 1:1, 1:2, 1:3, and 1:6 oligomer:F-actin ratios in per complex ratios. These ratios would be 4.5 μM : 3 μM, 3 μM : 3 μM, 1.5 μM : 3 μM (oligomer : F-actin) in terms of total protein concentration ratios with trimer experiments. These ratios were 6 μM : 3 μM, 3 μM : 3 μM, 2 μM : 3 μM and 1 μM : 3 μM for protein concentration in dimer experiments (oligomer : F-actin).

2.4 Myosin Co-sedimentation Assay

The binding activity of actin oligomers with full-length myosin was observed through co-sedimentation assays (Figure 9). Actin oligomers bound to full-length myosin thick filaments in the absence of ATP should be present in the pellet after spinning. A
Figure 9: Co-sedimentation Assay. Proteins were added to G-buffer and 10X poly. Then they were spun down and the top half of the supernatant was labeled as “S” and the bottom half was labeled as “K1”. The pellet was washed for any non bound ADPr-oligomer to come out in the supernatant labeled “K2”. 100 μl of F-buffer was added for another wash and spun. The supernatant is labeled “K3” while the pellet was re-suspended in 100 μl of F-buffer and labelled “P”.
The final concentration of 10 μM actin slurry and equimolar myosin were incubated for 1 hour in G-buffer (0.2 mM ATP, 0.2 mM CaCl₂, 2 mM Tris pH 7, 0.2 mM β-ME) and 10X poly (10 times polymerization buffer, 10 mM EGTA, 500 mM KCl, 20 mM MgCl₂, 250 mM Tris pH 7.5) (100 μl reaction) in 250 μl centrifuge tubes from Beckman Coulter. Samples were centrifuged at 95,000 rpm in a TLA 100 Beckman Coulter centrifuge with a TLA-100 Fixed Angle Rotor (TL-TB-003AM) for 15 min. The top 50 μl of supernatant was labelled as “S” and the bottom 50 μl was labelled as “K1”. The pellet was washed with 100 μl of F-buffer (1 mM EGTA, 50 mM KCl, 2 mM MgCl₂, 25 mM Tris pH 7.5) and centrifuged at 95,000 rpm for 15 min. This supernatant was marked as “K2” and the pellet was washed in F-buffer and centrifuged at the same speed as above. After the centrifugation spin, the supernatant was labelled “K3” and the pellet was re-suspended in 100 μl of F-buffer (Figure 9). Fractions were run on an SDS polyacrylamide gel and stained with Coomassie stain.

2.5 Native S1 with ADPr-oligomers

To observe direct interaction between the ADPr-dimer/trimer with myosin S1, 10 μl of reactions with 2:1 molecular ratio of S1:oligomers were run on a native gel with oligomers or S1 alone as control. The gel was silver stained.
Chapter 3 – Results

3.1 Protein Purification

3.1.1 ADPr-Oligomer Purification

Purification of actin was followed by modification of methods to obtain ADPr-oligomers adapted from (Perieteanu et al., 2010) (Figure 8, Section 2). F-actin is crosslinked with p-N,N’-phenylenebismaleimide between Cys-374 of one actin subunit and Lys-191 of another subunit, resulting in a series of crosslinked actin oligomers. The actin oligomers are then ADP-ribosylated on Arg-177, rendering the oligomers non-polymerizable. Polymerization salts are added and the reaction is centrifuged to remove any non-ribosylated actin. The ribosylated oligomers present in the supernatant after the spin are called the “slurry” (mixture of ADPr-oligomers). This slurry was passed over a gel filtration column to fractionate oligomers according to their size. Fractions containing mixed oligomers (monomer and dimer, dimer and trimer) were pooled and re-run on the gel filtration column. Pure fractions of dimer and trimer were pooled and concentrated (higher order oligomers are difficult to isolate individually). A purification gel for tracking progress was run using fractions collected throughout the purification protocol (Figure 10). Three monomeric bands were observed in ADPr-monomer fraction which could be due to proteolysis of N- or C-terminus.

Starting with 1 kg of lean turkey meat, the first extraction yielded around 90 gm of acetone powder. For an ADPr-actin oligomer preparation, 45 gm of acetone powder yielded 150 mg of ADPr-slurry and 50 mg of ADPr-monomer, 15 mg of ADPr-dimer, and 1 mg of ADPr-trimer after isolation. Concentration of slurry was measured with a Bradford assay according to subunits of actin. The drawbacks of this method are low
Figure 10: Progress of Purification for ADPr-Oligomers. Fractions run on 8% SDS-PAGE gel stained by Coomassie show the purification of ADPr-oligomers. Purification of oligomers was monitored by collecting fractions at each step. After crosslinking, oligomers were seen in the crosslinked (X-link) fraction including dimer, trimer and higher order. Supernatant (ADPrS) and Pellet (ADPrP) fractions after ADP ribosylation of crosslinked actin oligomers are shown. 100% ribosylation was not achieved; non-ribosylated oligomers polymerized and settled in the pellet. Oligomers purified by gel filtration chromatography show pure ADP-ribosylated monomers (ADPr-M), dimers (ADPr-D) and trimers (ADPr-T). Lower dimers (LDimer) are two monomers with crosslinking between Cys-Cys obtained by the fast reaction of pPBM with the cysteines in these monomers. UDimer is the upper dimer formed by Cys-Lys reaction. Three monomeric bands might be a result of proteolysis on the N-terminal or C-terminal of monomeric actin, these could be due to presence of proteolytic enzymes in lysate during photox purification.
yields and the amount of time it takes to purify actin oligomers in quantities suitable for experiments. Starting with muscle tissue, a typical preparation of ADPr-trimer takes 14 days to complete. However, by modifying the original protocol to dedicate actin extractions from acetone powder to crosslinking (Figure 8, Section 2), 2 days were saved in the preparation time. The concentration of slurry was measured with a Bradford on a subunit concentration basis.

3.1.2 Photox Purification

Photox, the enzyme responsible for ribosylation of actin, was isolated from inclusion bodies from E. coli Rosetta cells transformed with the photox gene encoding an N-terminal His tag using a chelating Sepharose fast flow column with zinc-charged resin. Fractions were collected using four different washes with increasing concentrations of imidazole (Figure 11A). Fractions containing photox were pooled and concentrated to 0.5 mg/ml.

To check the activity of photox enzyme, the increase in fluorescence detected by excitation at 305 nm and emission at 405 nm from the transfer of ADPr from ε-NAD⁺ to G-actin was monitored over 30 min (Figure 11B). The base fluorescence level of 25 μM of G-actin with 125 μM of ε-NAD⁺ was measured for 2 min, followed by the addition of 0.5% v/v photox. This reaction was monitored for 30 min and a 30-fold increase in fluorescence was observed, confirming photox activity.
Figure 11: Photox Purification and Activity Confirmation. A) Purification SDS-PAGE Lysate (L) from denatured inclusion bodies was loaded onto a chelating Sepharose column charged with zinc. Binding buffer fractions with 5 mM imidazole (5) initiate the elution of photox. Buffer 1 (20 = 20 mM imidazole), Buffer 2 (40 = 40 mM imidazole) and Elution buffer (100 = 100 mM imidazole) fractions show elution of photox (46 kDa) (MW = molecular weight marker). B) Activity of photox A baseline was achieved with 25 μM G-actin and 125 μM ε-NAD⁺ in 100 μl for 2 min. Photox 0.5% v/v was added to the solution and an increase in fluorescence was seen with time signifying the increasing amount of G-actin that was ribosylated.
Figure 12: Myosin S1 Purification and Activity Confirmation. A) Purification of myosin S1. Coomassie-stained 8% SDS-PAGE gel of full-length myosin (FM) cleavage with 0.03 mg/ml of papain. After 10 min, the reaction is stopped with 1 mM iodoacetic acid. Myosin S1 (95 kDa) is separated in the supernatant (S/N) from the rest of full-length myosin that sediments down in pellet (P) after centrifugation at 50,000 rpm for 15 min. B) Activity of myosin S1. 10 μM of F-actin was reacted with equimolar freshly-cleaved myosin S1 and spun at 95,000 rpm for 15 min. Supernatant (S/N) and Pellet (P) fractions were run on an 8% SDS-PAGE gel (S1+F) stained with Coomassie. Both S1 and F-actin were present in the pellet. 0.1 M ATP was added to the pellet fraction and spun again. Myosin S1 detached from F-actin and was present in the Supernatant (S/N) (S1+F+A) whereas F-actin was found in the pellet (P).
3.1.3 Myosin S1 Purification

Myosin S1 head from rabbit skeletal full length myosin was isolated following the procedure from Margossian and Lowey (1982). Full-length myosin (300 mg) yielded only 28 mg of soluble myosin S1 (Figure 12A). Losses were due to the high-viscosity and incomplete proteolysis of full-length myosin that were difficult to retrieve.

To test the activity of S1, equimolar myosin S1 was incubated with 10 μM F-actin in the absence of ATP at room temperature, and centrifuged at 95,000 rpm for 15 min. Myosin S1 co-sedimented with F-actin in the pellet (P/S1+F in Figure 12B). When the pellet fraction was re-suspended in 0.1 M ATP and centrifuged, active myosin S1 was found in the supernatant (S/N/S1+F+A).

3.2 Myosin ATPase Assays

Myosin has an intrinsic ATPase activity that is greatly enhanced in the presence of F-actin. According to the model of the crossbridge cycle, full-length myosin binds to F-actin and generates force through the hydrolysis of ATP (Figure 6), releasing inorganic phosphate (P$_i$). Measuring the release of this P$_i$ was used to assay the increase in myosin ATPase activity in the presence of actin. A previous graduate student in the Dawson lab observed a reduction in the baseline intrinsic myosin ATPase activity in the presence of ADP$_r$-trimer (preliminary unpublished data by Dr. Alex Periteanu). My experiments followed up this observation. I first started investigating the behaviour of myosin in the presence of all oligomers in the slurry. It should also be noted that the experiment was optimized with a constant final concentration of myosin at 0.125 mg/ml for all my ATPase assays.
<table>
<thead>
<tr>
<th>Component</th>
<th>(V_{\text{max}}) (μM/min)</th>
<th>(K_M) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-actin</td>
<td>34.14 ± 1.5</td>
<td>1.65 ± 0.3</td>
</tr>
<tr>
<td>Slurry</td>
<td>25.90 ± 0.7</td>
<td>12.02 ± 1.6</td>
</tr>
</tbody>
</table>

Figure 13: Myosin ATPase Activity Assay of F-actin vs F-actin with Slurry/Slurry only. **A)** Full length myosin ATPase activity in the presence of F-actin and/or F-actin with slurry Protein concentration given is for slurry (closed circles, N=6) or F-actin (closed triangles, N=12, and open circles, N=3 included in the inset). 5 μM slurry is present in all reactions measuring myosin ATPase activity stimulated by F-actin in the presence of slurry. The data for slurry is truncated to 50 μM (0-100 μM actual) for better visualization of data for F-actin and F-actin + slurry. Error bars are standard error of the mean. **B)** \(V_{\text{max}}\) and \(K_M\) values. Comparison of F-actin and only slurry.
3.2.1 F-actin vs F-actin with Slurry/ Slurry alone

To observe the impact of F-actin and slurry on myosin ATPase activity, increasing concentrations of F-actin or slurry were added to 0.125 mg/ml (250 nM) of full-length myosin and the released P_i was measured over 40 min (Figure 13A). Alternatively, the impact on myosin ATPase activity with increasing concentrations of F-actin in the presence of 5 μM slurry was measured (Figure 13A inset).

A previous study in the Spudich lab observed low precision in measuring ATPase activity at higher concentrations of F-actin, which was solved by adding gelsolin (Sommese et al., 2013). Gelsolin was added here to sever filaments and reduce viscosity, but I saw a decrease in ATPase activity and increased precipitation after a concentration of 20 μM of F-actin, making it difficult to determine accurate values of P_i released above 20 μM. However, no issues were observed with increasing concentrations of slurry, likely because the ADPr-oligomers are soluble.

The data were fitted to a Michaelis-Menten rectangular hyperbolic curve and the $V_{\text{max}}$ and $K_M$ values were determined (Figure 13B). An increase in $K_M$ and decrease in $V_{\text{max}}$ was seen when slurry was present in the assay. For the slurry added to F-actin, there was a decrease in ATPase activity induced by F-actin compared to F-actin alone.

3.2.2 F-actin vs ADPr-Oligomers

To investigate myosin ATPase activity in the presence of the short oligomers in the slurry, I isolated ADPr-dimer and ADPr-trimer. The limiting reagent for the assays was ADPr-trimer. Enough trimer to perform an ATPase assay with three reactions each with 5 μM trimer (in terms of subunit complex) was isolated; therefore, all the other actin
**Figure 14: Myosin ATPase Activity Assay for Individual ADPr-Oligomers.** Individual ATPase activities of (5 μM) F-actin, ADPr-dimer, ADPr-trimer and myosin basal activity (without actin). The 5 μM concentration is on the basis of per complex of dimer and trimer because we believed each complex to possess one binding site. On a per subunit basis, the concentrations are 15 μM for ADPr-trimer and 10 μM for ADPr-dimer. N=3, error bars mean standard error of the mean. ** mean significantly different compared to myosin basal activity. P-values of oligomers and F-actin or myosin basal activity compared to normal F-actin activity in a two tailed t-test. Dimer = 0.182, Trimer = 0.059 and F-actin = 0.00033**
oligomers were brought to the same concentration. It was observed that ADPr-trimer and ADPr-dimer provide almost no increase in myosin activity (Figure 14). Conversely, myosin activity was not inhibited by either trimer or dimer.

### 3.2.3 F-actin with ADPr-Oligomers

A decrease in the \( V_{\text{max}} \) ATPase activity was seen when slurry was added to F-actin (Figure 13A). To determine the contribution of individual oligomers on normal F-actin activated ATPase activity, I added individual oligomers complex to 5 μM of F-actin in a 1:8 ratio (0.625 μM complex concentration) (Figure 15). On a per subunit basis, the concentration was 0.625 μM monomer, 1.25 μM of dimer and 1.88 μM of trimer. In these experiments, F-actin and ADPr-oligomers were added together to myosin. No significant decrease in F-actin activity was observed, suggesting that none of the ADPr-oligomers decreased or inhibited the normal actin induced myosin ATPase activity.

To address the question of whether the ratio of F-actin:oligomer controlled inhibition of actin activated myosin activity, I measured the myosin ATPase activity in the presence of 3 μM of F-actin mixed with 1.5 μM, 1 μM or 0.5 μM of trimer (4.5 μM, 3 μM or 1.5 μM actin subunit concentration, respectively). Therefore, the total actin protein concentration was 4.5 μM, 6 μM and 7.5 μM. The concentration of F-actin examined was decreased because of limited ADPr-trimer reagent. An increase in myosin ATPase activity was seen with increasing concentration of added trimer (Figure 16). However, this increase was not statistically significant because of p-values above 0.05 (the closest is 1:2 with a p-value of 0.07) (Figure 16).
Figure 15: Myosin ATPase Activity Assay with Addition of ADPr-Oligomers to F-actin. In 1:8 molar concentration ratio of ADPr-oligomer complex:F-actin (0.625 μM: 5 μM). In terms of subunits, the concentration is 0.625 μM of monomer, 1.25 μM of dimer and 1.88 μM of trimer. No significant enhancement or inhibition was observed after addition of ADPr-oligomers to F-actin activity (F+M = F-actin+Monomer, F+D = F-actin+Dimer, F+T = F-actin+Trimer). N=3, error bars are standard error of the mean. P-values for oligomers added to 5 μM F-actin ATPase assay compared to normal ATPase activity at 5 μM concentration of F-actin. F-actin+Myosin = 0.449, F-actin+Dimer = 0.242 and F-actin+Trimer = 0.341.
Figure 16: Myosin ATPase Activity Assay with Increasing ADPr-Trimer. ATPase activity of myosin induced by 3 μM F-actin and increasing concentration of ADPr-trimer added to it. A pattern of increase in ATPase activity is seen. N=3, error bars are standard error of the mean. P-values for total protein concentration 4.5 μM = 0.356, 6 μM = 0.132 and 7.5 μM = 0.067.
Figure 17: Myosin ATPase Activity Assay with Increasing ADPr-Dimer and Linear Regression Plot. A) ATPase activity of myosin induced by 3 μM F-actin as control and increasing concentration of ADPr-dimer. An irregular increase is observed but no pattern or significant increase is seen. N=3, error bars are standard error of the mean. ** show significantly different activity from control F-actin. P-values for ATPase activities of F-actin compared with ADPr-dimer added to F-actin in different ratios. Total protein concentration 4 μM = 0.008**, 5 μM = 0.1591, 6 μM = 0.2635 and 9 μM = 0.1407. B) Linear regression plots for increasing trimer and dimer concentrations Regression plots for increasing ADPr-trimer (empty circles) and ADPr-dimer (full circles). The slope shows change in myosin activity per subunit of added trimer (0.6508) (R² = 0.978). The slope for added dimer is 0.2353 (R² = 0.275)
Varying ADPr-dimer: F-actin concentration ratios were also used to test the effect of concentration on the ATPase activity of myosin. Here, I used 3 μM of F-actin mixed with 3 μM, 1.5 μM, 1 μM or 0.5 μM of ADPr-dimer complex (i.e. 6 μM, 3 μM, 2 μM and 1 μM actin subunit concentration). Here, the total protein concentration was 9 μM, 6 μM, 5 μM and 4 μM. An insignificant increase in activity was seen (Figure 17) with large error bars. It is difficult to imagine why the 4 (1:6) μM total protein concentration was statistically significant while the 1:8 ratio of dimer:F-actin had no effect on myosin ATPase activity from Figure 15. This might be due to experimental error which is why the experiment needs to be repeated in future to confirm results. Lastly, we obtained a regression plot from Sigma Plot to observe the increase in actin-activated myosin ATPase activity with the increase in dimer and trimer. We found a good R² value for trimer but not dimer. Comparing the increase in activity of normal F-actin (3.2 μM of P_i released/min/μM) with trimer (0.6508 μM of P_i released/min/μM), shows us that activity is slow with addition of trimer compared to F-actin itself.

3.3 Co-sedimentation Binding – Slurry

I performed co-sedimentation assays of slurry with myosin to detect the binding of ADPr-oligomers to myosin (Figure 18A). Slurry was readily available in large quantities before starting the gel filtration purification to obtain individual oligomers. Therefore, I centrifuged soluble slurry with non-soluble full-length myosin to observe co-sedimentation of slurry components with myosin following Figure 9 in Section 2. Slurry alone and myosin alone were used as controls. A series of washes were included in the process to avoid oligomers from being trapped in pockets formed by the meshwork of
full-length myosin filament in pellets. Any trapped oligomers would be extracted during these washes in the supernatant (K2, K3).

Densitometry of the slurry control (Sl Cntl) K1 fraction and experimental (Sl + My) K1 fraction was carried out using *Image J*. The slurry control (Sl Cntl) K1 fraction was considered the total (T) of the soluble oligomers. The experimental (Sl + My) K1 fraction was considered \( x \). Therefore, total-\( x \) was the value of oligomers not in the K1 fraction of Sl + My. This value was divided by the total to convert into percentage values and a graph was generated from these values. The experiment (Sl + My) showed approximately 20% monomers, 37% lower dimers, 60% upper dimers, 75% trimers and 80% tetramers that were not in the supernatant fraction (K1) of the experiment (Figure 18B). The contrast of the gels was increased and faint bands of lower and upper dimers were seen in the pellet. However, I did not observe a corresponding concentration of dimers in the pellet as observed on the densitometry graph. Gels were run previously with K2 and K3 fractions and faint bands of upper and lower dimer were seen in K2, but no bands were seen in K3 after increasing contrast.

This assay had several drawbacks. First, the washes obscured the certainty of direct binding of actin oligomers to full-length myosin. Also, binding of trimer could not be directly observed due to full-length myosin and trimer running around the same molecular mass. Secondly, the supernatant was divided into “S” and “K1” in the first step which makes it more complicated to judge the presence of oligomers at the top and bottom half of the initial supernatant (S+ K1). Therefore, I decided to use a direct method for detection of binding with S1 on a native gel.
Figure 18: Co-sedimentation of Slurry with Full Length Myosin/Densitometry with Image J. A) Co-sedimentation SDS-PAGE 10 μM slurry and myosin were mixed. The pellet was washed according to Figure 9 and fractions were collected. Slurry (Sl Cntl) and myosin alone (My Cntl) were used as controls. K1 fractions were run beside the pellet fractions to observe the portions of slurry that co-sedimented with full-length myosin. M = ADPr-monomer, UD/UDimer = ADPr-Upper dimer, LD/LDimer = ADPr-Lower Dimer, T = ADPr-Trimer, H = ADPr-Higher oligomers B) Densitometry of the Slurry control (Sl Cntl) K1 fraction against experimental (Sl + My) K1 fraction using Image J. Slurry control (Sl Cntl) K1 fraction was considered T=total control as all of it is soluble, experimental (Sl + My) K1 fraction was considered x. Therefore, T-x was a value of oligomers not in K1 fraction of Sl + My. This value was divided by Total to convert into percentage values and a graph was generated from these values. N=8, error bars are standard error of the mean.
3.4 Native Gel S1 with ADPr-oligomers

To further investigate interactions between myosin and ADPr-dimer and trimer, a native gel was used. I ran an S1 control (S1) with ADPr-dimer (D) and trimers (T) with (S1+D, S1+T) or without S1 added to it (Figure 19). A positive control with F-actin and S1 and S1 with ATP were run because ATP would release the interaction. The entire S1 band seems to be diminished because of attachment to F-actin. Because the molar ratio of S1:actin monomer was 2:1, there is still some S1 in the lane. Densitometry showed the presence of 48% of control S1 in the S1+F lane. When 0.1 M ATP was added, the S1 was released from F-actin and S1 appears as in the S1 control fraction. The dimer band is partially diminished but an obvious new dimer + S1 band is not seen. Densitometry of dimer showed the presence of 43% of control dimer (D) in the experimental (S1+D) lane. For the trimer, we see the same band intensity of S1 and a separate trimer (S1+T) band which is slightly shifted. This shifting could just be due to the slow running of unbound trimer that has to pass through the gel from the presence of S1 in the lane. Here, quantification gave 82% of trimer to be present in S1+T with T as control (100%).
Figure 19: Native Gel with S1 and Oligomers. Molar concentration ratio of S1:oligomers (complex was 2:1 (6 μM:3 μM). ADPr-dimer and ADPr-trimer were observed for interactions with myosin S1. An F-actin control for this experiment was used with and without ATP. F-actin contol (F) is not itself visible on the gel due to its high molecular weight. If F-actin binds to S1 there would be a decrease in band intensity (seen in F+S1). Densitometry analysis showed 48.6 ± 7.7% of control (S1) present in (S1+F) that means almost half of the S1 was bound by F-actin (considering the 2:1 S1:F-actin ratio). Addition of ATP would release S1 from F-actin leading to S1 control-like bands F+S1+A. There is a change in band intensity of ADPr-dimer with S1. For ADPr-trimers there was a shift in position of the trimer band but that could be due to resistance from S1 in the same lane. Densitometry analysis by Image J shows presence of 43.6 ± 6.4% of control dimer (D) in the experiment (S1+D) and 82.7 ± 3.6% of control trimer (T) in the experiment (S1+T). Here, N=2 and errors are standard error of mean.
Chapter 4 – Discussion

To build a protein complex for X-ray crystal studies, we need a short non-polymerizing oligomeric structure of actin. This short actin oligomer must also bind to myosin. To test the ability of ADPr-dimer and ADPr-trimer to interact with myosin, I performed multiple assays.

4.1 Myosin ATPase Assays

4.1.1 Comparing F-actin with Slurry Activity

A previous graduate student in the Dawson lab compared the activity of myosin in the presence of individual oligomers or F-actin, and found inhibition of basal myosin activity. To reproduce this preliminary result, I compared the activity of F-actin with slurry due to its availability in large amounts. I also added 5 μM slurry to increasing concentrations of F-actin to observe any changes that slurry might cause to normal F-actin activated myosin ATPase activity.

Myosin ATPase activity was decreased with slurry compared to F-actin. The $V_{\text{max}}$ of slurry was 25.9 μM/min which was lower than that of F-actin at 34.1 μM/min. The $K_M$ value for slurry (12.02 μM) was much higher than for F-actin (1.65 μM).

Long-range inter-subunit interactions in actin are important for activity associated with myosin ATPase (Oosawa et al., 1975). Changes in conformation, like elongation of the F-actin filament, are seen when the myosin head binds to F-actin (Tsaturyan et al., 2005; Uyeda et al., 2011). Short oligomers, like ADPr-dimer and ADPr-trimer, lack long range inter-subunit interactions as they are bound by crosslinker and are ribosylated. From the results, we see a decrease in $V_{\text{max}}$ and increase in $K_M$ values. A low $V_{\text{max}}$ could
signify a low turnover rate \( (k_2) \) from the equation \( V_{\text{max}} = k_2 [E_T] \), since total enzyme concentration \( [E_T] \) stays the same. Alternately, a high \( K_M \) and low \( k_2 \) could mean that either \( k_1 \) \( (k_{\text{on}}) \) decreases or \( k_{-1} \) \( (k_{\text{off}}) \) increases following \( K_M = k_2 + k_{-1}/k_1 \). Thus, either conversion of enzyme \( [E] \) and substrate \( [S] \) to complex \( [ES] \) is slow or the reverse reaction is faster. Considering the above factors, the ratio of \( k_{-1} \) \( (k_{\text{off}}) \) to \( k_1 \) \( (k_{\text{on}}) \) would increase, increasing \( K_d \) for the reaction; thus, there is a possibility of low affinity between actin and myosin. I hypothesize that myosin took longer than usual to recognise binding sites for activity in slurry because of a lack of long-range interactions in slurry oligomers.

When 5 μM of slurry was added to F-actin, I saw a decrease in activity. If the two components (F-actin and slurry) were acting independently, then we could be able to add the activity of each component together to get the total activity, up to the \( V_{\text{max}} \) of the enzyme; however, I don’t observe this additive effect. I rationalize this on the basis of increased protein concentration. Individually, 2.5 μM of F-actin gives ~20 μM/min and the same amount of slurry gives 7 μM/min. But together, the activity is only around 20 μM/min rather than 27 μM/min. Therefore, the two components are not acting independently; rather, the two components are either affecting each other or they are interacting somehow in the assay.

4.1.2 F-actin vs ADPr-Oligomers

I repeated the experiment of the previous graduate student, examining the ATPase activity of myosin in the presence of purified ADPr-oligomers. From the results obtained, both dimer and trimer do not elicit significantly different activities than myosin basal activity. These data suggest that these oligomers do not inhibit myosin activity;
moreover, these results suggest that myosin does not interact with these oligomers at all, supporting the idea from section 4.1.1 that higher $K_M$ and low $V_{max}$ of slurry are the result of no interaction or low affinity interactions with slurry. Monomers, dimers, and trimers that make up around 75% of slurry protein may not be interacting with myosin. Therefore, the effective concentration of myosin active oligomers is only 25% of the actin. This model suggests that the myosin activity seen in the presence of slurry is primarily from higher-order oligomers (tetramers, pentamers, etc.) that contain partial long-range interactions. These results were different from Dr. Perieeteanu’s results which did not look reliable due to high error bars.

4.1.3 F-actin with ADPr-Oligomers

The ADPr-oligomers were added to 5 μM of F-actin individually at a 1:8 protein complex ratio to observe any inhibition effect in the presence of F-actin. Here, F-actin and slurry were added together to myosin. Monomer, dimer and trimer did not show any effect on normal F-actin activated myosin ATPase activity at this concentration ratio. These data further support the hypothesis that none of these oligomers interact with myosin or inhibit the interaction of F-actin and myosin. Addition of slurry and F-actin to myosin together might be able to cap F-actin before since ADPr-actin has the ability to bind to barbed end of F-actin (Wegner and Aktories, 1988). One thing to keep in mind is that here the F-actin and oligomers were added together. In case the F-actin was added to myosin after adding the oligomers, the oligomers might have had time to bind to myosin to inhibit activity.
I examined the impact of higher concentrations of purified oligomers on F-actin activated myosin activity. Increasing the concentration of trimer led to an interesting observation of increased myosin ATPase activity with increased concentration of trimer, which proves that the previous decrease in ATPase activity of F-actin +Slurry (5 μM) was not due to competitive inhibition of ADPr-oligomers with F-actin. Since trimer did not produce any myosin ATPase activity all by itself, how did myosin ATPase activity increase when trimer was mixed with F-actin?

Previous studies in Dawson lab have shown that a trimer can cap the barbed end of F-actin and inhibit further addition of monomers to this end (Perieteanu et al., 2010). I believe this attachment of trimer to the barbed end of F-actin might change the conformation of the trimer, making it myosin-active, forming a new binding site. With the increasing concentration of trimer, more trimer may be bound to the same end of F-actin, increasing the length of F-actin and the number of binding sites for myosin. Linear regression plots show that the increase in activity due to F-actin is higher than the increase in activity with addition of trimer, possibly due to the short oligomeric length.

I also studied the behavior of increasing dimer concentration on the F-actin activated ATPase of myosin. Here, there was a constant increase in ATPase activity of myosin throughout the increase in dimer concentration with large error bars. These data could mean that no matter how much dimer is present, only a certain fraction of the dimer caps the barbed end of F-actin. The presence of two types of pPBM-crosslinked dimers was illustrated by Mockrin and Korn (1981) and Steinmetz et al. (1997) due to different types of connections (Cys-Lys, Cys-Cys), (Section 1.6 in Chapter 1). The conformation of some of crosslinked actin dimer might be similar to F-actin (Mockrin and Korn, 1981).
Perhaps only one of the two kinds of dimers is interacting with the F-actin present. In addition, it might be difficult for dimers to cap due to greater inter-subunit flexibility compared to trimer with its three preset subunits.

4.2 Co-sedimentation of Slurry with Full Length Myosin

Myosin co-sedimentation was carried out with slurry to investigate the binding of ADPr-oligomers in slurry. Densitometric analysis showed an average of 20% monomers, 37% lower dimers, 60% upper dimers, 75% trimers and 80% tetramers were lost from the soluble K1 fraction in the experiment. These data contrast to our ATPase results suggesting no interaction between dimers, trimers and full length myosin individually. Also, the pellet fraction did not show the expected slurry components lost from supernatant fraction (K1) considering that faint dimer bands were found in one of the washes K2 but none in K3.

As noted above, ADPr-oligomers can cap actin filaments (Periétanu et al., 2010; Wegner and Aktories, 1988). It is possible that higher order oligomers (tetramer, pentamer, etc.) might have been de-ribosylated and could bind short oligomers. Another possibility is that higher order oligomers behave like F-actin due to the presence of partial long-range interactions, since long-range interactions aid in interaction with myosin. Ribosylated trimer and dimer can cap the barbed end, changing their conformation to F-actin-like and increasing the length of F-actin. Higher order oligomeric studies are difficult due to poor separation by gel filtration. The reason for the non-corresponding percentage of dimers in the pellet compared to the fractions lost in the supernatant could
be due to oligomeric fractions being trapped in buffer pockets that were removed with washes in between collecting the fractions (K2).

4.3 Native Gel Myosin S1 with ADPr-Oligomers

Incubating purified ADPr-dimers and ADPr-trimers with myosin S1 and running these on a native gel led me to believe there might be partial binding of dimers to S1 and almost no binding with trimers. Previous studies in the Dawson lab by Scott Morrison (Morrison et al., 2010) have shown that longitudinal dimers have a strong affinity for myosin S1; however, I worked with lateral dimers. Lateral dimer studies by Kim et al. (2002) showed no activity or interaction with myosin which is supported by our native gel. Also, lateral crosslinked dimers do not have a conformation like native F-actin (Sawaya et al., 2008). The hydrodynamic radii of ADPr-trimers showed them to be more open than in the Oda model. This conformational change in trimer could be due to the presence of ADP ribose on the inner sides of the trimer as suggested by Perieleanu et al. (2010). Thermal stability measurements demonstrated lower values for dimer and trimer compared to modified and unmodified actin. Moreover, decreased nucleotide affinity proved how different these oligomer structures are from native actin.

It is possible that either the upper or lower dimer is an F-actin derived lateral dimer because the band intensity of dimer has decreased to 43% of the control dimer, but did not completely disappear. Also, dimers have a more flexible conformation because of only one crosslinking molecule, unlike trimers. On the other hand, a small shift is observed with trimer, but this shift might be a result of trimer being resisted by the presence of myosin S1 on the gel during electrophoresis compared to the control. A
combination of past and present results could lead us to the conclusion that ADPr-dimers or trimers might not be pertinent structures representative of F-actin to build an actomyosin complex. One question still remains: why was the myosin ATPase activity of F-actin with 5 μM slurry reduced when we saw an increase in the myosin ATPase activity of F-actin with trimer and dimer present? Previous work has shown that ADPr-monomers cap F-actin (Wegner and Aktories, 1988). I propose that the presence of ADPr-monomers in the slurry bind to the barbed ends of F-actin or higher order oligomers in slurry to inhibit the binding of trimer to F-actin. To test this, we would incubate ribosylated ADPr-monomers with F-actin and add trimers to a myosin ATPase activity assay later to determine if the presence of ADPr-monomer inhibits the increase in myosin ATPase activity of F-actin in the presence of trimers.

4.4 Proposed Slurry (F-actin) Model

From the above observations, I propose a working model explaining the interaction between short actin oligomers and myosin: short oligomers that by themselves do not bind or lead to any ATPase activity of myosin. But with all the oligomers present, there is an interaction and myosin ATPase activity is seen.

Interaction of higher order oligomers or F-actin with myosin changes the long range interactions in the actin filament. Moreover, induction of polymerization was seen with ribosylated dimer (longitudinal) when in the presence of myosin S1 and gelsolin (Morrison et al., 2010). Long-range inter-subunit interactions are important for binding of myosin to F-actin and give ATPase activity (Oosawa 1975). Short oligomers might be interacting with the higher order oligomers on their barbed end after this conformational
Figure 20: New Model with Trimer. A) Addition of ADPr-trimer based on model. Change in conformation of F-actin/higher order oligomers, followed by capping of the barbed end by a trimer (green) (B). C) The addition of more trimers (yellow) due to increase in concentration of trimers increases the length of filament and therefore, binding sites for myosin. Trimers are restricted on their flexibility due to presence of three subunits and less space to move with the crosslinker.
Figure 21: New Model with Dimer A) Addition of ADPr-dimer based on model. Change in conformation of F-actin/higher order oligomers, followed by capping of barbed end by a dimer that exposes the right residues for capping the barbed end despite the flexibility of the oligomer (B). C) The addition of only structure specific dimers (green) increases the length of filament, addition of dimers not suitable to actin filament (pale yellow) is ambiguous.
change. So, after addition of trimer or dimer to F-actin there is a change in their structure, converting small oligomers to a more F-actin-like structure. Myosin might bind the newly bound oligomers at the novel F-actin barbed end site. Therefore, addition of oligomers increased the ATPase activity of F-actin (Figure 20, Figure 21).

Co-sedimentation results also support the fact that in the presence of higher order ADPr-oligomers (tetramer, pentamer, etc. (slurry)) the trimer and dimer could have an affinity for full-length myosin. But individually, partial or no binding was seen, similar to results of the native gel with S1 using F-actin as a positive control.
Chapter 5 - Conclusions and Future Directions

5.1 Conclusions

Taken together, my data does not support the hypothesis that ADPr-trimer inhibits myosin ATPase activity; rather, my data suggests that purified ADPr-trimer might not interact with myosin at all. The preliminary results shown by a previous student were not reliable due to the high error bars present and a series of data that represent different behavior of ADPr-trimer than predicted. The interactions between myosin and trimer were seen when in the presence of higher order ADPr-oligomers or F-actin and may be the result of trimer addition to these longer actin polymer structures. This situation is not suitable for the generation of a short, specific actomyosin complex for protein structural determination. Recent studies have implemented the production of high resolution structures using cryo EM which shows a near native state of proteins. The cryo EM study performed by Behrmann was at 8 Å and is a high resolution structure (Behrmann et al., 2012). We hope that the advances in the field of EM can lead us to accurate answers regarding the mechanism of the crossbridge cycle (Li et al., 2013). These advances could overcome the need for symmetrical patterns of proteins required for X-ray crystallography.

5.2 Future Directions

A short F-actin for myosin binding is could still be helpful for assessment. Alternate methods of crosslinking and ribosylation can be used to prepare a more stable complex. A previous graduate student in the Dawson lab, Scott Morrison, tested four different kinds of longitudinal dimers (Morrison et al., 2010). One of these was a
ribosylated dimer made up of mutant Q41C monomer combined with wild type actin which showed a positive interaction with myosin S1, exhibiting low $K_D$ values. Although this dimer may only give an insight into partial interactions between actin and myosin, it might prove to be more stable than other structures. The production of this dimer has been previously achieved in the Dawson lab; purification of S1-dimers and subsequent crystal trials are obvious next steps.
References


