THE ROLE OF KEY AMINO ACIDS IN THE COLD-ADAPTATION OF THE COILED-COIL PROTEIN TROPOMYOSIN

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A thesis submitted in partial fulfillment of the requirements for the degree of Master's of Science

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December, 2011

St. John's Newfoundland

Abstract

The structural basis of cold-adaptation in a rod-shaped (α-helical, coiled-coil) protein tropomyosin was investigated by site-directed mutagenesis. An α-type tropomyosin from Atlantic salmon skeletal muscle having twenty amino acid substitutions compared to a warm-blooded counterpart (rabbit) was used as a model. Two adaptive-strategies were elucidated. The conformational stability of tropomyosin was shown to be enhanced by the presence of a polar amino acid, threonine-179, within the hydrophobic core and the presence of a pair of closely-spaced glycines at positions 24 and 27. The specific details are outlined below in point form:

1) Four mutants of Atlantic salmon fast skeletal muscle alpha-tropomyosin were engineered using the QuikChange Lightning site directed mutagenesis kit. Mutations were chosen in order to investigate the role of a pair of unique glycines near to the amino-terminal end as well as threonine 179 which occurs in a core position of the coiled coil. The four mutants were: Gly24Ala, Gly27Ala, Thr179Ala and a double mutant, Gly24Ala/Gly27Ala. Mutations were confirmed by DNA sequencing.

2) Recombinant (mutated and non-mutated) tropomyosins were obtained by expression in BL21 cells and induction with isopropylthiogalactopyranoside. Enriched protein was isolated, without exposure to organic solvents or high temperatures, by salt-induced precipitation and chromatography on ion exchange column and hydroxyapaptite columns.

3) Far-UV circular dichroism was used to investigate the conformational stability of the recombinant tropomyosins (0.1M NaCl, 20mM sodium phosphate, 1-2 mM dithiothreitol, 0.01 % mass/vol NaN_3). The observed melting temperatures of the three glycine mutants were similar to each other and that of the non-mutated recombinant tropomyosin: Gly24Ala, 36.9 °C; Gly27Ala, 37.3 °C; Gly24AlaGly27Ala, 38.1 °C and non-mutated, 37.0 °C. However, the Thr179Ala mutant showed significant stabilization, 40.7 °C.

Melting profiles of the four tropomyosin mutants and non-mutated recombinant tropomyosin showed that the four mutants displayed more cooperative unfolding profiles compared to the non-mutated protein.

4) Limited chymotrypsin digestion (buffer: 50 mM NH_4HCO_3 , 0.1 M NaCl, 1 mM DTT, pH 8.5), as monitored by SDS PAGE, revealed that the non-mutated tropomyosin is more susceptible to proteolysis than the Thr179Ala mutant (37 °C, ~1:500 enzyme:substrate mole ratio). After 30 min, none of the intact non-mutated protein was detected in the reaction mixture whereas not all of theThr179Ala mutant had been digested.
At ~25 and ~10 °C, the difference in the rate of digestion between the two, was not as significant.

5) From points 3 and 4 it can be concluded that threonine in the 179 position of salmon tropomyosin is destabilizing compared to the alanine in the same position of rabbit skeletal alpha-tropomyosin.

6) Sequencing of two fragments from the chymotrypsin digestion of non-mutant recombinant tropomyosin indicates that the initial cleavage site is between Leu11 and Lys12.

7) Omp-T digestion (buffer: 0.1 M NaCl, 50 mM sodium phosphate, 5 mM EDTA, 1 mM DTT, pH 7.0) patterns, as monitored by SDS PAGE, were compared between Gly24Ala, Gly27Ala, Gly24AlaGly27Ala, and non mutated tropomyosin at ~10, ~25 or ~37 ^{o}C. At all three temperatures, non-mutated tropomyosin was digested faster compared to the glycine mutants, which were different to each other. The observed rate of breakdown decreased in the order: Gly27Ala > Gly24Ala > Gly24AlaGly27Ala, indicating that both glycines influence the conformational stability of the amino-terminal region and that Gly24Ala is more influential than Gly27Ala.

Note: Some of the above findings were reported in preliminary form at this year's Biophysical Society Meeting (Fudge, K.R. and Heeley, D.H (2011) A mutant of Atlantic salmon fast muscle tropomyosin. 55th Biophysical Society (Baltimore)). A full manuscript is in preparation for submission to Biochemistry.

Acknowledgements

First, I would like to thank my supervisor, Dr. David Heeley, for allowing me the opportunity to work is in his lab and for his guidance, encouragement and advice throughout this thesis. I would also like to thank my supervisory committee, Dr. Ross McGowan and Dr. Mike Morrow for their suggestions and guidance throughout this research project. I would like to express my gratitude for the staff at CREAIT - Dr. Elizabeth Perry and Dr. Ed Yaskowiak - for their help and advice on DNA sequencing. I am grateful to Ms. Donna Jackman for her assistance and suggestions in the initial stages of my research. I would like to thank Dr. Mike Hayley for his advice on circular dichroism and his help in creating the graphs. My appreciation goes to Mr. Craig Skinner for his technical support throughout this research project. I am grateful to the technicians at the Advanced Protein Technology Center (Sick Children's Hospital, Toronto) for sequencing protein fragments. I would like to express my gratitude to my former lab mate, Robert Mercer, for his suggestions when I first started my research. I want to thank my lab mate, Tolu, for all of the help, support and advice she has given me, both inside and outside of the lab. I would like to acknowledge NSERC and the School of Graduate Studies at Memorial University of Newfoundland for providing funding for this research endeavor. Finally, I would like to thank my parents, brother, sister and friends for all of their support throughout the past couple of years – without you, I never would have made it this far.

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List of Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
β-ME	β-mercaptoethanol
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
cDNA	Complimentary deoxyribonucleic acid
dH_2O	Deionized water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ε_{280} 1 mg/ml	Extinction coefficient of a 1 mg/ml protein at 280 nm
IPTG	Isopropyl β-D-1 thiogalactopyranoside
LB	Luria broth
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	Messenger ribonucleic acid
MWCO	Molecular weight cut off
NMR	Nuclear magnetic resonance
Omp-T	Outer membrane protease T
PCR	Polymerase chain reaction
pI	Isoelectric precipitation point
PMSF	phenylmethylsulfonyl fluoride
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOC	Super Optimal broth with Catabolite repression
TEMED	N, N, N', N' – tetramethylethylenediaminutese
TnC	Troponin C
TnI	Troponin I
TnT	Troponin T
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet

Chapter 1
Introduction

The purpose of this thesis was to establish a role for key amino acids in the cold-adaptation of the rod-shaped protein, tropomyosin. Tropomyosin is an α-helical coiled-coil and an important regulatory component in the muscle thin filament. This research used as a model tropomyosin from Atlantic Salmon. Four recombinant mutants were prepared by Quik change lightning site-directed mutagenesis. Mutations focused on one site (specifically, residue-179) within the almost-continuous hydrophobic seam that is principally responsible for stabilising the coiled-coil as well as a pair of glycines (residues 24 and 27) in the amino terminal region that are solvent exposed. Temperature induced unfolding experiments involving circular dichroism showed that changing one amino acid (specifically, threonine-179 to alanine) could significantly affect the conformational stability of tropomyosin. The spectroscopic findings were confirmed by limited proteolytic digestion (chymotrypsin and outer membrane protease T) and extended by Edman-based sequencing of fragments in order to locate sites having altered molecular flexibility.

The introduction of the thesis will start by briefly describing the structure of the striated muscle and the mechanism of muscle contraction. The thesis will then describe the major proteins in the sarcomere: myosin, actin, troponin and tropomyosin. Following the brief introduction of the proteins, tropomyosin will be explained in more detail including its amino acid sequence and the sites of chymotrypsin and Omp-T digestion.

The thesis will then outline some of the current knowledge of psychrophilic (low-temperature) proteins, the reasoning behind preparing a chosen tropomyosin mutant, and a depiction of the goals of the thesis.

1.1 Structure of Striated Muscle

Muscle is contractile tissue that is responsible for movement in animals. It is composed of specialized cells which are responsible for its contraction and regulation. Muscle tissues have adapted to perform a variety of functions which has resulted in three types of muscle tissue: smooth, cardiac and skeletal. Smooth muscle is non-striated and is responsible for involuntary contraction in the walls of blood vessels, respiratory tract and gastrointestinal tract. Cardiac muscle is striated muscle that is responsible for the involuntary contractions of the heart. Skeletal muscle is striated muscle that is responsible for voluntary contraction.

Muscle sarcomeres (Figure 1A), which are generally 2-3 μm long and 1-2 μm in diameter, link end-to-end in order to form long thin strands called myofibrils (Squire, 1997, review). These myofibrils run parallel to each other and form a muscle fibre (Squire, 1997, review). At high magnification, the myofibrils appear striated due to the overlapping thick (15 nm diameter) and thin filaments (6 nm diameter). The thin filaments are comprised of actin, tropomyosin and troponin and the thick filaments are made up of myosin (Figure 1B and 1C). Located at either end of the sarcomere is the Z line which links successive sarcomeres within the myofibrils. It also serves as the attachment point of the thin filaments. In between two Z lines are two I bands which are

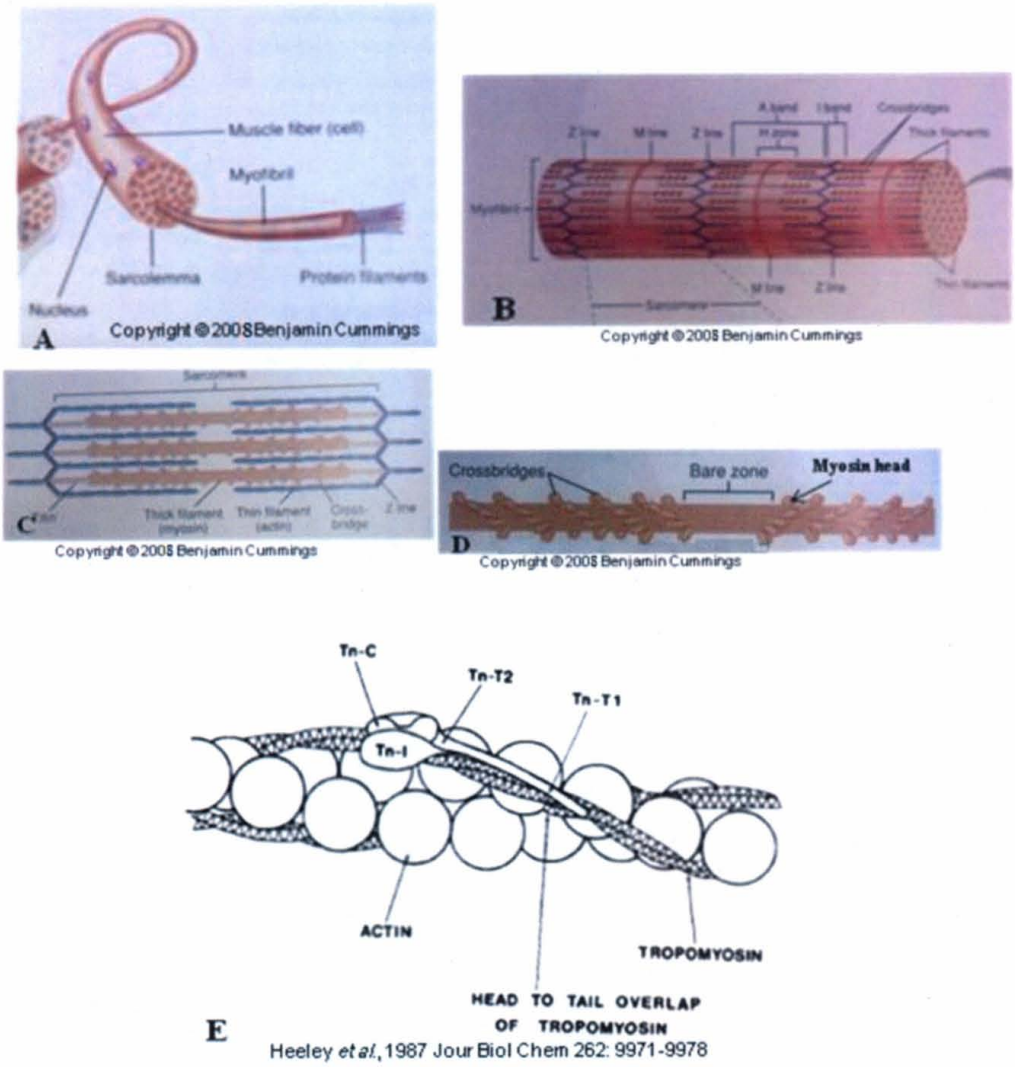


Figure 1: The architecture of the skeletal muscle

A schematic representation of (A) the muscle fibre, (B) the myofibril, (C) the sarcomere, (D) the thick filament and (E) the major proteins of the thin filaments.

on either side of an A band. The A band is darker than the I band because the former is comprised of overlapping thick and thin filaments whereas the latter is comprised of thin filaments only (Huxley and Hanson, 1954; Aidley, 1991, pg 248-249, review) (Figure 1B and 1C).

Skeletal muscle has been divided into three categories based on the following criteria: number of mitochondria present, importance of oxidation and glycolytic pathways in respiration, myosin ATPase activity, contraction time of the fibres and the fibres resistance to fatigue. The three types of skeletal muscle are slow oxidative, fast glycolytic and fast oxidative fibres. Slow oxidative fibres have a high mitochondrion content, low glycogen content, and a long contraction time (Barnard *et al.*, 1971). Their red colour is due to the high concentrations of myoglobin, they are small in diameter and they contain a higher supply of oxygen so they can sustain aerobic activity. In comparison, the fast glycolytic fibres have a high glycogen content, short contraction time and a low mitochondrion content. They have a larger diameter and have a lighter colour due to a decreased amount of myoglobin. The fast oxidative fibres are an intermediate of the other two fibres. These fibres have a high mitochondrion content, high glycogen content and a short contraction time. They also have a high resistance to fatigue and are red due to their higher myoglobin content. The fast oxidative and fast glycolytic fibres constitute the majority of skeletal muscle in vertebrates (Barnard *et al.*, 1971).

1.2 Mechanism of Contraction

Both the troponin complex (troponin C, troponin I and troponin T) and tropomyosin are involved in the regulation of muscle contraction, although the exact mechanism is not yet known. According to the steric blocking model, tropomyosin completely covers the myosin binding site on actin in the relaxed muscle (Lehman *et al.*, 1995) and it therefore acts as a relay between the troponin complex and actin (Potter *et al.*, 1985). Following an action potential, Ca^{2+} is released from the sarcoplasmic reticulum which increases the free Ca^{2+} in the cytoplasm. These Ca^{2+} ions bind to the specific sites on troponin C (TnC) which causes a conformational change on the TnC. This conformational change leads to increased binding of TnC to troponin I (TnI) (relayed by troponin T), which results in a decrease in binding between TnI and actin. Therefore, there is a decrease in the inhibition that TnI has on the actomyosin MgATPase (Zot and Potter, 1987, review; Farah and Reinach, 1995, review; Perry 1996, review; Lehman and Craig, 2008, review). It is believed that the binding of calcium ions to the troponin complex shifts the tropomyosin on actin, thereby exposing the myosin binding sites of actin (Vibert *et al.*, 1997).

Using interference microscopy Huxley and Niedergerke showed that during shortening or stretching the length of the A-bands remained constant while that of the I-bands changed (Huxley and Niedergerke, 1954). Phase contrast examination revealed that the addition of ATP to myofibrils in order to cause contraction resulted in the simultaneous shortening of the I-band and the H-zone (Huxley and Hanson, 1954). These results led to the proposal of the sliding filament theory (Figure 2) in which the thick

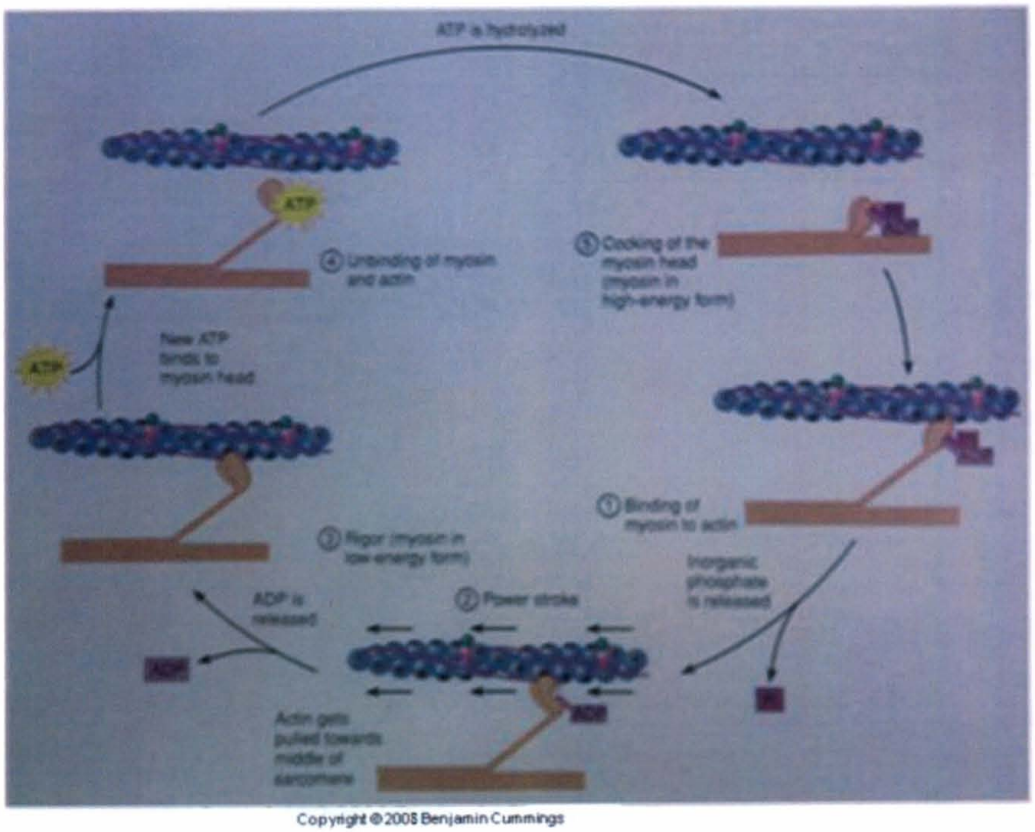


Figure 2: Diagram of the sliding filament theory of muscle contraction

(myosin) and thin (actin and tropomyosin) filament move past each other. In this theory, myosin undergoes a conformational change so that it detaches and reattaches to actin. In the absence of ATP, the myosin cross-bridge is strongly attached to actin. The binding of ATP causes the dissociation of actomyosin and the ATP is hydrolyzed to ADP and P_i which 'cocks' the myosin head. This hydrolysis initiates a weak association of the myosin head to actin and is followed by the release of P_i which is associated with the power stroke. Dissociation of ADP then prepares for another contraction (Huxley and Simmons, 1971; Huxley 1974; Lymn and Taylor, 1971).

1.3 Major Sarcomeric Proteins

The major proteins in the sarcomere are myosin, actin, troponin C, troponin I, troponin T and tropomyosin. Although not discussed below, muscle also contains a host of other proteins which are required for various aspects of filament structure including, anchoring (eg α-actinin), end 'capping' (e.g. tropomodulin and CapZ) and spatial organization (e.g. titin and nebulin) as well as enzymes for the production of ATP and creatine phosphate.

1.3.1 Myosin

Myosin, an enzyme that hydrolyzes ATP (Engelhardt and Ljubimowa, 1939), accounts for greater than 50% of the total protein in the muscle myofibril (Furukawa *et al.*, 1972). In this tissue it is a hexameric protein containing a number of functional and structural domains. Sedimentation experiments first suggested the correct molecular weight of 470,000 Da (Lowey and Cohen, 1962). Later research showed that there are

two heavy chains (Mr ~ 230,000 Da) (Gershman *et al.*, 1969; Lowey *et al.*, 1969), two regulatory light chains (M_r ~ 16,000 – 20,000 Da) (Lowey and Risby, 1971; Weeds and Lowey, 1971) and two essential light chains (M_r ~ 16,000 – 20,000 Da) (Lowey and Risby, 1971; Weeds and Lowey, 1971). The six chains are arranged to form two globular heads attached to an α-helical coiled coil tail (Slayter and Lowey, 1967). The C-terminal portion of the heavy chains is mainly α-helical and forms the myosin tail. The N-terminal part of the heavy chains forms the heads. Each globular head also has one essential and one regulatory light chain associated with it, and contains the ATP, actin and divalent cation-binding sites and is the site of ATP hydrolysis (Gazith *et al.*, 1970). Crystal x-ray diffraction reveals that the head is approximately 50% α-helical in structure (Rayment *et al.*, 1993). The α-helical tails have the typical coiled coil heptapeptide repeat that is described later in more detail in section 1.3.4 (Crick, 1953).

Myosin molecules aggregate to form a bipolar filament structure which makes up the thick filament (Huxley, 1963) (Figure 1D). Lowey *et al.* (1969) hypothesized that the thick filament is formed because most of the myosin tail is water insoluble while the remainder of the tail and the globular heads are water soluble. The structural role of the insoluble myosin tail appears to be the organization of the thick filament (Lowey *et al.*, 1969). This packing arrangement results in the middle of the thick region being a head free region and a region where actin-myosin crossbridges cannot be formed (Harford and Squire, 1986). Importantly, the globular heads project out from the body of the filament (Figure 1D). The organization of the myosin chains has two implications: 1) the myosin

polarity is reversed in each half of the filaments and 2) the ATPase and actin binding sites are on the exterior of the filament (Aidley, 1991 p 265-269, review).

1.3.2 Actin

Actin was accidentally discovered in Szeged, Hungary in 1942 by F.B. Straub (1942). It is a pervasive protein which is found in nearly all eukaryotic cells and is the main component of the thin filament (Kabsch and Vandekerckhove, 1992, review). Muscle actin can exist as either a monomer (G-actin) or as a filamentous polymer (F-actin). G-actin, Mr ~ 42,000 Da, consists of 375 amino acids (Elzinga *et al.*, 1973) and the sequence has been highly conserved throughout evolution. Sequencing of cyanogen bromide peptides revealed that residue 73 is a 3-methylhistidine (Elzinga, 1971). It is believed that this amino acid is incorporated into the protein by enzymatically adding the methyl groups at residue 73 with S-adenosylmethionine acting as the methyl group donor (Asatoor and Armstrong, 1967). The first three codons in the skeletal muscle actin gene code for Met-X-Asp (X is usually Cys but it can be Gly or Ala) whereas the mature protein terminates with an acetyl-Asp (Zakut *et al.*, 1982). Research on the *Drosophila melanogaster* actin suggests a possible mechanism for removing the initial two N-terminal amino acids of actin: the methionine is removed by the ribosomal amino peptidase, followed by acetylation of the cysteine, removal of the acetylated cysteine and finally the acetylation of the aspartic acid residue (Rubenstein and Martin, 1983).

Kabsch *et al.* (1990) first determined the atomic structure of G-actin in complex with DNase I at 2.8 and 3.0 Å resolutions in the presence of either ATP or ADP. In the structure, the nucleotide was situated in a cleft between a small and large domain. The

small domain consists of residues 1-144 and 338-375 and the large domain is made up of residue 145-337. Each domain can be further divided into two subdomains. The small domain is comprised of subdomain 1 (residues 1-32, 70-144 and 338-375) and subdomain 2 (residues 33-69). The large domain encompasses subdomain 3 (residues 145-180 and 270-337) and subdomain 4 (residues 181-269). They determined that subdomain 1 contains a five-stranded β-sheet surrounded by five helices and subdomain 2 consists of a three-stranded antiparallel β-pleated sheet with a helix connecting the two edge-strands. Subdomain 3 is a five-stranded β-sheet surrounded by three helices and subdomain 4 contains a two-stranded anitparallel β-sheet and four α-helices.

Monomeric actin (G-actin) can aggregate to form a filamentous polymer (F-actin) in the presence of Ca^{2+} and Mg^{2+} and higher ionic strengths. Upon polymerization, the bound molecule of ATP is hydrolyzed to leave bound ADP (Szent-Gyorgyi, 1951). Murakami *et al.* (2010) recently proposed a model detailing the formation of filamentous acting and the hydrolysis of ATP. They hypothesized that a newly incorporated actin monomer enables the rotation of the outer domain of the penultimate actin molecule. An interaction between this actin and the neighbouring actin orients the subdomain 2 of the former so that its DNase I loop reaches outward. Part of this loop fits into a hydrophobic cleft while another part causes a downward shift to a proline-rich loop. This shift triggers ATP hydrolysis and P_i is released. F-actin is the form involved in contraction and it is composed of two strands that are arranged in a double helix. F-actin, as determined by electron microscopy, is helical with 13 actin molecules per 6 turns and a repeat of approximately 360 Å (Holmes *et al.*, 1990). In the non-muscle system, the polymerization

of actin is dynamic and the monomers attach and detach from the ends of the filament at different rates (Wegner and Engel, 1975). The ends are labeled as the barbed (plus) end or the pointed (minus) end. Actin monomers will bind at either end, but it has a preference to attach at the barbed end and to detach at the pointed end (Woodrum *et al.*, 1975). Formation of F-actin stimulates the actin ATPase but the release of phosphate is slower than the formation of the filament resulting in an ATP-actin cap at the barbed end. Monomeric actin containing ADP and phosphate accumulate in the rest of the filament (Carlier M-F, 1991, review).

Warm-blooded vertebrates contain six actin isoforms that are tissue specific: two striated muscle actins (cardiac and skeletal), two smooth muscle actins (vascular and visceral) and two non-muscle actins (β and γ) in all non-muscle cells (Kabsch and Vandekerckhove, 1992, review).

1.3.3 Troponin

Troponin, discovered in 1965 (Ebashi and Kodama, 1965), is a complex of three regulatory proteins (Ebashi *et al.*, 1968). The molecular masses of the three troponin subunits were first determined from the amino acid sequences of the proteins obtained from rabbit skeletal muscle: troponin C (TnC, M_r = 18,000 Da) (Collins *et al.*, 1977), troponin T (TnT, M_r = 30,500 Da) (*et al.*, 1977a) and troponin I (TnI, M_r = 21,000 Da) (Wilkinson and Grand, 1975). TnC is the calcium binding subunit, TnT binds to tropomyosin and TnI is the inhibitory subunit (Greaser and Gergely, 1971, 1973). The

atomic structure of most of the complex, the so-called core, has been reported in both Ca^{2+} - bound and free froms (Takeda *et al.*, 2003; Viongradova *et al.*, 2005).

1.3.3.1 Troponin C

Troponin C is the smallest of the three troponin subunits containing about 160 residues and has a pI of 4.1-4.4 (Hartshorne and Dreizen, 1973). It is a dumbbell-shaped protein with a long central helix connecting the amino and carboxy-terminal domains (Sundaralingam *et al.*, 1985). Greater than 65% of the amino acids are in α-helical conformation (Herzberg and James, 1985). It utilizes the common helix-loop-helix (HLH) Ca^{2+} binding motif where a 12 residue loop is flanked by two α-helical segments (Collins *et al.*, 1977). Troponin C is capable of binding one or two Ca^{2+} ions, depending on the isotype, in low-affinity Ca-specific sites and two Ca^{2+} ions in high affinity sites (which can also bind Mg^{2+} ions) (Potter and Gergely, 1975). The two high-affinity binding sites (III and IV, $K_a \sim 10^7\ M^{-1}$) are located at the C-terminal (structural domain) and the low-affinity binding sites (I and II, $K_a \sim 10^5 - 10^6\ M^{-1}$) are located at the N-terminal (regulatory domain) (Sundaralingam *et al.*, 1985; Pearlstone *et al.*, 1997). It is the latter which serve as the calcium sensor.

1.3.3.2 Troponin I

Troponin I is blocked by an N-acetyl group and it has an isoelectric point of 9.3. The rabbit skeletal TnI is comprised of 179 amino acids (Wilkinson and Grand, 1975). It inhibits actomyosin ATPase and is capable of binding to actin, tropomyosin, TnC and TnT (Zot and Potter, 1987, review). The atomic structure of TnI is not yet known,

however, biochemical, chemical and biophysical investigations have shown that the protein can be divided into three regions. The N-domain (~residues 1-95) interacts with the C domain of TnC when sites III and IV are occupied by Ca^{2+} (strong interaction) or Mg^{2+} (weaker interaction) (Farah *et al.*, 1994). This interaction is partially responsible for anchoring TnC to other members of the troponin complex and has both Ca^{2+} -dependent and –independent components (Pearlstone *et al.*, 1997). The second domain (~residues 96-116) is mainly responsible for actomyosin ATPase inhibitory activity in the absence of TnC and TnT. Residue 105-115 is the minimal length required for inhibition to occur (Talbot and Hodges, 1981). In the absence of Ca^{2+}, it is believed that this region is bound to actin and when exposed to Ca^{2+}, it forms a complex the TnC (Pearlstone *et al.*, 1997). Residues 117-156 have a critical regulatory role for the Ca^{2+} dependent interaction of the third domain (~residues 117-181) with the N domain of TnC (Farah and Reinach, 1995, review).

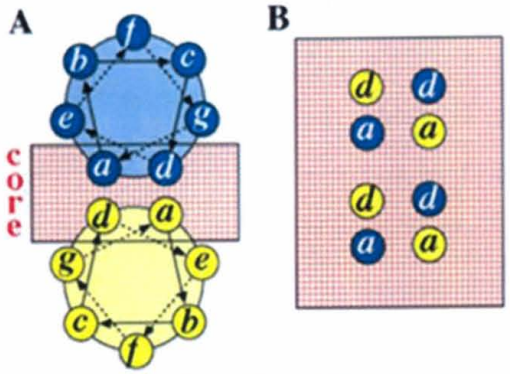
1.3.3.3 Troponin T

Troponin T is acetylated at its N-terminus (Pearlstone *et al.*, 1977b) and the major isoform found in the rabbit skeletal muscle consists of 259 amino acids and has a pI of 9.1 (Pearlstone *et al.*, 1976; Pearlstone *et al.*, 1977b). It is required for the Ca^{2+} sensitive inhibition of ATPase activity (Greaser and Gergely, 1971). This subunit directly interacts with TnI, TnC and tropomyosin and it links the troponin complex to tropomyosin (Zot and Potter, 1987, review). Also, TnT influences the binding of actin and tropomyosin in the presence and absence of Ca^{2+} (Heeley *et al.*, 1987). The N-terminal domain of TnT interacts with tropomyosin (Jackson *et al.*, 1975; Mak and Smillie, 1981b; Pearlstone and

Smillie, 1981; Pearlstone and Smillie, 1982) whereas the globular C-terminal domain interacts with tropomyosin, TnI and TnC (Malnic *et al.*, 1998).

1.3.4 Tropomyosin

Tropomyosin was first isolated in 1946 (Bailey, 1946). It is found in skeletal, cardiac and smooth muscle and (at lower quantities) in non-muscle tissues (Smillie, 1979). The muscle tropomyosin contains 284 amino acids whereas as the non-muscle form is shorter with approximately 247 amino acids (Smillie, 1979). The protein is a coiled-coil dimer (Crick, 1953; Cohen and Szent-Gyorgyi, 1957; Woods, 1967) and Smillie (1979) determined that a subunit of α-tropomyosin from rabbit fast skeletal muscle has a molecular weight of 33,000 Da. Sarcomeric tropomyosin has a diameter of approximately 20 Å and is approximately 400 Å long (Hitchcock-DeGregori, 2008, review). In a muscle, tropomyosin molecules are arranged in a head-to-tail manner along the length of the actin filament (Figure 1E). It was proposed that the N- and C-terminal ends of the 284-residue tropomyosin overlap by nine residues (McLachlan and Stewart, 1975). A more recent study of an NMR structure of the overlap between tropomyosin molecules indicates that 11 residues on the N-terminal of one molecule fits into the cleft at the C-terminal of another molecule that is created when the two strands spread apart (Greenfield *et al.*, 2006). Astbury *et al.* (1948) showed that tropomyosin exhibited a distinct periodicity that was characteristic of the k-m-e-f (keratin-myosin-epidermin-fibrinogen) group of proteins. The diffraction patterns of tropomyosin were a result of a left-handed coiled-coil structure involving two right-handed α-helices that were at a 20° angle to each other (Crick, 1953) (Figure 3C). In order for this to occur, a strip of non-



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C

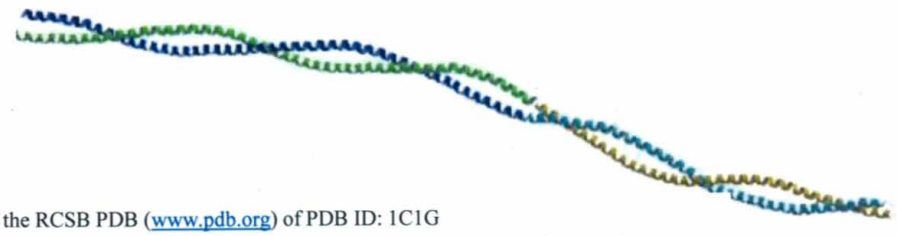

Image from the RCSB PDB (www.pdb.org) of PDB ID: 1C1G

Whitby FG, Phillips Jr GN (2000) Crystal structure of tropomyosin at 7 Angstroms resolution. *Proteins* 38: 49-59

Figure 3: The Structure of Tropomyosin

(A) Amino acid positions of the coiled coil tropomyosin as viewed from an axial position. Residues '*a*' and '*d*' are typically hydrophobic and help keep the two helices in contact with each other. Residues '*g*' and '*e*' are typically charged amino acids that stabilize the protein through ionic interactions. Residues '*b*', '*c*' and '*f*' are usually charged. (B) Side view in which the cylinders represent the helical back bone. (C) Molecular ribbon diagram of a tropomyosin dimer.

polar residues run down one side of each of the two α-helices. The helices wind around each other to form the coiled-coil that is stabilized by the "knobs and holes" packing arrangement (Crick, 1953). In order to create the non-polar sides of the helices, tropomyosin can be thought of as having a heptapeptide sequence (*a b c d e f g*) where '*a*' and '*d*' are non polar amino acids. Residues '*a*' and '*d*' on one strand are in contact with residues '*a*' and '*d*' on the other strand and acidic and basic residues are located on the exterior of the coiled coil (Figures 3A and 3B). The remaining residues are usually polar or ionic and salt bridges between residues '*e*' and '*g*' often appear in order to further stabilize the coiled-coil structure (McLachlan and Stewart, 1975). Johnson and Smillie (1975) have shown that the two subunits are in register and in parallel due to the fact that it is possible to form an intramolecular disulfide bond between the cysteines at position 190.

Tropomyosin has an unbroken series of 40 heptads that is known to be unusual for fibrous proteins (Parry, 1975). As well, additional stabilization is added to the protein because the charged residues in positions '*e*' and '*g*' on neighbouring chains form salt bridges which results in the two chains being in-register (McLachlan and Stewart, 1976). Most tropomyosin molecules also have an unusually high number of alanine resdiudes in the core region compared to other α-fibrous proteins. In vertebrate skeletal muscle, as many as ≈ 35% of the *'d'* position residues are alanines (Conway and Parry, 1990). Brown *et al.* (2001) describes seven alanine clusters in the tropomyosin molecule. These clusters which cause the chains to pack closer together (compared to the remainder of the molecule) are staggered which results in a bend. The bend is important as it allows the

tropomyosin to wind around the actin filament (Brown *et al.*, 2001; Singh and Hitchcock-DeGregori, 2003).

Tropomyosin is highly α-helical with the N-terminal half of the molecule having the greatest amount of helical content. Progressively less helical content exists towards the C-terminal (Smillie *et al.*, 1980). Consistent with this, Greenfield *et al.* (2003) showed that the N-terminal forms a continuous coiled-coil up to residue 279 and that the last five residues are in a random conformation. It has been shown by Phillips *et al.* (1980) and confirmed by Brown *et al.* (2001) that the N-terminal part of tropomyosin is more rigid compared to the C-terminal part. Li *et al.* (2002) determined that the C-terminal section of tropomyosin (residues 254-284) does not form a coiled coil and that the two helices splay apart at this end of the molecule. The site of splaying involves a small group of destabilizing core residues including Gln263 ('*d*') and Tyr267 ('*a*') that pack asymmetrically at the interface of the helices (Li *et al.*, 2002). The mid-region of tropomyosin has three core alanines in a row (residues 151-158) which causes a 6^{o} bend in the coiled coil and there are also isolated alanines which create gaps along the protein (Brown *et al.*, 2005).

More recently, Gly126 ('*g*') and Asp137 ('*d*'), both of which are strongly conserved, have been shown to impart flexibility and instability to the middle region of the tropomyosin molecule (Nevzorov *et al.*, 2011; Sumida *et al.*, 2008).

1.3.4.1 Tropomyosin Isoforms

Tropomyosin is present in virtually all eukaryotic cells. The forms of the protein which have been found in non-muscle tissues are shorter in length by one (platelet, Lewis *et al.*, 1983) two (yeast Tpm1, Liu and Bretscher, 1989) or three (yeast Tmp2, Drees *et al.*, 1995) actin binding sites compared to those in muscle, with a few noted exceptions (MacLeod *et al.*, 1985). The first evidence that there are multiple forms of tropomyosin came from electrophoresis separations performed in the presence of SDS (Cummins and Perry, 1973). Two variants were observed, so-called α and β. The basis of the separation is not due to a true difference in mass but net charge which affects the interaction with SDS. Protein sequencing showed that 39 amino acid differences exist between these isoforms in rabbit skeletal muscle tropomyosin, with the C-terminal half being more divergent (Mak *et al.*, 1980). Further, β-tropomyosin is predicted to have a higher net negative charge at neutral pH than α due to Ser229Glu and His276Asn (Mak *et al.*, 1980). The sequence analysis also confirmed a difference in cysteine content (Cummins and Perry, 1974). Subsequently, four tropomyosin genes were discovered in mammals from which at least 20 isoforms are expressed by alternative promoters and RNA processing (Pittenger *et al.*, 1994). In humans, the four genes have been labeled TPM1, TPM2, TPM3 and TPM4 (Cutticchia and Pearson, 1993). TPM1 and TPM2 correspond to the genes that are responsible for the α- and β-tropomyosins located in skeletal muscle (Helfman *et al.*, 1986; Ruiz-Opazo and Ginard 1987). TPM1 contains 15 exons, and through alternative splicing, can give rise to at least eight other isoforms including smooth muscle α-tropomyosin. The β-tropomyosin gene contains nine exons (five of

which are in all isoforms) and codes for skeletal and smooth muscle tropomyosin and fibroblast tropomyosin isoforms (Perry, 2001, review). The TPM3 gene codes for the 248 residue fibroblast tropomyosin and a 284 residue slow twitch skeletal muscle α-tropomyosin (TM3) (Clayton *et al.*, 1988; Gunning *et al.*, 1990; Smillie, 1996). The TPM4 gene contains eight exons and codes for fibroblast TM4 (Lees-Miller *et al.*, 1990; Macleod *et al.*, 1987). It should be noted that the tropomyosin which was selected for study in this thesis specifically, salmon fast skeletal, is of the α-type, as determined by sequencing (Heeley *et al.*, 1995).

1.3.4.2 Post-Translational Modifications

Skeletal muscle tropomyosin undergoes two post-translational modifications which are: N-terminal acetylation and phosphorylation of serine283 (Hodges and Smillie, 1973; Mak *et al.*, 1978). The acetylation of the N-terminal methionine is necessary for the tropomyosin to have a strong binding affinity to actin (Urbancikova and Hitchcock-DeGregori, 1994). The absence of acetylation in β-tropomyosin causes an eight-fold reduction in actin affinity and its absence in α-tropomyosin results in a 40-fold reduction in actin affinity (Coulton *et al.*, 2006). Although acetylation is not necessary for homo- or heterodimer formation, it appears to stabilize the coiled-coil (Greenfield *et al.*, 1994). Covalently bound phosphate was first detected in tropomyosin from the skeletal muscle of a frog that had been injected with ^{32}P orthophosphate (Ribulow and Barany, 1977). Subsequently it has been found in the striated muscle tropomyosins of rabbit, chicken, fish, rat and mouse (Montarras *et al.*, 1981; Heeley and Hong, 1994; Heeley *et al.*, 1982). One theory for the role of phosphorylation of tropomyosin suggests that it has

significance for myofibrillogenesis (Heeley *et al.*, 1982), while another proposes that it plays a role in stabilizing the head-to-tail interaction during polymerization (Mak *et al.*, 1978; Heeley *et al.*, 1989). Heeley (1994) demonstrated that tropomyosin phosphorylation increases the steady-state rate of hydrolysis of actomyosin MgATPase. Later research using phosphorylated and unphosphorylated shark tropomyosin confirmed this activation (Hayley *et al.*, 2008). It is important to note that the phosphorylation is unique to striated tropomyosin because smooth and non-muscle isoforms do not have a serine at position 283 (Sanders and Smillie, 1985).

1.3.4.3 Interactions with Self and other Proteins

Tropomyosin is associated with the I-band and thin filament of skeletal muscle and it interacts with actin, troponin T (Corsi and Perry, 1958; Perry and Corsi, 1958; Eisenberg and Kielly, 1974). Tropomyosin lies along the narrow groove of the actin helix (McLachlan and Stewart, 1976). One tropomyosin molecule spans seven polymerized actin monomers even though tropomyosin contains 14 quasiequivalent repeating regions of acidic and non-polar residues that could serve as binding sites (Stewart and McLachlan, 1975). In a tropomyosin dimer, there are 28 acidic zones on the surface which are arranged in 14 opposing pairs to produce four sets of seven zones which are at 90^{o} to each other (Stewart and McLachlan, 1975). Tropomyosin binds cooperatively to actin, but the ionic conditions that are necessary *in vitro* depend on the species (Yang *et al.*, 1977; Yang *et al.*, 1979; Lehrer and Morris, 1982). Mg^{2+} is important in forming the actin-tropomyosin complex and it is thought that Mg^{2+} bridges form between carboxylate groups on both proteins (Martonosi, 1962). Local instability and side chain flexibility of

tropomyosin is necessary for actin binding (Singh and Hitchcock-DeGregori, 2006). Singh and Hitchcock-DeGregori (2006) predicted that in addition to the flexible coiled-coil, a specific recognition site on the surface of the coiled-coil is also important for actin binding. McLachlan and Stewart (1976) first showed that tropomyosin had seven periods that were quasi-equivalent for actin binding. Further research by Singh and Hitchcock-DeGregori (2007) illustrated that these periods also had specific regulatory functions. Period 5 (residues 166-207) was the most conserved period and it was also the most important for binding to filamentous actin (Hitchcock-DeGregori *et al.*, 2002). It is thought that period 1 is also very important for maximal binding affinity to actin because it is the only other period that contains alanine clusters embedded within the consensus repeats (Singh and Hitchcock-DeGregori, 2007).

Troponin T is responsible for the interaction of the troponin complex with tropomyosin (Zot and Potter, 1987, review). It is a stable complex that forms independently of calcium but is sensitive to the ionic strength of its surroundings (van Eerd and Kawasaki, 1973; Jackson *et al.*, 1975). Tropomyosin and TnT interact with a 1:1 molar ratio (Greaser *et al.*, 1972). There are two tropomyosin interaction sites on TnT. First site is located within the N-terminal half of TnT, between residues 71-151 (numbering system corresponding to rabbit skeletal protein) and the second site is within the C-terminal half of TnT (residues 159-259) (Jackson *et al.*, 1975; Ohtsuki, 1979). It is generally believed that the majority of the C-terminal fragment of TnT is needed for interaction with tropomyosin (Pearlstone and Smillie, 1981; Jackson *et al.,* 1975). Site 1

on TnT is thought to bind to the C-terminal part of tropomyosin whereas site 2 binds near Cys190 (Pato *et al.*, 1981b; Chong and Hodges, 1982, Morris and Lehrer, 1984)).

1.4 Atlantic Salmon Tropomyosin

The bulk of the biochemical tropomyosin literature described above, pertains to mammalian and avian sources of the protein. Less work has been carried out with tropomyosin from cold dwelling organisms such as fish. Despite a wide phylogenetic separation and a substantial difference in growth temperature, the number of substitutions that exist between tropomyosins from fish and higher (warm-blooded) vertebrates is small. For example, the sequence of Atlantic salmon fast myotomal tropomyosin (below), inferred from its cDNA sequence (accession number NP_001117128) (Heeley *et al.*, 1995), contains a mere 20 amino acid replacements compared to rabbit alpha (accession number NM_001105688) (Stone and Smillie, 1978). Yet NP_001117128 has been shown to vary significantly from the mammalian version in terms of its conformational stability (Goonasekara and Heeley, 2008). Further, both halves of salmon tropomyosin were found to be of comparatively lower stability (Goonasekara and Heeley, 2008).

```
mdaikkkmqm lkldkenald raegaegdkk aaedkskqle ddlvalqkkl
kgtedeldky seslkdaqek levaektatd aeadvaslnr riqlveeeld
raqerlatal tkleeaekaa desergmkvi enraskdeek melqdiqlke
akhiaeeadr kyeevarklv iiesdlerte eraelsegkc seleeelktv
tnnlksleaq aekysqkedk yeeeikvltd klkeaetrae faersvakle
ktiddledel yaqklkykai seeldnalnd mtsi
```

Of the 20 substitutions (Table 1), only one affects a core amino acid specifically, residue-179 which is a threonine in salmon and an alanine in rabbit. In the amino-terminal half, there is a unique pair of glycines at positions 24 and 27. These substitutions were viewed as potentially important determinants of the unique properties of salmon tropomyosin.

1.5 Sites of Proteolytic Digestion in Tropomyosin

Throughout this study Omp-T and chymotrypsin were used to study the digestion patterns of tropomyosin. Pato *et al.* (1981a) examined the digestion patterns of rabbit striated α-tropomyosin by limited chymotrypsin proteolysis. They showed chymotrypsin initially cleaved tropomyosin on the C-terminal side of Leu-169. This residue is in the core of the protein and was believed to be fairly inaccessible to the enzyme. The researchers proposed that this residue is in a destabilized area due to the presence of several bulky hydrophobic residues at positions 169-172 (-Leu-Val-Ile-Ile-). They also speculated that an aspartic acid (Asp175) in a *'g'* position results in an electrostatic repulsion with Glu180 which contributes to that region's instability.

The Omp-T digestion patterns of muscle tropomyosin were first explored by Goonasekara *et al.* in 2007. Edman sequencing was used to prove that Omp-T cleaved between Lys6 and Lys7 on salmon skeletal tropomyosin. The researchers showed that the cleavage occurred at the same site on tropomyosin from several different sources including shark skeletal trunk muscle, bovine heart, chicken breast, chicken gizzard and rabbit skeletal muscle. Goonasekara *et al.* (2007) also demonstrated that salmon skeletal

Table 1: Differences between the rabbit and salmon fast muscle tropomyosin

Position	Heptapeptide designation	Salmon	Rabbit
24	c	Gly	Gln
27	f	Gly	Ala
35	g	Lys	Arg
42	g	Asp	Glu
45	c	Ala	Ser
63	g	Ser	Ala
73	c	Val	Leu
77	g	Thr	Lys
111	f	Thr	Gln
132	f	Asn	Ser
135	b	Ser	Gln
143	c	Leu	Ile
145	e	Asp	Glu
157	c	Glu	Asp
179	d	Thr	Ala
191	b	Ser	Ala
229	e	Thr	Ser
247	b	Ala	Thr
252	g	Thr	Ser
276	c	Asn	His

Shaded boxes denote substitutions of interest that were mutated in the current research.

muscle tropomyosin is more susceptible to digestion than the rabbit counterpart and that the unacetylated salmon tropomyosin is completely digested at 30 min whereas the N-acetylated salmon tropomyosin was mainly intact at this time.

1.6 Psychrophilic Proteins

Organisms can be placed in three categories based on the surrounding temperature that they thrive on. Mesophiles thrive in temperatures of 17-40 °C, thermophiles thrive at temperatures of greater than 50 °C, and psychrophiles thrive at temperatures below 15 °C (Gerday *et al.*, 1997; Greaves and Warwicker, 2007). Most of the research that has been carried out on proteins from psychrophilic organisms has been from the standpoint of globular proteins. It has been shown, in a given family of globular proteins, that compared to mesophiles and thermophiles, psychrophiles have increased loop sizes and decreased numbers of ion pairs, hydrogen bonds and hydrophobic contacts (Greaves and Warwicker 2009; Greaves and Warwicker 2007; Gianese *et al.*, 2002). These differences provide psychrophilic proteins with the necessary flexibility to function at low temperature. They also account for the fact that such proteins are generally unstable at mesophilic temperatures and higher.

Specific amino acid differences often occur when comparing psychrophiles to thermophiles: arginine and glutamine are replaced by lysine and alanine on exposed α-helices sites, valine is replaced by alanine at buried regions on α-helices, there is a significant increase in alanine and asparagine at exposed sites and a decrease in arginine and the buried sites of β-strands are depleted of valine (Gianese *et al.*, 2001). Some

research has been done on the stability of the non-globular protein tropomyosin which confirms that the core position amino acids ('*a*' and '*d*') are critical to its stability. Hayley *et al.* (2008) reported that shark tropomyosin (psychrophile) has three core amino acid substitutions compared to rabbit tropomyosin (Stone and Smillie, 1978). In each case, the equivalent amino acid is replaced by a hydroxyl-containing one, with the associated loss of two alanines: Thr179Ala, Ser190Cys and Ser211Ala. Examination of shark and rabbit tropomyosin indicates that the shark isoform has a reduced conformational stability through most of its structure compared to the rabbit isoform which is a result of the core amino acid substitutions (Hayley *et al.*, 2011).

1.7 Mutant Tropomyosins

Four mutants of salmon fast muscle tropomyosin were used throughout this research project – three single mutations and one double mutation (Table 1). Each mutant involved the site-directed mutagenesis of a core amino acid and was selected for a specific reason. Three of the mutants (Gly24Ala, Gly27Ala and Gly24Ala/Gly27Ala) were chosen because of the unique double glycines that are present at the N-terminal of the salmon tropomyosin (Jackman et al., 1996). Glycine residues are more common on the C-terminal half of α-helices and in loops, (Richardson and Richardson, 1988). Research indicates that glycine allows for a more flexible protein compared to alanines because the former can adopt more phi and psi angles (Creighton, 1993; Serrano *et al.*, 1992). The salmon tropomyosin glycines were mutated to alanines for a couple of reasons. Primarily, alanine was chosen because it is the standard replacement. Also, in

rabbit skeletal tropomyosin there is an alanine at position 27 instead of a glycine. All three glycine mutants were made to examine both the individual and additive effect of that residue on the conformational stability.

The threonine mutation was of interest because it is the only substitution (salmon vs. rabbit α) that occurs at a core position on the tropomyosin molecule (Heeley *et al.*, 1995). It is also important to note that this isomorphism places a polar side chain in a region of known instability (Lehrer, 1978). As a result of having a threonine in position 179, there are three consecutive '*d*' amino acids that contain destabilizing amino acids (Ile at 172 and Ser at 186). This threonine occurs in a region (172-192) of the protein that is known to be susceptible to familial hypertrophic cardiomyopathy causing mutations (Thierfelder *et al.*, 1994; Golitsina *et al.*, 1997; Bing *et al.*, 1997). The threonine residue was mutated to an alanine because alanine is the residue that is present at the same position in rabbit skeletal tropomyosin (Stone and Smillie, 1978).

1.8 Goals of Study

The main goal of this study was to characterize the influence that particular amino acids in salmon fast muscle tropomyosin have on the protein's flexibility. The three residues that were of interest were Gly24, Gly27 and Thr179. It was hypothesized that the Thr179 increases flexibility because it is a hydrophilic amino acid located in a predominantly hydrophobic core region of the protein. The glycines were hypothesized to enhance flexibility owing to the hydrogen side-chain which allows for a greater range of phi and psi angles.

The effect that these residues have on the conformational stability of salmon tropomyosin was investigated by characterising the consequence of mutating a given residue to alanine using heat-induced unfolding in conjunction with circular dichroism and limited proteolysis in conjunction with Edman-based sequencing. The findings described herein offer insight into the factors which modulate conformational stability of rod-shaped proteins in psychrophiles, which have not been studied to the same extent as globular proteins.

The specific goals are enumerated below:

1) Use site directed mutagenesis to create four mutant salmon fast skeletal tropomyosin: Gly24Ala, Gly27Ala, Gly24Ala/Gly27Ala, Thr179Ala
2) Isolate enriched proteins samples using salt-precipitation and ion-exchange and hydroxyapatite chromatography
3) Use a circular dichroism spectrophotometer to monitor the heat-induced unfolding of the four mutant and the non-mutant recombinant tropomyosins in order to determine their melting temperatures
4) Use Omp-T digestion to compare the rates of N-terminal cleavage of the non-mutant tropomyosin, Gly24Ala, Gly27Ala and Gly24Ala/Gly27Ala at 10, 25 and 37 ^{o}C
5) Use chymotrypsin digestion to compare the cleavage patterns of the non-mutant and Thr179Ala tropomyosin
6) Sequence electroblotted fragments in order to determine the initial site of chymotrypsin cleavage

Chapter 2
Material and Methods

2.1 pH Measurements

A refillable combination calomel glass electrode (Futura^{TM} , 12 x 130 mm, Beckman) attached to a Beckman Φ32 pH meter was used for all pH measurements. The electrode was calibrated daily using standard solutions of pH 7.00 and pH 4.00.

2.2 Electrophoresis

SDS PAGE was performed according to the methods described by Laemmli (1970) using 12% (w/v) acrylamide (BioRad) diluted from a stock solution (30% w/v acrylamide and 0.8% bis acrylamide) with separating buffer (0.75 M Tris-HCl, 0.21% w/v SDS, pH 8.8) (total volume of 20 ml). Polymerization was achieved with the addition of 17.5 µl N, N, N', N' – tetramethylethylenediaminutese (TEMED: Promega) and 120 µl of a 10% (w/v) solution of ammonium persulfate (APS: Promega). Stacking gels were diluted to 3% (w/v) from the same stock solutions (total volume of 5.5 ml) and polymerized with 80 µl of APS and 6 µl of TEMED. A BioRad Protean II apparatus was used for electrophoresis. Gels were generally 7.0 cm long, 10.0 cm wide and 0.75 mm thick. All samples were dissolved in SDS sample buffer (bromophenol blue, glycerol, SDS and DTT). Electrophoresis was carried out at a voltage between 120-180V until the bromophenol blue dye ran off the gel. Gels were stained in 0.25% (w/v) Coomassie Brilliant Blue R-250 (BioRad), 50% (v/v) ethanol (or methanol) and 10% (v/v) acetic acid and then destained in 15% (v/v) acetic acid and 20% (v/v) ethanol (or methanol). A

volume of 10 µl of Precision Plus Protein Unstained Standards (BioRad, cat # 161-0363, 10-250 kDa) were used to estimate the molecular weight of the protein.

2.3 Dialysis

Dialysis was performed using either narrow (10 mm width; 12,000 - 14,000 Da MWCO) or wide (50 mm width; 6,000 - 8,000 Da MWCO) dialysis tubing (Spectrum, California). Dialysis tubing was prepared by heating to ~70 °C in ~1.8 L of 10 mM sodium bicarbonate and 1 mM EDTA. This was followed by extensive washing (3-4 changes) of deionized water (dH_20). The tubing was left to soak overnight in dH_20 and was then stored at ~4 °C in 70% (v/v) ethanol. Prior to use, the dialysis tubing was thoroughly washed with dH_20.

2.4 Spectroscopy

2.4.1 UV-Visible Absorbance

Protein concentrations were determined using UV absorbance measurements in either a Beckman DU-64 spectrophotometer or an Agilent 8453 Diode Array spectrophotometer. Tropomyosin was dialyzed (narrow dialysis tubing, 12,000 - 14,000 Da MWCO) overnight at ~ 10 °C against a given buffer and the undissolved protein was removed by centrifugation at 12,000 rpm (Eppendorf microcentrifuge 5415D, F45-24-11 rotor) for 2 min. For the Beckman spectrophotometer, the instrument was calibrated using dH_20 and the absorbance of both the dialysis buffer and the protein sample was measured at relevant wavelengths. Measurements were between 0.1-1.0 absorbance units. For the

Diode Array spectrophotometer, the instrument was calibrated with air, and the absorbance of dH_20, dialysis buffer and protein were measured at relevant wavelengths. An extinction coefficient, ε_{280} 1 mg/ml, of 0.25 was used after correction for scatter by subtracting 1.5 x A_{320} (Johnson and Taylor, 1978). The molar mass of tropomyosin was taken to be 66,000 g/mole.

2.4.2 Circular Dichroism

Approximately 10 mg of freeze-dried tropomyosin (either mutated or non-mutated) was dissolved in 2 ml of buffer (0.1 M KCl, 20 mM potassium phosphate, 0.01% Na azide, 1 mM EGTA , 1.5 mM DTT, pH 7.0) and dialyzed (narrow dialysis, 12,000 - 14,000 Da MWCO) overnight against 1 L of the buffer in the cold room. Following dialysis, the sample was centrifuged to remove any insoluble protein. A protein concentration of either 1 or 2 mg/ml sample was used. Spectra were recorded using a Jasco 810 spectropolarimeter. Mutant and non-mutant tropomyosins were heated from 5 ^{o}C to 65 ^{o}C in jacketed cells of varying light path (0.1 mm or 0.2 mm) whilst simultaneously measuring the ellipticity at 222 nm. The temperature was controlled by a CTC-345 circulating water bath. The temperature increase was performed at a rate of either 30 or 60 ^{o}C/hr. The scanning speed of the instrument was set at 100 nm/min with normal sensitivity. The melting temperature was obtained by determining the temperature of the normalized data at 50% unfolding.

2.4.3 Nanodrop

A Thermo Scientific Nanodrop 2000 machine was used to determine the concentration of DNA. The machine was first blanked using 2 µl of nuclease free water and then the absorbance of the sample was measured. Absorbances were measured at 260 and 280 nm and the ratio of the two absorbance measurements indicated the nucleic acid purity of the sample.

2.5 Site-directed mutagenesis

2.5.1 Oligonucleotide Primers

Custom-made oligonucleotide primers were obtained from Operon (Huntsville, Alabama). All primers had a melting temperature between 73 and 75 °C. The underlined nucleotide is the one that differs from the original cDNA. The following primers were used for the site-directed mutagenesis:

Mutation: Gly24Ala	5′ TGGACAGAGCTGAGGCAGCCGAGGGAGACAAGA 3′ FORWARD 5′ TCTTGTCTCCCTCGGCTGCCTCAGCTCTGTCCA 3′ REVERSE
Mutation: Gly27Ala	5′ GCTGAGGGAGCCGAGGCAGACAAGAAGGCAG 3′ FORWARD 5′ CTGCCTTCTTGTCTGCCTCGGCTCCCTCAGC 3′ REVERSE
Mutation: Gly24Ala/Gly27Ala	5′ GCTGAGGCAGCCGAGGCAGACAAGAAGGCAG 3′ FORWARD 5′ CTGCCTTCTTGTCTGCCTCGGCTGCCTCAGC 3′ REVERSE
Mutation: Thr179Ala	5′ AGTGATCTGGAACGTGCAGAGGAGCGCGCTGAG 3′ FORWARD 5′ CTCAGCGCGCTCCTCTGCACGTTCCAGATCACT 3′ REVERSE

2.5.2 Purification of pTRC99A Plasmid from BL21 Cells

In this section, all centrifugation steps were performed in an Eppendorf microcentrifuge 5415D, F45-24-11 rotor at room temperature (RT). BL21 cells (60 µl) with the expression vector pTrc99A (Pharmacia, 27-5007-01), which contains the Atlantic Salmon fast skeletal muscle tropomyosin cDNA, (Jackman *et al.*, 1996) was used to inoculate 6 ml of LB broth containing 25 µg/ml chloramphenicol and 100 µg/ml ampicillin was incubated overnight at 37 °C with agitation. The DNA from the overnight culture was purified using the Wizard plus miniprep kit (Promega) in accordance with manufacturer's instructions. The cells were sedimented at 12,000 rpm for 2 min and resuspended in 300 µl of cell resuspension solution. Cell lysis solution (300 µl) was added and the samples were inverted several times. Neutralization solution (300 µl) was added to and the sample was again inverted several times. Samples were pelleted by centrifugation at 15,000 rpm for 10 min and the supernatant was purified using the resin provided in the kit. The mini-column was washed using the wash solution provided followed by centrifugation at 12,000 rpm for 2 min. 50 µl of nuclease-free H_2O was used to elute the DNA followed by a short centrifugation at 12,000 rpm.

2.5.3 Site Directed Mutagenesis

Site-directed mutagenesis was carried out by means of the polymerase chain reaction using the QuikChange Lightning Site-Directed Mutagenesis kit (Stratagene). All reagents, unless otherwise stated, were provided in the kit. Double-stranded DNA template (10-100 ng), containing the Atlantic Salmon tropomyosin cDNA (Jackman *et al.*, 1996) was mixed with 5µl of 10X reaction buffer, 1 µl of dNTP mix, 1.5 µl of

QuikSolution reagent and 125 ng of each of the two custom-made oligonucleotide primers (Operon). The sample was diluted to a final volume of 50 µl with sterile dH_2O and 1 µl of QuikChange Lightning enzyme was added. DNA was amplified using a MJ Research Peltier Thermal Cycler 200 machine using the following parameters:

Segment	Cycles	Temperature	Time
1	1	95 °C	2 min
2	18	95 °C	20 sec
		60 °C	10 sec
		68 °C	2.5 min
3	1	68 °C	5 min

Following amplification, the reaction mixture was digested by addition of 2 µl of *Dpn* I restriction enzyme and incubation at 37 °C for 5 min.

2.5.4 Transformation of DNA with XL10-Gold Ultracompetent Cells

β-mercaptoethanol (2 µl) was added to 45 µl of XL10-Gold ultracompetent cells in a pre-chilled tube and incubated on ice for 2 min. 2 µl of *Dpn* I treated reaction mixture was added to the cells and incubated on ice for 30 min. After 30 min, it was heat pulsed in a 42 °C water bath for 30 sec followed by incubation on ice for 2 min. 0.5 ml of NZY^+ broth (preheated in a water bath to 42 °C) was added to the reaction mixture and incubated at 37 °C for 1 hr with shaking. 100 and 200 µl of the transformation reaction was plated onto LB agar plates containing 25µg/ml chloramphenicol and 100 µg/ml ampicillin. The plates were incubated overnight at 37 °C. Following overnight incubation, a colony from the plate was used to inoculate 5 ml of LB broth containing 25µg/ml chloramphenicol and 100 µg/ml ampicillin. The culture was incubated overnight at 37 °C with shaking.

2.5.5 Transformation of DNA with BL21 Cells

450 µl of 0.1M $CaCl_2$ was added to 50 µl of competent BL21 cells in pre-chilled tubes. 10-100 ng of DNA (purified from XL10-Gold ultra competent cells, using the same procedure for purification of DNA from BL21 cells) was added and the sample was incubated on ice for 30 min. The sample was heat shocked for 60 sec at 42 °C and incubated on ice for 2 min. 900µl of preheated (42 °C) Super Optimal broth with Catabolite repression (SOC) medium (Invitrogen) was added and the transformation reaction was incubated at 37 °C for 1 hr with shaking. The cells were concentrated by centrifugation at 12,000 rpm for 2 min and the entire transformation reaction was spread onto LB agar plates containing 25µg/ml chloramphenicol and 100 µg/ml ampicillin. The plates were incubated overnight at 37 °C. A white colony was used to inoculate 10 ml of LB broth containing 25 µg/ml chloramphenicol and 100 µg/ml ampicillin. The cell culture was incubated at 37 °C overnight with continuous agitation. The cell culture was stored at -80 °C in equi-volume glycerol.

2.6 DNA Sequencing

Mutations were confirmed by DNA sequencing at CREAIT (Memorial University of Newfoundland, NL, Canada). The primers (from Operon) used to sequence the tropomyosin insert of the expression vector were:

M13/pUC reverse	5′ AGCGGATAACAATTTCACACAGG	3′
pBad-rev	5′ ATCAGACCGCTTCTGCGTTC	3′

CREAIT uses an Applied Biosystems ABI3730x1 sequencer that is automatic except for some preliminary set-up. For each sample, 2 µl of 5x sequencing buffer, 0.5 µl of sequencing mix, 3.2 pmol of a primer, DNA template (at least 500 pmol) and nuclease-free water to obtain a volume of 20 µl was added at RT. Samples were mixed by vortexing briefly in a table top microcentrifuge. pGEM was set up as a control. A 9800 thermocycler (Applied Biosystems) was used for PCR amplification using the following program: six minutes at 96 °C, 25 cycles at 96 °C for 10 seconds each, 50 °C for five seconds, 60 °C for four minutes and then 4°C until the samples were removed from the thermocycler. The samples were again briefly centrifuged at RT.

The Agencourt CleanSEQ system (Agencourt Bioscience) was used to purify the PCR product for sequencing. Briefly, 10 µl of Agencourt CleanSEQ magnetic beads was added to each sample followed by 62 µl of 85% ethanol (vol/vol) and mixed thoroughly. Samples were placed onto a magnetic plate (Agencourt SPRIPlate 69R). When the solution was clear (3-5 min), it was aspirated from the sample and discarded. A 100 µl aliquot of 85% ethanol (vol/vol) was added to the sample and after 30 seconds, the ethanol was discarded. This ethanol wash was performed again and the sample left to air dry for 10 min. Deoinized water (40 µl) was added to the sample after which it was removed from the magnetic tray. After 5 min, the sample was returned to the magnetic tray to isolate the DNA. After 5 min, 35 µl of sample was loaded into the ABI 3730x1 for sequencing. Sequencing results were analyzed using FinchTV from Geospiza and compared to the cDNA of Atlantic Salmon fast muscle tropomyosin (Accession # NM_001123656).

2.7 Expression of Tropomyosin in Bacteria

In this section, unless otherwise stated, centrifugation was performed in a Beckman J6-HC centrifuge at 4 °C. Bacterial expression of the recombinant tropomyosin (mutated and non-mutated) was performed as described by Jackman *et al.* (1996). Briefly, 40 ml of LB broth containing 25 μg/ml chloramphenicol and 100 μg/ml ampicillin was inoculated with 400 μl of cells containing the mutated tropomyosin DNA. The cell culture was incubated overnight at 37 °C with agitation. 4 L of LB broth containing 25 μg/ml chloramphenicol and 100 μg/ml ampicillin was inoculated with the 40 ml of overnight cell culture.

The culture was incubated at 37 °C with shaking and the absorbance at 600 nm was measured. When the absorbance reached 0.6 – 0.8, expression of tropomyosin was induced using 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) (overnight, 37 °C, with shaking). The following morning, the cells were pelleted in a centrifuge at 4,000 rpm for 20 minutes. The cells were dispersed in 100 ml of 0.2 M NaCl, 50 mM MOPS, 1 mM DTT at pH 7.0 and passed through a French pressure cell at RT. The lysate was mixed with 1200 ml of the above buffer and stirred for 15 min (RT) following the addition of PMSF (saturated, in 95% ethanol). Samples were centrifuged at 4,000 rpm for 20 min. The supernatant was pI precipitated (4.6) and centrifuged at 4,000 rpm for 30 min.

The pellets were dispersed in 800 ml total of buffer (0.2 M NaCl, 50 mM Tris, 0.5 mM EDTA, 0.25 mM DTT pH 7.9) at ~10 °C for 15 min (with PMSF). Samples were centrifuged at 4,000 rpm for 20 min in a Beckman J6-HC centrifuge. The supernatant

precipitated by ammonium sulfate (45%) followed by centrifugation at 4,000 rpm for 30 min in a Beckman J6-HC centrifuge. Centrifugation was followed by 70% ammonium sulfate precipitation of the supernatant. The sample was then centrifuged at 8,000 rpm for 45 min in a Beckman J2-21 centrifuge (JA-10 rotor) at 4 °C.

The pellets were dissolved in dH_2O and dialyzed (wide dialysis, 6,000 - 8,000 Da MWCO) against dH_2O (containing ammonium bicarbonate and mercaptoethanol) at ~ 8 °C for 2 days with 4 water changes (for a total of 20 L). Finally, the dialyzed sample was lyophilized.

2.8 Enrichment of Tropomyosin

2.8.1 Ion-Exchange Chromatography

Approximately 300 mg of protein was dissolved in 40 ml of filtered starting buffer (75 mM NaCl, 30 mM Tris, 1 mM DTT, pH8.0, ~10 °C). A 2.5 x 14 cm Q Sepharose Fast Flow column (volume of ~ 70 ml) (Amerisham Biosciences) was equilibrated with ~300 ml of the above buffer (filtered) at a flow rate of ~15 ml/hr, in the cold room. The pre-spun protein solution was loaded onto the column at a rate of ~ 20 ml/hr. The column was then washed with ~ 100 ml of starting buffer, at the same rate. The protein was eluted from the column by a salt gradient of 75 – 500 mM NaCl. Elution occurred at a rate of ~ 30 ml/hr and fractions were collected every 9 min. The collected fractions were analyzed via absorbance at 280 nm and conductivity measurements (CDM 80 conductivity meter

from Radiometer) to determine which fractions contained tropomyosin. Fractions believed to contain tropomyosin were confirmed via SDS 12% PAGE.

2.8.2 Hydroxyapatite Chromatography

A 2.5 x 16 cm hydroxyapatite column (volume of ~ 80 ml) (BioRad) was equilibrated at room temperature with 300 ml of filtered starting buffer (1M NaCl, 0.01% Na Azide, 30 mM sodium phosphate, 1 mM DTT, pH 7.0) at a rate of ~15 ml/hr. The combined tropomyosin containing fractions from the Fast Q column were directly loaded onto the column at 15 ml/hr. The column was then washed with ~100 ml of start buffer. A sodium phosphate gradient of 30 mM to 250 mM was used to elute the protein at a rate of ~ 30 ml/hr. Fractions were collected every 9 min. SDS 12% PAGE and absorbance measurements at 280 nm were used to determine the tropomyosin containing fractions. These fractions were combined and dialyzed (wide dialysis tubing, 6,000 – 8,000 Da MWCO) (in the cold room) over two days against dH_20 (containing ammonium bicarbonate and mercaptoethanol) while changing the water four times (for a total of 20L). The sample was then lyophilized. The enrichment of the protein was assessed by SDS 12% PAGE.

2.9 Proteolytic digestion

2.9.1 Omp-T Digestion

Outer membrane protease-T (Omp-T) digestion of tropomyosin was performed as described by Goonasekara *et al.*, (2007). Approximately 13 mg of protein was dissolved in 1 ml of buffer (0.1 M NaCl, 50 mM sodium phosphate, 5 mM EDTA, 1 mM DTT, pH

7.0) and dialyzed (narrow dialysis tubing, 12,000 – 14,000 Da MWCO) overnight against 1 L of buffer in a cold room. 100 µl of *E. coli* JM109 cells were used to inoculate 10 ml of LB broth and incubated overnight at 37 °C with shaking. Following incubation, the cultures were centrifuged and the pellets were dispersed in 1 ml of buffer. The reactions were performed at ~10, ~ 25 or ~ 37 °C. Usually, 500 µg of dissolved protein would be digested in 1 ml of dispersed cells. 100 µl of reaction mixture was stopped by 1 min centrifugation at 12, 000 rpm in a Eppendorf Centrifuge 5415D. The supernatant was added to dissolving solution and then boiled. Digestion patterns were analyzed on a SDS 12% polyacrylamide gel.

2.9.2 Chymotrypsin Digestion

Limited proteolysis by chymotrypsin was carried out as described by Pato *et al.* (1981). 2 mg of chymotrypsin was dissolved in 2 mM HCl and dialyzed (narrow dialysis tubing, 12,000 – 14,000 Da MWCO) overnight. Generally ~ 10 mg of tropomyosin was dissolved in 2 ml of buffer: 50 mM NH_4HCO_3, 0.1 M NaCl, 1 mM DTT, pH 8.5. The protein was dialyzed (narrow dialysis tubing, 12,000 – 14, 000 Da MWCO) overnight against 1L of buffer in the cold room. 400 µg of tropomyosin was digested with 0.6 µg of chymotrypsin (Worthington) (~1:500 enzyme to substrate mole ratio) at 37 °C for 30 min. At varying time points, 15 µl of reaction mixture was removed and inhibited using ~1 µM lima bean trypsin inhibitor (Worthington) (dissolved in 20 mM Tris, pH 7.5). Digestion patterns were analyzed on a SDS 12% polyacrylamide gel.

2.10 Western Blotting

Western blotting was performed on a chymotrypsin digestion of non-mutant recombinant tropomyosin (described in section 2.9.2) as described by Heeley and Hong (1994). The protein was transferred from SDS-polyacrylamide gels to polyvinylidene diflouride membrane (Bio-Rad) in a BioRad mini-Trans-Blot Electrophoresis Transfer cell. Blotting was carried out in a buffer of 10 mM CAPS, 10% (v/v) methanol, pH 11.00 at 60 V for 3 hrs. The membrane was briefly stained (2 min) in 40 % (v/v) methanol, 0.1% Coomassie Brilliant Blue R-250 and destained in 50% (v/v) methanol (< 5 min). The membrane was then left to air dry. Edman-based sequencing was performed in the Advanced Protein Technology Center at Sick Children's Hospital, Toronto.

2.11 Calculating Mean Residue Ellipticity

The mean residue ellipticity (θ) of tropomyosin was calculated using the following formula:

$$[\theta] = \frac{\theta_{obs} \times 115 \text{ g/mole}}{10 \times L \times C}$$

Where: θ_{obs} is the observed ellipticity in degrees at 222 nm
L is the length of the cell in cm
115 g/mole is the average residue molar mass
10 is for centimole
And C is the protein concentration in g/cm^3

Chapter 3
Results and Discussion

3.1 Purification of DNA from BL21 Plasmids

The initial step in creating the tropomyosin mutants involved the isolation of the pTrc99A expression vector (with tropomyosin insert) from the BL21 cells. A Nanodrop (Thermo Scientific) was used to measure the concentration of the DNA. The Nanodrop gives the ratio for the measurements of 260/280, which indicates the nucleic acid purity. A ratio between 2.0 – 2.2 indicates an acceptable level of protein content. The DNA samples that were used had a 260/280 ratio of 1.8-2.1. The concentrations of the DNA were determined to be between 80-120 ng/µl.

3.2 Nucleotide Sequencing

The mutations of the cDNA of tropomyosin had to be confirmed prior to any work being done with them. The nucleotide sequencing was carried out in CREAIT and the results were compared to that of *Salmo salar* (Atlantic Salmon) fast myotomal muscle tropomyosin mRNA (accession # L25609). For each mutant, four samples were submitted for sequencing and only the samples that contained the desired mutated nucleotide base were used for further analysis.

Sequencing of the nucleotides from the four mutant tropomyosins (by CREAIT) confirmed that site-directed mutagenesis had mutated the nucleotides of interest. For the Gly24Ala mutant, nucleotide 152 was mutated from a guanine to a cytosine (Figure 4). In

Figure 4: Confirmation of the Gly24Ala tropomyosin mutation compared to the cDNA of Atlantic salmon fast muscle tropomyosin

Only a partial DNA sequence is shown to confirm the mutation. The mutated base (# 152) is in bold type, underlined and circled in red.

```
105 GCAGATGCTC AAGCTCGACA AGGAGAATGC CTTGGACAGA GCTGAGGCAG
    |||||||||| |||||||||| |||||||||| |||||||||| |||||||||
    GCAGATGCTC AAGCTCGACA AGGAGAATGC CTTGGACAGA GCTGAGGGAG

155 CCGAGGGAGA CAAGAAGGCA GCAGAGGACA AGAGCAAACA GCTCGAGGAT
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    CCGAGGGAGA CAAGAAGGCA GCAGAGGACA AGAGCAAACA GCTCGAGGAT

205 GACTTGGTAG CTCTGCAGAA GAAGCTGAAG GGAACAGAGG ATGAGTTGGA
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GACTTGGTAG CTCTGCAGAA GAAGCTGAAG GGAACAGAGG ATGAGTTGGA

255 CAAGTACTCT GAGTCTCTTA AGGATGCACA GGAGAAACTT GAGGTGGCTG
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    CAAGTACTCT GAGTCTCTTA AGGATGCACA GGAGAAACTT GAGGTGGCTG

305 AGAAGACAGC CACGGACGCT GAGGCCGATG TCGCTTCCCT TAACAGACGT
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    AGAAGACAGC CACGGACGCT GAGGCCGATG TCGCTTCCCT TAACAGACGT

355 ATCCAGCTAG TTGAGGAGGA GTTGGATCGT GCTCAGGAGC GGCTGGCAAC
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    ATCCAGCTAG TTGAGGAGGA GTTGGATCGT GCTCAGGAGC GGCTGGCAAC

405 TGCCCTGACC AAGCTGGAGG AGGCTGAGAA GGCGGCTGAT GAGTCTGAGA
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    TGCCCTGACC AAGCTGGAGG AGGCTGAGAA GGCGGCTGAT GAGTCTGAGA

455 GAGGCATGAA GGTCATTGAG AACAGGGCCT CCAAGGATGA GGAGAAGATG
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GAGGCATGAA GGTCATTGAG AACAGGGCCT CCAAGGATGA GGAGAAGATG

505 GAGCTGCAGG ATATCCAGCT GAAGGAGGCC AAGCACATCG CTGAGGAGGC
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GAGCTGCAGG ATATCCAGCT GAAGGAGGCC AAGCACATCG CTGAGGAGGC

555 CGACCGCAAA TACGAGGAGG TTGCCCGTAA GCTGGTCATC ATTGAGAGTG
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    CGACCGCAAA TACGAGGAGG TTGCCCGTAA GCTGGTCATC ATTGAGAGTG
```

Figure 5: Confirmation of the Gly27Ala tropomyosin mutation compared to the cDNA of Atlantic salmon fast muscle tropomyosin

Only a partial DNA sequence is shown to confirm the mutation. The mutated (# 161) is in bold type, underlined and circled in red.

```
105 GCAGATGCTC AAGCTCGACA AGGAGAATGC CTTGGACAGA GCTGAGGGAG
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GCAGATGCTC AAGCTCGACA AGGAGAATGC CTTGGACAGA GCTGAGGGAG

155 CCGAGGCAGA CAAGAAGGCA GCAGAGGACA AGAGCAAACA GCTCGAGGAT
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    CCGAGGGAGA CAAGAAGGCA GCAGAGGACA AGAGCAAACA GCTCGAGGAT

205 GACTTGGTAG CTCTGCAGAA GAAGCTGAAG GGAACAGAGG ATGAGTTGGA
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GACTTGGTAG CTCTGCAGAA GAAGCTGAAG GGAACAGAGG ATGAGTTGGA

255 CAAGTACTCT GAGTCTCTTA AGGATGCACA GGAGAAACTT GAGGTGGCTG
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    CAAGTACTCT GAGTCTCTTA AGGATGCACA GGAGAAACTT GAGGTGGCTG

305 AGAAGACAGC CACGGACGCT GAGGCCGATG TCGCTTCCCT TAACAGACGT
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    AGAAGACAGC CACGGACGCT GAGGCCGATG TCGCTTCCCT TAACAGACGT

355 ATCCAGCTAG TTGAGGAGGA GTTGGATCGT GCTCAGGAGC GGCTGGCAAC
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    ATCCAGCTAG TTGAGGAGGA GTTGGATCGT GCTCAGGAGC GGCTGGCAAC

405 TGCCCTGACC AAGCTGGAGG AGGCTGAGAA GGCGGCTGAT GAGTCTGAGA
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    TGCCCTGACC AAGCTGGAGG AGGCTGAGAA GGCGGCTGAT GAGTCTGAGA

455 GAGGCATGAA GGTCATTGAG AACAGGGCCT CCAAGGATGA GGAGAAGATG
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GAGGCATGAA GGTCATTGAG AACAGGGCCT CCAAGGATGA GGAGAAGATG

505 GAGCTGCAGG ATATCCAGCT GAAGGAGGCC AAGCACATCG CTGAGGAGGC
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GAGCTGCAGG ATATCCAGCT GAAGGAGGCC AAGCACATCG CTGAGGAGGC

555 CGACCGCAAA TACGAGGAGG TTGCCCGTAA GCTGGTCATC ATTGAGAGTG
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    CGACCGCAAA TACGAGGAGG TTGCCCGTAA GCTGGTCATC ATTGAGAGTG
```

double mutant, Gly24Ala/Gly27Ala, showed that the guanines at position 152 and 161 were mutated to cytosines (Figure 6). For the Thr179Ala mutant, nucleotide 617 was mutated from cytosine to guanine (Figure 7).

3.3 Expression of Tropomyosin

Isopropyl β-D-1 thiogalactopyranoside (IPTG) (500 μM) was used to induce the expression of tropomyosin from cultures of BL21. Small scale (approximately 8 ml of bacteria) induction of tropomyosin was carried out prior to large scale (4 L) induction to ensure that the tropomyosin would be expressed.

Electrophoretic analysis (Figure 8) shows that tropomyosin expression was indeed induced by IPTG and that it was now one of the major protein components. Enrichment of tropomyosin was obtained by isoelectric point precipitation and salt-induced precipitation followed by ion exchange and hydroxyapatite chromatography. Figure 9 shows the elution profile of protein from a Q Sepharose Fast Flow column with a salt gradient of 75-500 mM NaCl. Electrophoresis (not shown) confirmed that the protein was eluting in fractions 126-152. These fractions were pooled and loaded directly onto a hydroxyapatite column. This column works because negatively charged groups within tropomyosin are adsorbed by Ca^{2+} sites and then eluted by a phosphate gradient (10-250 mM phosphate). Tropomyosin eluted in fractions 122-145 (Figure 10) were pooled, dialyzed against dH_2O and lyophilized. To analyze the enrichment of the various mutants, the samples were analyzed electrophoretically using loadings of > 15 μg (Figure 11). At such a high loading a few trace contaminants are evident, but it is clear that the main component of the protein

Figure 6: Confirmation of the Gly24Ala/Gly27Ala tropomyosin mutation compared to the cDNA of Atlantic salmon fast muscle tropomyosin

Only a partial DNA sequence is shown to confirm the mutation. The mutated bases (# 152 and # 161) are in bold type, underlined and circled in red.

```
105 GCAGATGCTC AAGCTCGACA AGGAGAATGC CTTGGACAGA GCTGAGGCAG
    |||||||||| |||||||||| |||||||||| |||||||||| |||||||||
    GCAGATGCTC AAGCTCGACA AGGAGAATGC CTTGGACAGA GCTGAGGGAG

155 CCGAGGCAGA CAAGAAGGCA GCAGAGGACA AGAGCAAACA GCTCGAGGAT
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    CCGAGGGAGA CAAGAAGGCA GCAGAGGACA AGAGCAAACA GCTCGAGGAT

205 GACTTGGTAG CTCTGCAGAA GAAGCTGAAG GGAACAGAGG ATGAGTTGGA
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GACTTGGTAG CTCTGCAGAA GAAGCTGAAG GGAACAGAGG ATGAGTTGGA

255 CAAGTACTCT GAGTCTCTTA AGGATGCACA GGAGAAACTT GAGGTGGCTG
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    CAAGTACTCT GAGTCTCTTA AGGATGCACA GGAGAAACTT GAGGTGGCTG

305 AGAAGACAGC CACGGACGCT GAGGCCGATG TCGCTTCCCT TAACAGACGT
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    AGAAGACAGC CACGGACGCT GAGGCCGATG TCGCTTCCCT TAACAGACGT

355 ATCCAGCTAG TTGAGGAGGA GTTGGATCGT GCTCAGGAGC GGCTGGCAAC
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    ATCCAGCTAG TTGAGGAGGA GTTGGATCGT GCTCAGGAGC GGCTGGCAAC

405 TGCCCTGACC AAGCTGGAGG AGGCTGAGAA GGCGGCTGAT GAGTCTGAGA
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    TGCCCTGACC AAGCTGGAGG AGGCTGAGAA GGCGGCTGAT GAGTCTGAGA

455 GAGGCATGAA GGTCATTGAG AACAGGGCCT CCAAGGATGA GGAGAAGATG
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GAGGCATGAA GGTCATTGAG AACAGGGCCT CCAAGGATGA GGAGAAGATG

505 GAGCTGCAGG ATATCCAGCT GAAGGAGGCC AAGCACATCG CTGAGGAGGC
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GAGCTGCAGG ATATCCAGCT GAAGGAGGCC AAGCACATCG CTGAGGAGGC

555 CGACCGCAAA TACGAGGAGG TTGCCCGTAA GCTGGTCATC ATTGAGAGTG
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    CGACCGCAAA TACGAGGAGG TTGCCCGTAA GCTGGTCATC ATTGAGAGTG
```

Figure 7: Confirmation of the Thr179A tropomyosin mutation compared to the cDNA of Atlantic salmon fast muscle tropomyosin

Only a partial DNA sequence is shown to confirm the mutation. The mutated base (# 617) is in bold type, underlined and circled in red.

```
405 TGCCCTGACC AAGCTGGAGG AGGCTGAGAA GGCGGCTGAT GAGTCTGAGA
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    TGCCCTGACC AAGCTGGAGG AGGCTGAGAA GGCGGCTGAT GAGTCTGAGA

455 GAGGCATGAA GGTCATTGAG AACAGGGCCT CCAAGGATGA GGAGAAGATG
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GAGGCATGAA GGTCATTGAG AACAGGGCCT CCAAGGATGA GGAGAAGATG

505 GAGCTGCAGG ATATCCAGCT GAAGGAGGCC AAGCACATCG CTGAGGAGGC
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GAGCTGCAGG ATATCCAGCT GAAGGAGGCC AAGCACATCG CTGAGGAGGC

555 CGACCGCAAA TACGAGGAGG TTGCCCGTAA GCTGGTCATC ATTGAGAGTG
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    CGACCGCAAA TACGAGGAGG TTGCCCGTAA GCTGGTCATC ATTGAGAGTG

605 ATCTGGAACG TA-AGAGGAG CGCGCTGAGC TTTCAGAAGG CAAATGCTCT
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    ATCTGGAACG TACAGAGGAG CGCGCTGAGC TTTCAGAAGG CAAATGCTCT

655 GAGCTTGAGG AAGAGTTGAA AACTGTGACC AACAACCTGA AGTCACTGGA
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GAGCTTGAGG AAGAGTTGAA AACTGTGACC AACAACCTGA AGTCACTGGA

705 GGCCCAGGCT GAGAAGTACT CACAGAAGGA GGACAAGTAC GAGGAGGAGA
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GGCCCAGGCT GAGAAGTACT CACAGAAGGA GGACAAGTAC GAGGAGGAGA

755 TCAAGGTCCT CACCGACAAG CTGAAGGAGG CTGAGACTCG TGCTGAGTTC
    |||||||||| |||||||||| ||||||||||||||||||| ||||||||||
    TCAAGGTCCT CACCGACAAG CTGAAGGAGG CTGAGACTCG TGCTGAGTTC

805 GCTGAAAGAT CAGTAGCCAA ACTTGAGAAG ACCATCGACG ACTTGGAAGA
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    GCTGAAAGAT CAGTAGCCAA ACTTGAGAAG ACCATCGACG ACTTGGAAGA

855 TGAGTTGTAT GCCCAGAAAC TGAAGTACAA GGCCATCAGC GAGGAGCTGG
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
    TGAGTTGTAT GCCCAGAAAC TGAAGTACAA GGCCATCAGC GAGGAGCTGG
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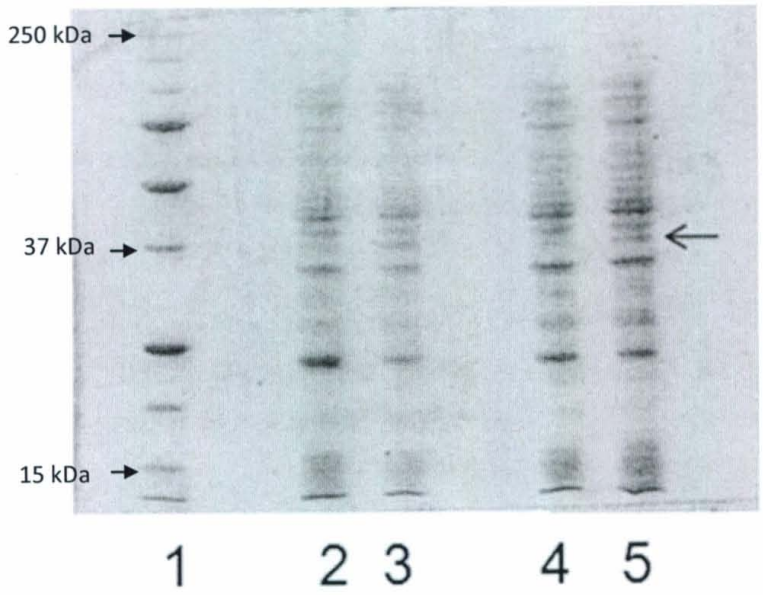


Figure 8: SDS PAGE analysis of a lysate of IPTG-induced BL21 cells containing Gly24Ala tropomyosin

5 ml of LB broth (containing 25 µg/ml of chloramphenicol and 100 µg/ml of ampicillin) was inoculated using 5 µl of BL21 cells containing the mutation. The samples were induced with 500 µM IPTG when the absorbance was between 0.6-0.8 at 600 nm and then incubated overnight at 37 °C. 100 µl of sample was pelleted and dissolved in 50 µl of SDS buffer. 4 µl (lanes 2 and 3) or 8 µl (lanes 4 and 5) were loaded onto a 12% (w/v) polyacrylamide gel which was stained in Coomassie Brilliant Blue R-250. Lane 1 is a collection of molecular weight markers; lanes 2 and 4 are uninduced BL21; lanes 3 and 5 are induced BL21. The arrow indicates the tropomyosin-containing band.

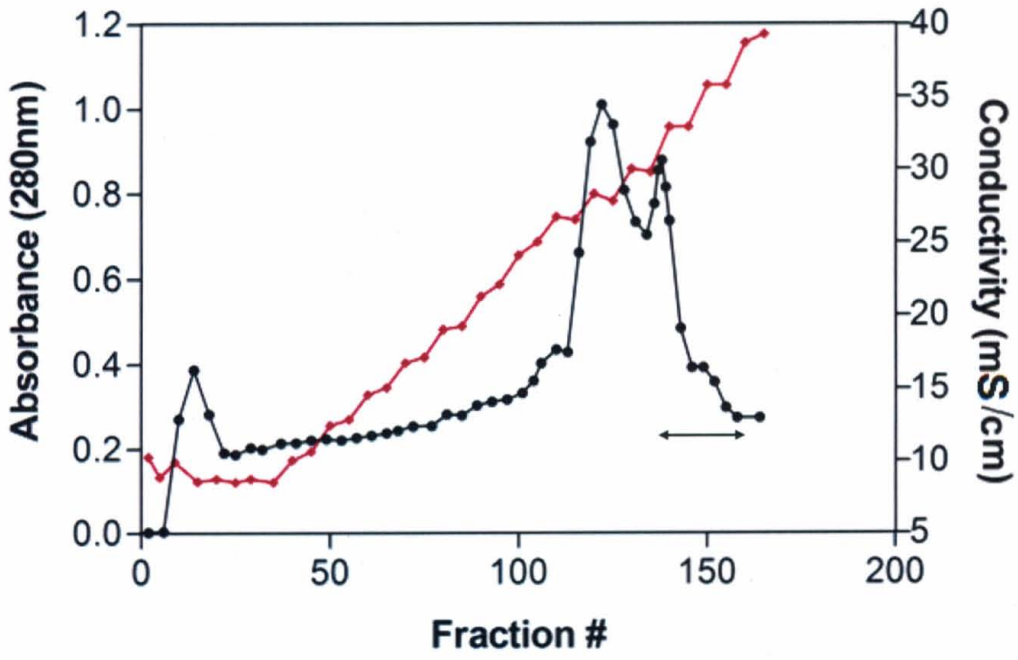


Figure 9: Q Sepharose Fast Flow chromatography profile of crude recombinant tropomyosin

Column dimensions, 2.5 x 14 cm; buffer, 30 mM Tris, 1 mM DTT, pH8.0; salt gradient, 75-500 mM NaCl; temperature, 10 °C. The absorbances (black circles) were measured at 280 nm using a Beckman DU-64 spectrophotometer. Conductivities (red diamonds) were measured using a Radiometer CDM 80 conductivity meter. Data corresponding to the isolation of the double mutant Gly24Ala/Gly27Ala were used to create the graph. Fractions 126-152 contained tropomyosin (indicated by a horizontal bar).

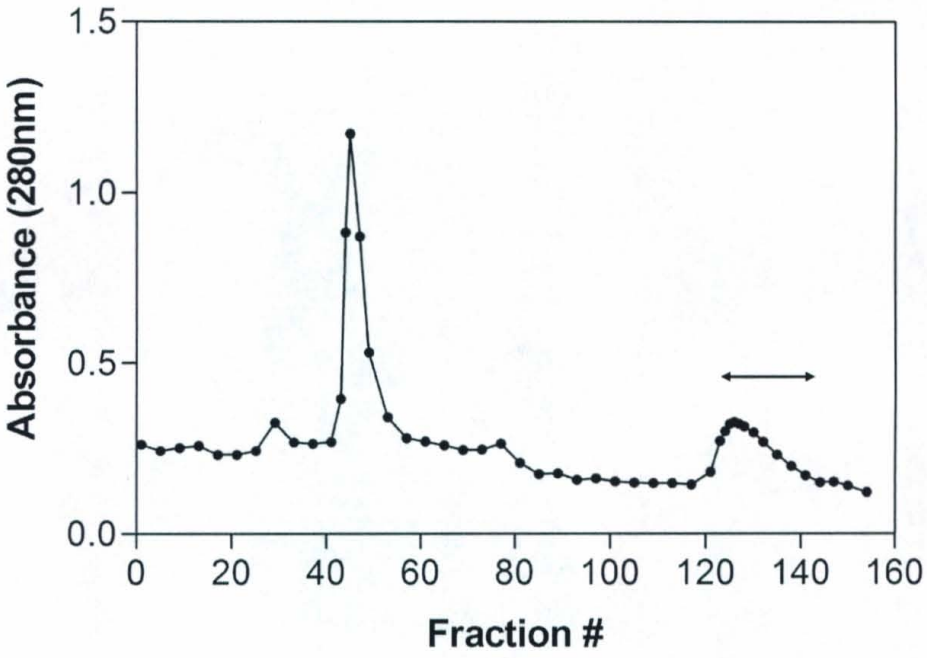


Figure 10: Hydroxyapatite chromatography profile of recombinant tropomyosin

Column dimensions, 2.5 x 16 cm; buffer, 1M NaCl, 0.01% Na Azide, , 1 mM DTT, pH 7.0; phosphate gradient, 30-250 mM sodium phosphate; temperature, ~25 °C. Again the absorbances from the double mutant Gly24Ala/Gly27Ala were used to create the graph. Fractions 122-145 contained tropomyosin (indicated by a horizontal bar).

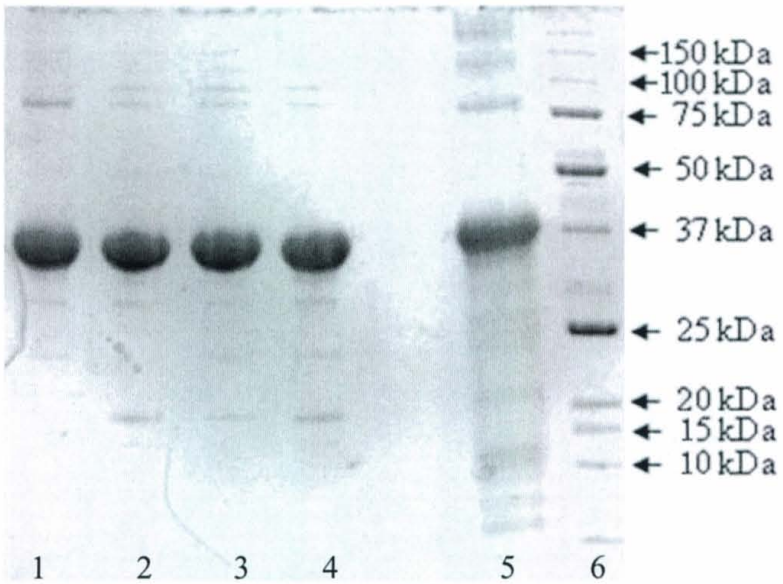


Figure 11: SDS PAGE analysis of highly-enriched mutant and non-mutant tropomyosins

Lane 1 – Gly24Ala; Lane 2 – Gly27Ala; Lane 3 – Gly24Ala/Gly27Ala; Lane 4 – Thr179Ala; Lane 5 – non-mutant recombinant tropomyosin; Lane 6 – molecular weight marker. More than 15ug of protein was loaded unto lanes 1-5. Lane 6 contained 10 µl of molecular weight markers The 12% (w/v) polyacrylamide gel was stained in Coomassie Brilliant Blue R-250.

samples is tropomyosin. The yield was ~ 20 mg of enriched tropomyosin per litre of growth medium.

3.4 Heat-Induced Unfolding

Circular dichroism was used to determine the melting temperatures of the four mutant tropomyosins and the recombinant non-mutant tropomyosin. Unfolding profiles were developed by monitoring the ellipticity at 222 nm as a function of temperature. The samples were exposed to temperatures from 5-65 °C, at a rate of 30 or 60 °C / hr. The average of the first 5 data points (5-6 °C) was taken to be the starting (100%) ellipticity and the average of the last 5 data points (64-65 °C) was taken to be the end (0%) ellipticity. Each sample was analyzed eight times, (except for Gly24Ala which was analyzed three times) and the results were averaged in order to create a normalized melting curve of the samples. The formula used to calculate the normalization was:

$$\frac{\theta - \theta_{end}}{\theta_{start} - \theta_{end}} \times 100$$

Where: θ is the ellipiticity of the sample at the particular temperature
θ_{end} is the average ending ellipticity and
θ_{start} is the average starting ellipticity

The melting temperature was taken to be the temperature at which there was a 50% signal change.

As can be seen in Figures 12-16 there is close agreement of the melting temperatures and the profiles amongst the samples for each of the four mutants and the non-mutant tropomyosin. Table 2 indicates that the standard deviations for the tropomyosins ranged from 0.12 °C (Gly24Ala) to 0.97 °C (Thr179Ala). Figure 14 and

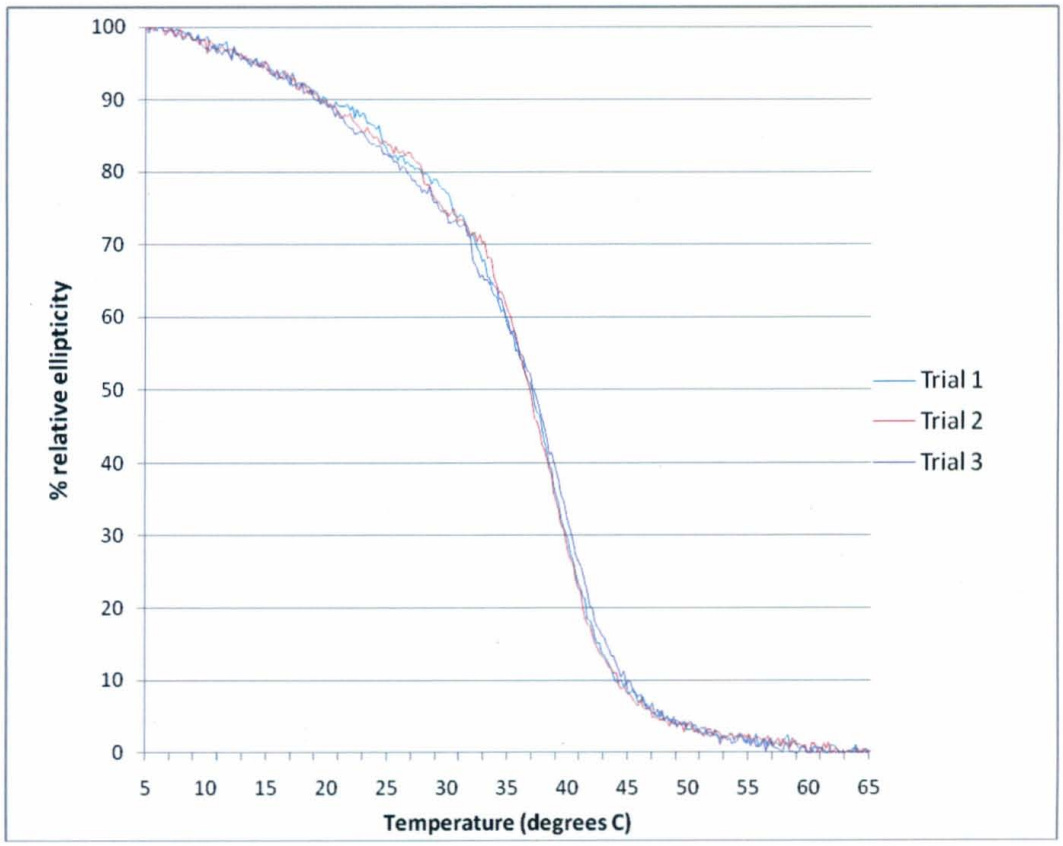


Figure 12: Normalized curve of the melting profiles of three samples of Gly24Ala tropomyosin

Unfolding was monitored at 222 nm from 5-65 °C in a 0.1 mm cell. 2 mg/ml of tropomyosin was dissolved in 0.1 M KCl, 20 mM potassium phosphate, 0.01% Na azide, 1 mM EGTA , 1.5 mM DTT, pH 7.0.

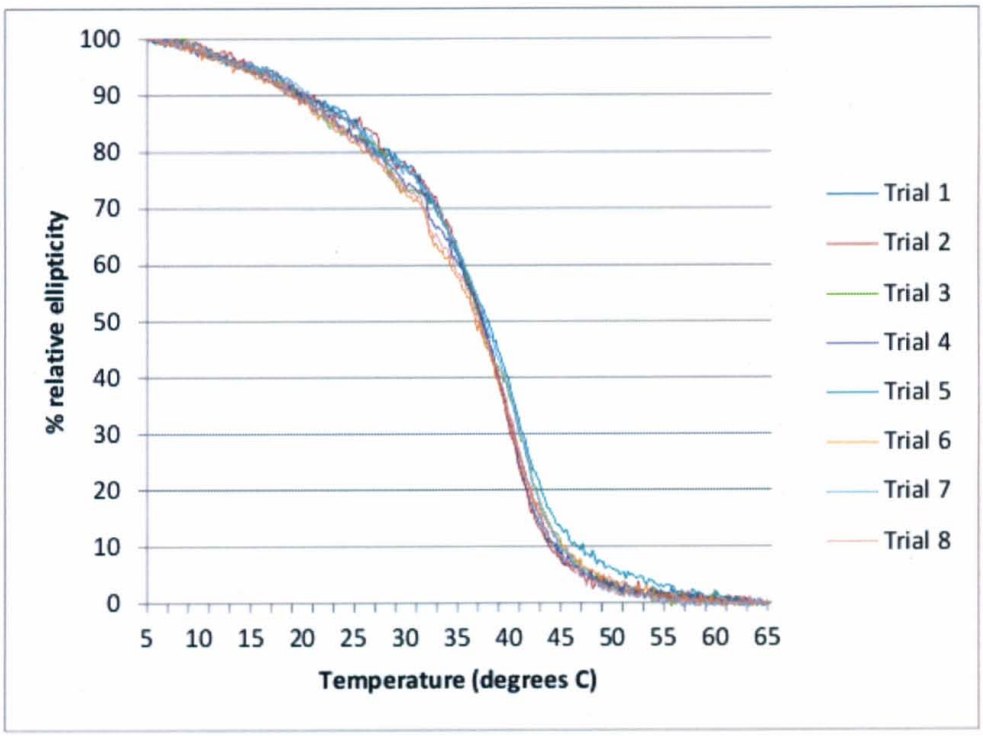


Figure 13: Normalized curve of the melting profiles of eight samples of Gly27Ala tropomyosin

Conditions as described in Figure 12.

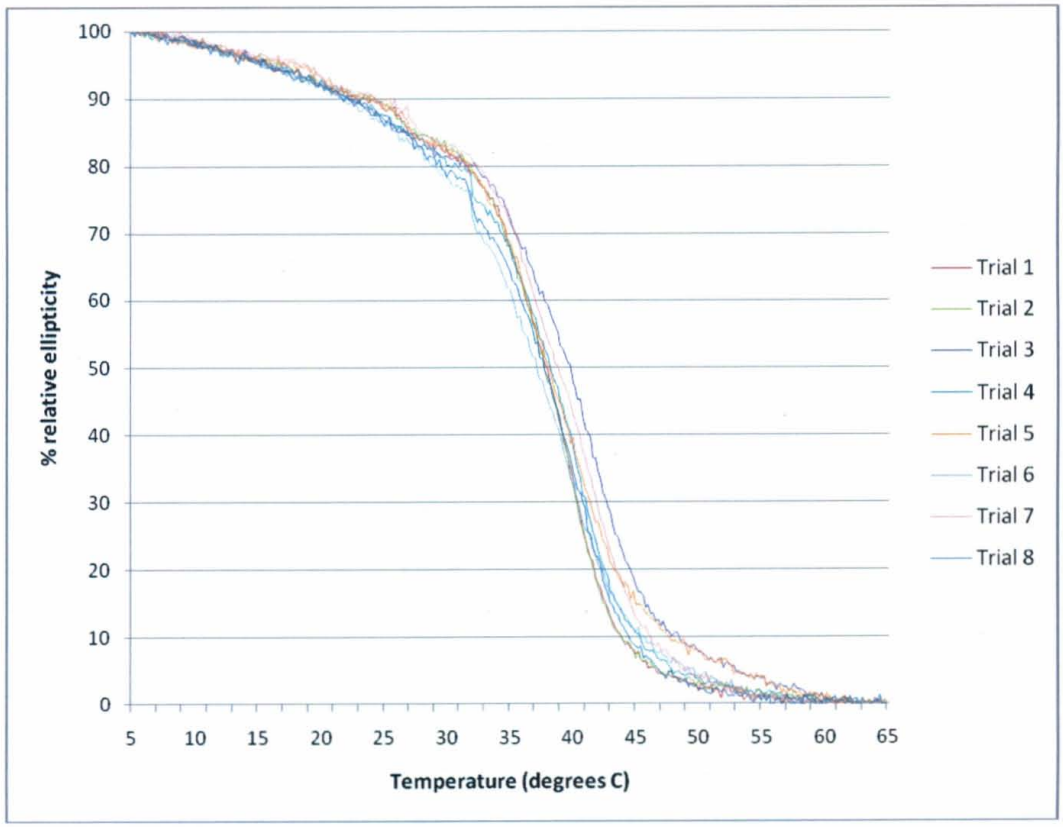


Figure 14: Normalized curve of the melting profiles of eight samples of Gly24Ala/Gly27Ala tropomyosin

Conditions as described in Figure 12.

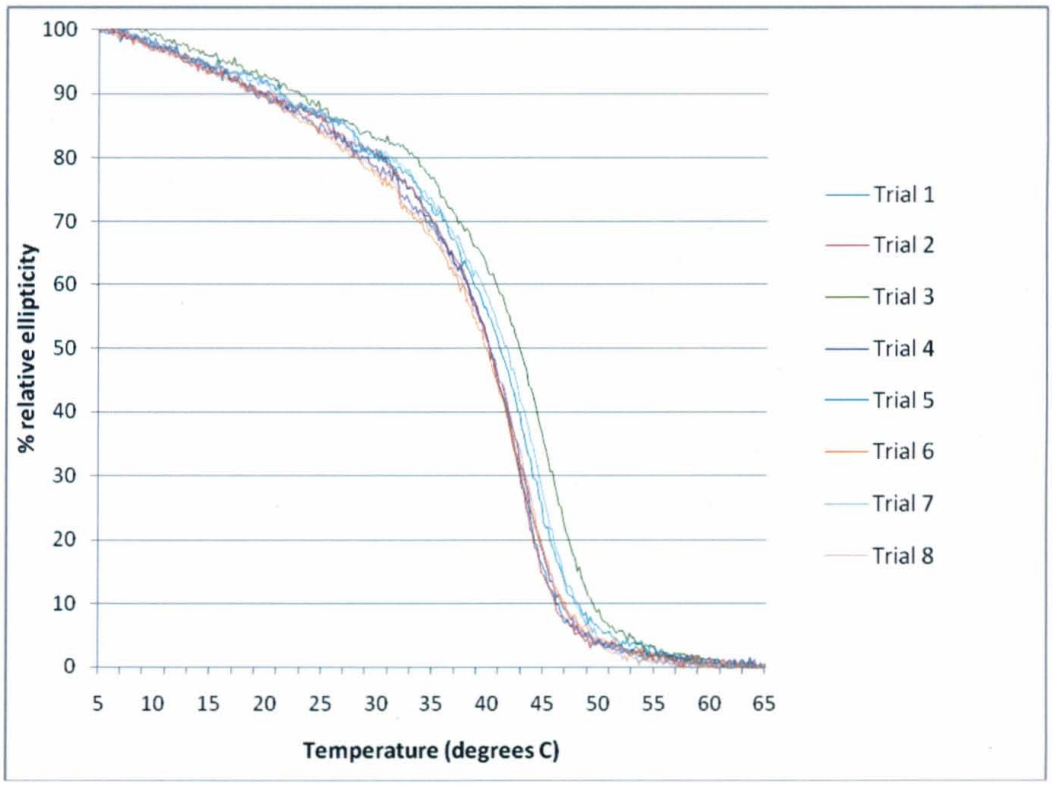


Figure 15: Normalized curve of the melting profiles of eight samples of Thr179Ala tropomyosin

Conditions as described in Figure 12.

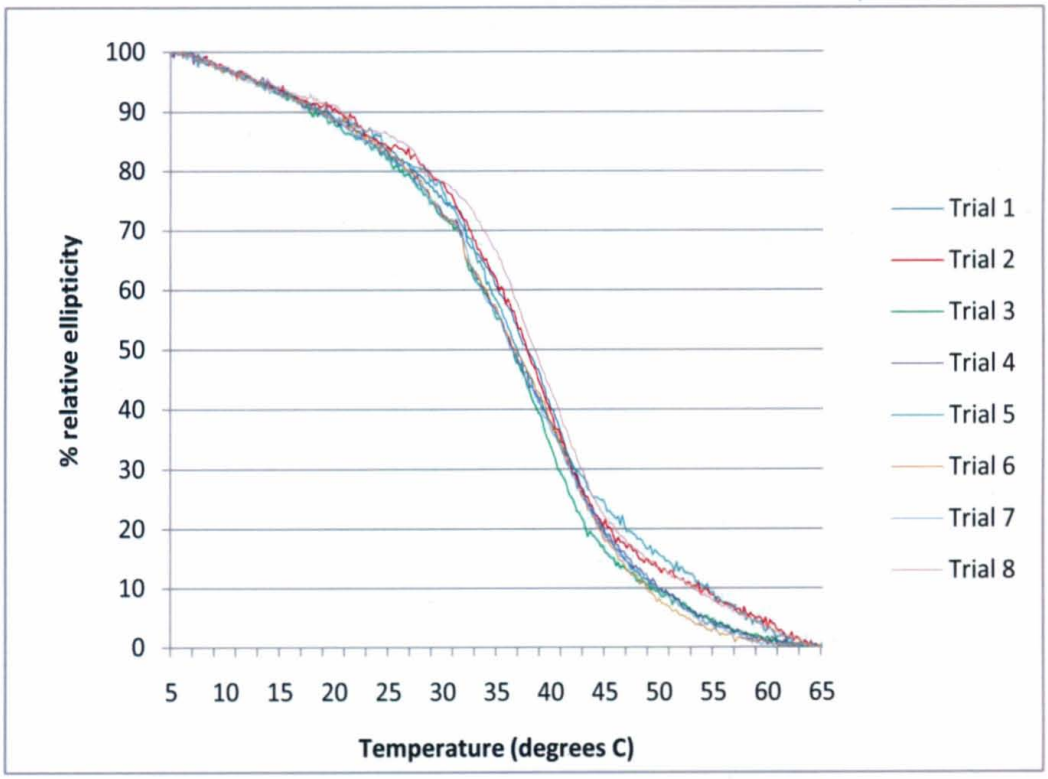


Figure 16: Normalized curve of the melting profiles of eight samples of non-mutant tropomyosin

Conditions as described in Figure 12.

Table 2: Average melting temperatures and standard deviations of the non-mutant and mutant salmon tropomyosins

	Average (°C)	Standard deviation
Non-mutated (n=8)	37.0	± 0.81
Gly24Ala (n=3)	36.9	± 0.12
Gly27Ala (n=8)	37.3	± 0.33
Gly24Ala/Gly27Ala (n=8)	38.1	± 0.73
Thr179Ala (n=8)	40.7	± 0.97

Table 2 indicates that Gly24Ala and Gly27Ala have very similar melting temperatures (36.9 and 37.3 °C respectively) which were both lower than that of Gly24Ala/Gly27Ala (38.1 °C). Comparing these three mutants to the control tropomyosin (melting temperature of 37.0 °C) it appears that a single mutation of glycine to alanine in either position 24 or 27 does not have a significant effect on the melting temperature of tropomyosin. However, the presence of both mutations does have a small effect because the melting temperature has increased by approximately 1 °C.

From the heat-induced unfolding experiments, it appears that glycine (salmon tropomyosin) as opposed to glutamine or alanine (rabbit tropomyosin) in positions 24 and 27, has a destabilizing effect on the protein. It was suggested by Goonasekara *et al.* (2008) that the two glycines at the N-terminus end of salmon tropomyosin would result in flexibility of the protein at natural temperatures. It is evident in Ramachandran plots (Creighton, 1993) that glycine allows the protein to adopt a larger range of phi and psi angles which results in a more flexible protein.

Thr179Ala mutant had a significantly higher melting temperature (Table 2) compared to the non-mutated tropomyosin and the Gly24Ala, Gly27Ala and Gly24Ala/Gly27Ala mutants. Comparing the 20 amino acid differences amongst salmon and rabbit tropomyosins (Table 1), the mutation of alanine (rabbit) to threonine (salmon) is the only one that occurs in either an '*a*' or a '*d*' position. It is also important to note that this results in a polar side chain and it occurs in a region of known instability (Lehrer, 1978). As a result of having a threonine in position 179, there are three consecutive '*d*' amino acids that contain destabilizing amino acids (Ile at 172 and Ser at 186). Thus

having the destabilizing threonine at residue 179 appears to reduce the stability of tropomyosin by lowering the melting temperature from 40.7 (without the threonine in position 179) to 37.0 °C.

Based on the results of the unfolding experiments, the conformational stability of the various tropomyosin changes in the following order: non-mutant (least stable) ≈ Gly24Ala ≈ Gly27Ala < Gly24Ala/Gly27Ala < Thr179Ala (most stable). In comparing the melting profiles of Gly24Ala, Gly27Ala, Gly24Ala/Gly27Ala, Thr179Ala and the non-mutated tropomyosin (Figures 17-20), it appears the mutated tropomyosins unfold more cooperatively compared to the non-mutated form.

3.5 Chymotrypsin Digestion

Limited proteolysis was used to examine the effect that a threonine at position 179 has on the conformational stability of Atlantic salmon tropomyosin compared to alanine in the same position, as occurs in rabbit tropomyosin. Chymotrypsin cleaves peptide bonds on the C-terminal side of phenylalanine, tyrosine, tryptophan, leucine and methionine. The initial cleavage site of chymotrypsin in rabbit tropomyosin is residue 169 (leucine) (Pato and Smillie, 1981), which is only 10 amino acids away from the threonine at residue 179.

Time studies were performed at temperatures of 10, 25 and 37 °C. At the highest temperature (Figure 21) both the Thr179Ala mutant and non-mutant are rapidly degraded to a large fragment which runs just under the band corresponding to intact protein on a

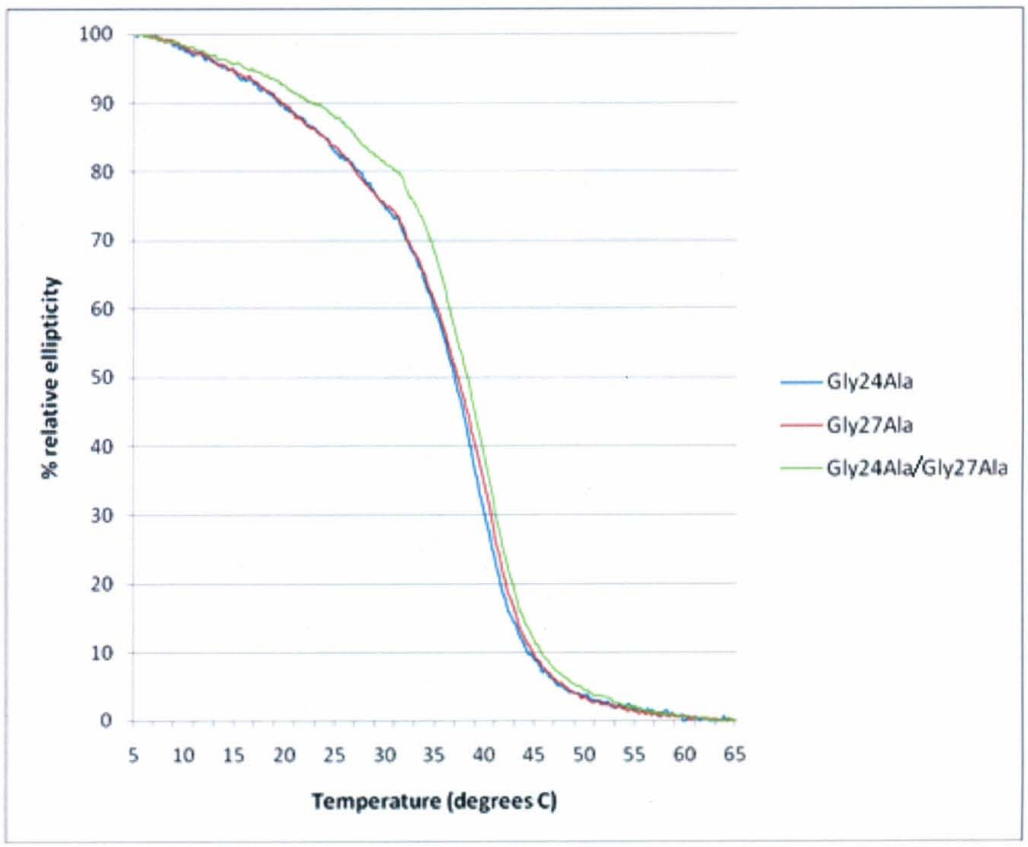


Figure 17: Comparison of the averaged, normalized melting curves of the three glycine mutants

The curves are color coded: Gly24Ala, blue; Gly27Ala, red and Gly24Ala/Gly27Ala, green.

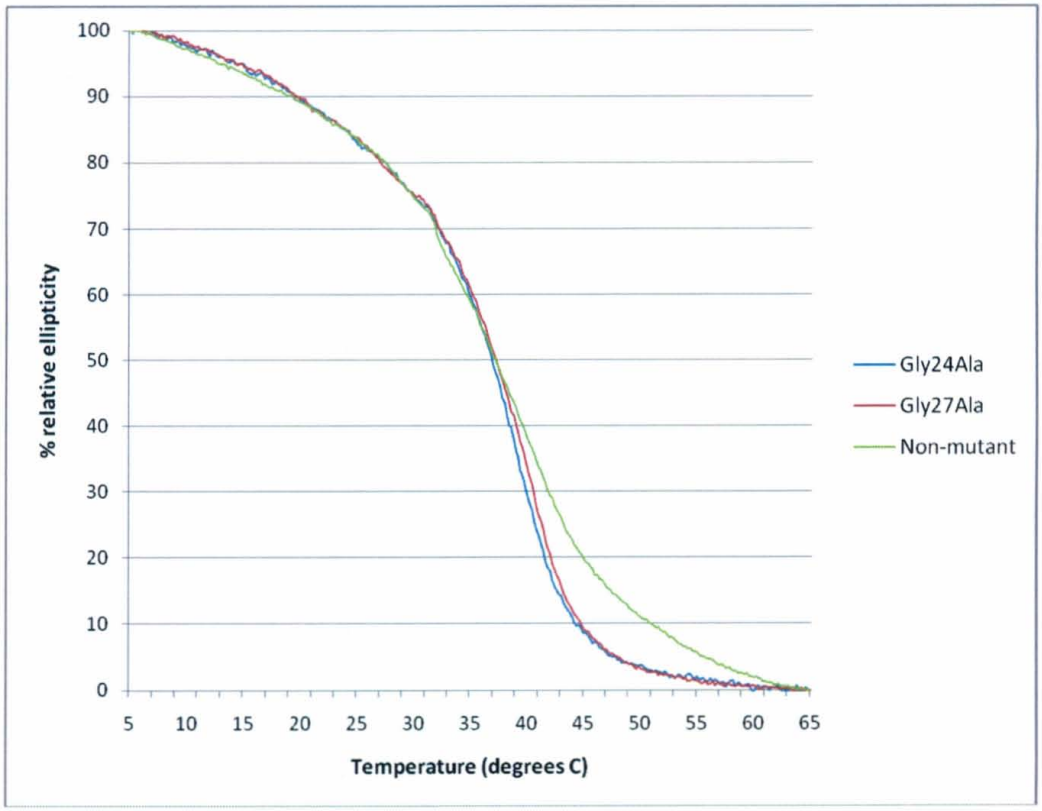


Figure 18: Comparison of the averaged, normalized melting curves of two glycine mutants and the non-mutant tropomyosin

The curves are color coded: Gly24Ala, blue; Gly27Ala, red and non-mutant, green

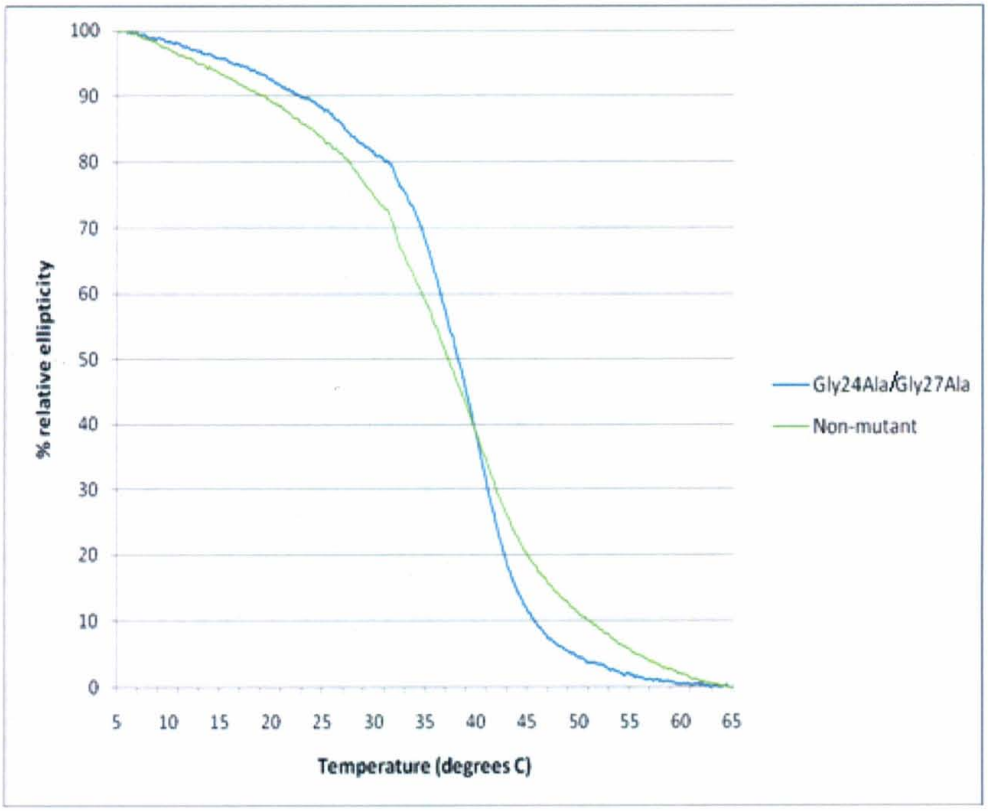


Figure 19: Comparison of the averaged, normalized melting curves of the double glycine mutant and the non-mutant tropomyosin

The curves are color coded: Gly24Ala/Gly27Ala, blue; and non-mutant, green

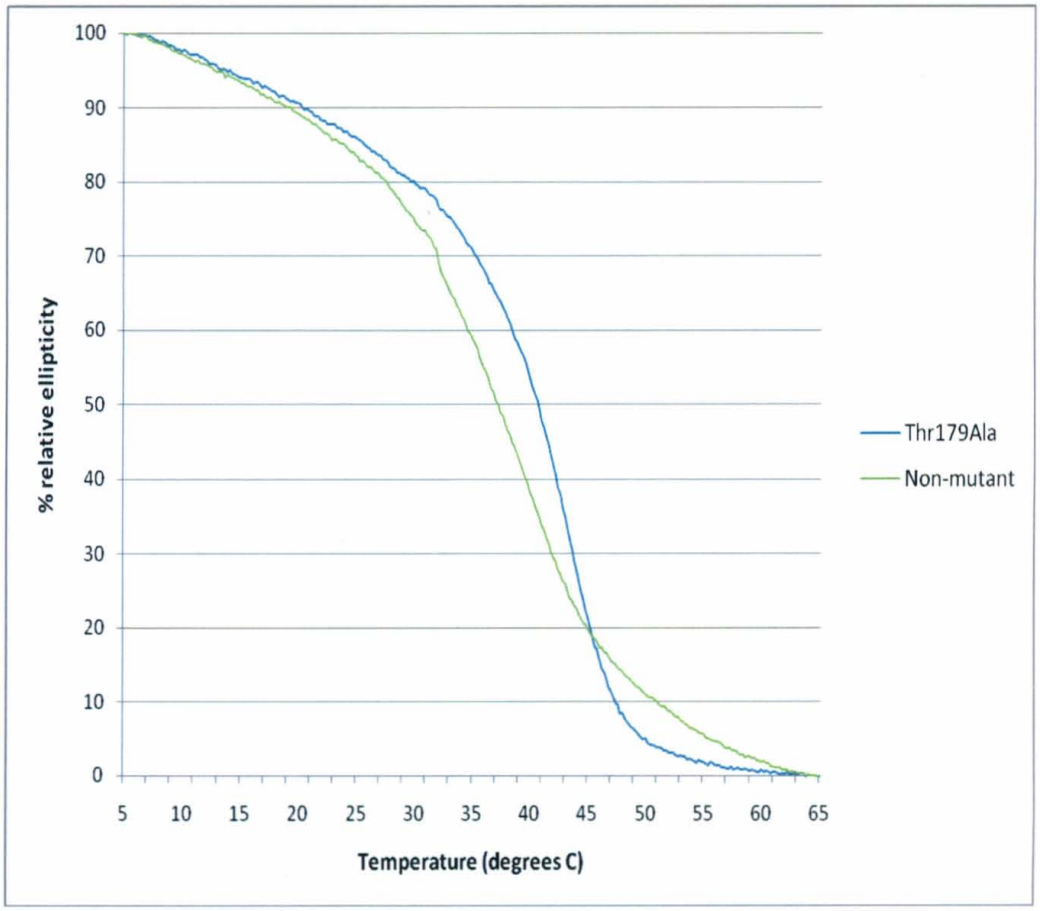


Figure 20: Comparison of the averaged, normalized melting curves of the threonine mutant and the non-mutant tropomyosin

The curves are color coded: Thr179Ala, blue; and non-mutant, green

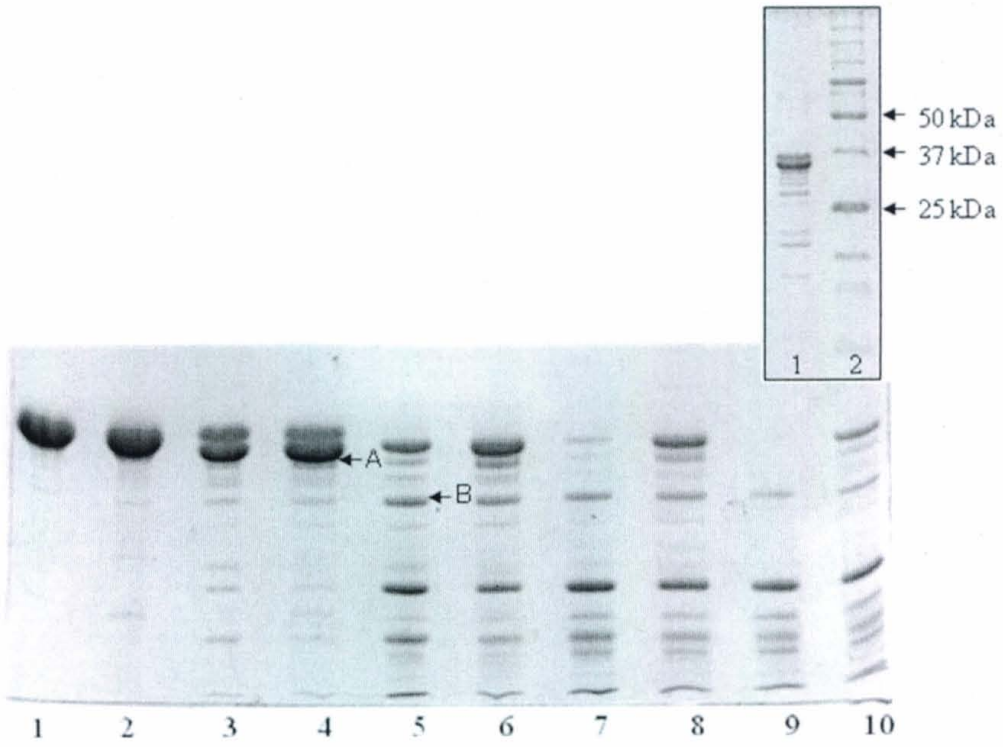


Figure 21: Comparison of the susceptibility of non-mutant and Thr179Ala tropomyosins to limited chymotryptic digestion at 37 °C.

Buffer: 50 mM NH_4HCO_3, 0.1 M NaCl, 1 mM DTT, pH 8.5. Enzyme to substrate mole ratio ~ 1:500. The reaction was stopped by mixing with lima bean trypsin inhibitor and freezing until preparation for electrophoresis. The two tropomyosins were digested side-by-side.

Non-mutated tropomyosin – Lanes 1, 3, 5, 7 and 9. T179A mutant – Lanes 2, 4, 6, 8 and 10. Lanes 1 and 2 have no chymotrypsin and the ensuing lanes represent increasing intervals of time (2, 10, 20 and 30 mins.) The entire 12% (w/v) polyacrylamide gel which was stained in Coomassie Brilliant Blue R-250 is shown.

The two fragments that were sequenced following western blotting are indicated as either A or B.

Insert: Lane 1 – limited chymotrypsin digestion of non-mutant tropomyosin, Lane 2 – molecular weight markers (BioRad).

12% SDS polyacrylamide gel (Figure 21, lanes 3 and 4), suggestive of a terminally-located cleavage event (ie removal of a small peptide). With continued incubation this fragment breaks down such that there are larger changes in mobility, suggestive of cleavage at 'internal' sites (ie well within the molecule). The major sub-fragment after 30 min is positioned halfway down the gel, just above the 20kDa marker (Figure 21, insert). Judging from the banding patterns changing the 179th amino acid has not led to the production of a unique fragment. However, it has altered the proteolytic susceptibility of some peptide bonds. For example, after 30 min the large fragment corresponding to the control tropomyosin has disappeared from the gel but that corresponding to the mutant is present in detectable amounts (Figure 21, lanes 9 and 10). These results, which depict a clear difference in the rate of digestion between the two tropomyosins are strong evidence to suggest that a threonine at position 179 in the salmon tropomyosin is destabilizing relative to alanine.

As would be expected, less extensive proteolysis is apparent at the lower temperatures, although the terminal cleavage is complete after 10 min for both tropomyosins (Figures 22 and 23). At 25 ^{o}C (Figure 22), the control (lanes 5, 7 and 9), but not the mutant (lanes 6, 8 and 10) is seen to contain small amounts of a lower Mr peptide in the middle of the gel. Figure 23 shows the same experiment repeated at 10 ^{o}C. Again the large fragment is rapidly produced, but in this instance there is no distinction in the rates of further degradation.

The results of these three experiments suggest that threonine 179 contributes to the conformational stability of tropomyosin. However, these effects are most

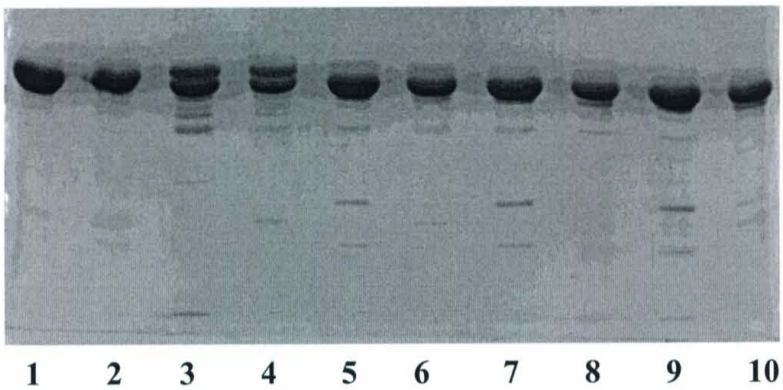


Figure 22: Comparison of the susceptibility of non-mutant and Thr179Ala tropomyosins to limited chymotryptic digestion at 25 °C.

The two tropomyosins were digested side-by-side. Buffer: 50 mM NH_4HCO_3, 0.1 M NaCl, 1 mM DTT, pH 8.5. Enzyme to substrate mole ratio ~ 1:250. The reaction was stopped by mixing with lima bean trypsin inhibitor and freezing until preparation for electrophoresis.

Non-mutated tropomyosin – Lanes 1, 3, 5, 7 and 9. T179A mutant – Lanes 2, 4, 6, 8 and 10. Lanes 1 and 2 have no chymotrypsin and the ensuing lanes represent increasing intervals of time (2, 10, 20 and 30 mins.) The entire 12% (w/v) polyacrylamide gel which was stained in Coomassie Brilliant Blue R-250 is shown.

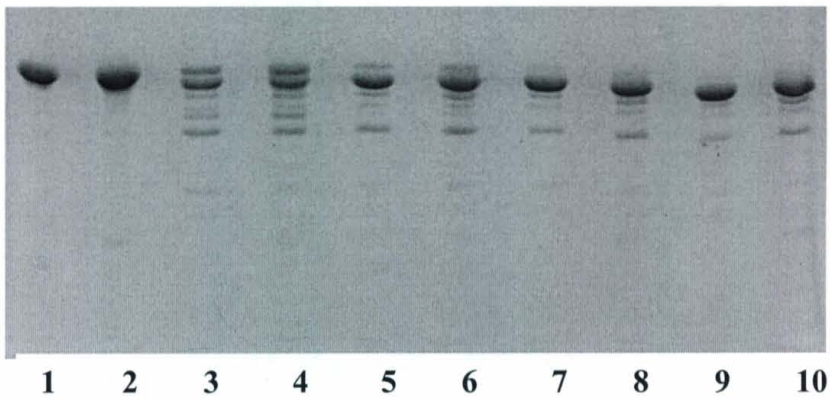


Figure 23: Comparison of the susceptibility of non-mutant and Thr179Ala tropomyosins to limited chymotryptic digestion at 10 °C.

Conditions as described in Figure 22 except that a temperature of 10 °C was used.

Non-mutated tropomyosin – Lanes 1, 3, 5, 7 and 9. T179A mutant – Lanes 2,4, 6, 8 and 10. Lanes 1 and 2 have no chymotrypsin and the ensuing lanes represent increasing intervals of time (2, 10, 20 and 30 mins.) The entire 12% (w/v) polyacrylamide gel which was stained in Coomassie Brilliant Blue R-250 is shown.

noticeable at a temperature near the melting temperature of the protein (~37 °C). The difference in the relative stability of the Thr179Ala tropomyosin and the non-mutant tropomyosin at the three temperatures is in good agreement with the results of the heat-induced unfolding experiments in section 3.4. When the tropomyosin is heated to 10 °C, the non-mutant tropomyosin is, on average, 97.2% folded, whereas Thr179Ala is 97.9% folded. At 25 °C, non-mutant tropomyosin is approximately 83.4% folded compared to Thr179Ala which is 85.7% unfolded. However, at 37°C the non-mutant tropomyosin is 50.6% folded whereas Thr179Ala is still 64.8% folded. These comparisons suggest that at temperatures below 25 °C all of the chymotrypsin sites are equally accessible on the non-mutant and Thr179Ala tropomyosin. However, at higher temperatures, some are differentially accessible (with the exception of the terminal site) such that there is enhanced breakdown of the mutant relative to the control.

3.6 Sequencing of Chymotrypsin Fragments

Sequencing was performed on two chymotrypsin digestion fragments of the non-mutant tropomyosin. Fragment A (as indicated in Figure 21), which in the previous section was alluded to as the large fragment and which is the major product at short incubation times was subjected to 11 cycles of sequencing and Fragment B (indicated in Figure 21), a fragment which increases in prominence at later times, underwent six sequencing cycles. The results from Fragment A were: Lys-Leu-Asp-Lys-Glu-Asn-Ala-Leu-Asp-Arg-Ala. Those results from Fragment B were: Lys-Leu-Asp-Lys-Glu-Asn. By comparing these results to the known sequence of salmon skeletal tropomyosin (Heeley *et*

al., 1995), it was determined that the N-terminal residue for both fragments was Lys12. These results show that the initial chymotrypsin cleavage site on recombinant salmon tropomyosin (at 37 °C) is between residues Leu11 and Lys12. Although chymotrypsin usually cleaves proteins on the C-terminal side of aromatic amino acids, it occasionally cleaves on the C-terminal side of leucine. The initial chymotryptic cleavage site on recombinant salmon tropomyosin is not the same as that of rabbit skeletal tropomyosin (Leu169) (Pato and Smillie, 1981). One possible explanation for this difference is that the recombinant tropomyosin is unacetylated. Research has shown that having an unacetylated methionine at residue 1 causes the protein to be more destabilized at the N-terminus (Hitchcock-DeGregori and Heald, 1987; Greenfield *et al.*, 1994; Frye *et al.*, 2010). This destabilization could be the reason as to why the recombinant salmon tropomyosin is initially cleaved by chymotrypsin near the N-terminus. A second explanation for the difference in initial cleavage is the presence of the double glycine in the salmon tropomyosin. However, this explanation is less likely since there is no difference in the site of Omp-T digestion (which cleaves near this site at residue 6) in salmon tropomyosin compared to rabbit tropomyosin (Goonasekara *et al.*, 2007).

While the non-flanking cleavage sites were not characterized in this work speculation is possible in the case of Fragment B. The electrophoretic mobility of B corresponds to an apparent molecular mass of ~26,000 Da (Figure 21 insert) which is consistent with the removal of some 40 amino acids from the carboxyl terminus. In this regard, there is a conserved phenylalanine at residue-241. Thus, it is possible (but obviously not proven) that fragment B is comprised of residues 12-241.

The initial internal cleavage site of Thr179Ala was not determined but it appears that it is the same as the non-mutant tropomyosin because the fragment sizes look to be identical. Figure 21 shows that following two minutes of digestion (lanes 3 and 4), the two samples had equal amounts of the large fragment. This result suggests that the initial cleavage site is not affected by Thr179.

3.7 Omp-T Digestion

Outer membrane protease-T (Omp-T) is a bacterial endoprotease (180,000 g/mole) having trypsin-like activity that selectively cleaves tropomyosin between Lys-6 and Lys-7 only (Goonasekera *et al.*, 2007). Removal of the amino-terminal hexapeptide results in a small, but detectable shift in electrophoretic mobility (Goonasekara *et al.*, 2007). Given the location of the scissile peptide bond, Omp-T digestion was carried out in order to determine if glycine (at res-24 and 27) influences the conformational stability of the N-terminal end of tropomyosin.

Figures 24, 25 and 26 show the Omp-T digestion patterns of Gly24Ala and non-mutant tropomyosin at 10, 25 and 37 °C respectively. Incomplete Omp-T digestion produces two closely spaced protein bands with no other bands appearing on the gel. At all three temperatures it is apparent that the non-mutant tropomyosin is more readily cleaved compared to the Gly24Ala mutant. For example, after one hour of digestion at 10 °C (Figure 24), the non-mutant tropomyosin is approximately 50% digested (lane 3) whereas the Gly24Ala mutant is largely intact (lane 4). Figure 25 indicates that after 30

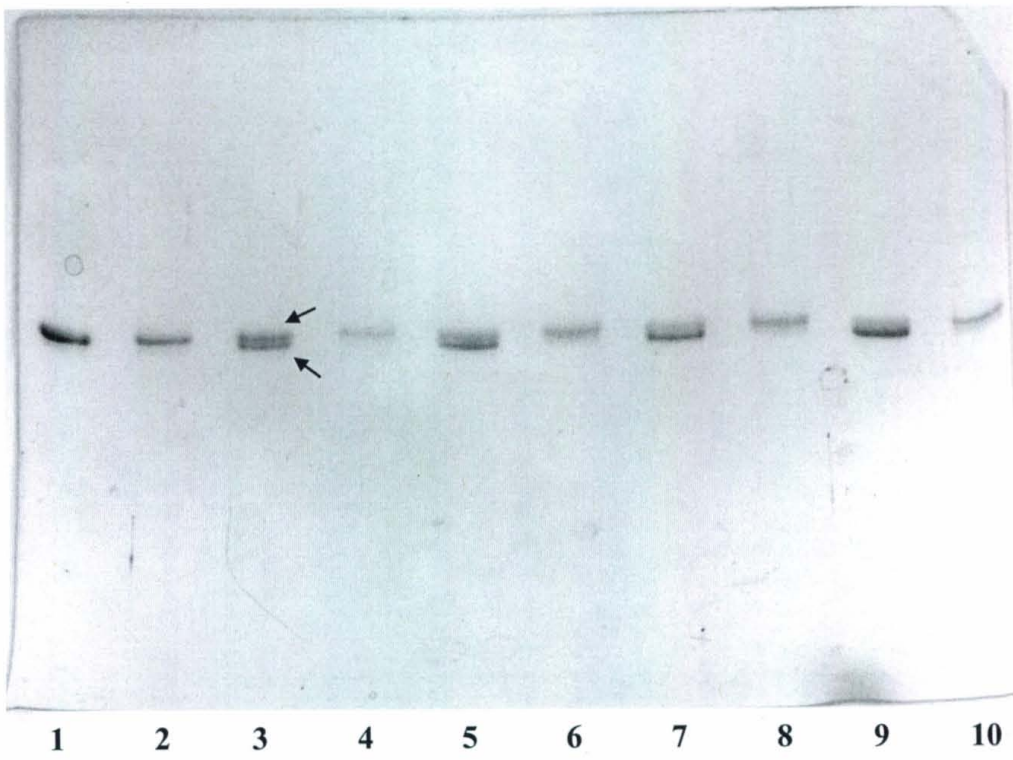
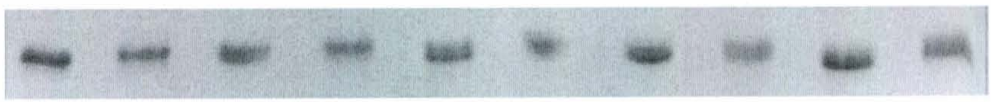


Figure 24: Comparison of the Omp-T digestion of non-mutant and Gly24Ala tropomyosins at 10 °C as analyzed by SDS PAGE

Two separate digestions were performed in parallel. 500 µg of a given tropomyosin was digested using a 1 ml suspension of JM109 cells as described in Materials and Methods. At set times aliquots of 100 µl of reaction mixture were removed and the reaction immediately stopped by centrifugation and boiling the supernatant in the presence of an equivolume of SDS sample buffer.

Approximately 1 µg of protein was analyzed on a SDS 12% (w/v) polyacrylamide gel which was stained with Coomassie Brilliant Blue R-250. The reason for the diffuse staining apparent in lane 4 is unknown. The full (non-sectioned) gel is presented to confirm that proteolysis had not occurred at multiple sites (Goonasekara et al 2008). Arrows in lane 3 correspond to intact tropomyosin and cleaved tropomyosin.

Lanes 1, 3, 5, 7 and 9 contain non-mutant tropomyosin. Lanes 2, 4, 6, 8, and 10 contain Gly24Ala. Lanes 1 and 2 contain non-digested protein and the subsequent lanes are from samples taken at 1, 2, 3 and 4 hour digestion times.



1 2 3 4 5 6 7 8 9 10

Figure 25: Comparison of the Omp-T digestion of non-mutant and Gly24Ala tropomyosins at 25 °C as analyzed by SDS PAGE

Conditions are as described in Figure 23, except that T = 25 °C. In this and all ensuing Figures only the tropomyosin-containing section of the 12% (w/v) polyacrylamide gel is shown.

Lanes 1, 3, 5, 7 and 9 contain non-mutant tropomyosin. Lanes 2, 4, 6, 8, and 10 contain Gly24Ala. Lanes 1 and 2 contain non-digested protein and the subsequent lanes are from samples taken at 5, 10, 20 and 30 min digestion times.

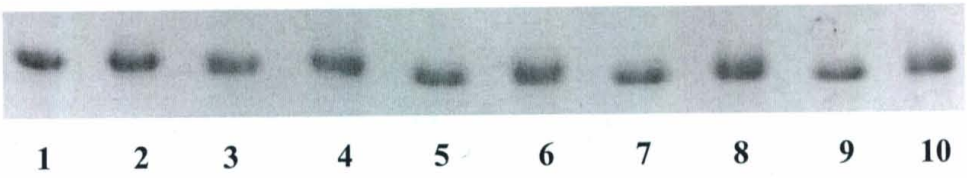


Figure 26: Comparison of the Omp-T digestion of non-mutant and Gly24Ala tropomyosin at 37 °C

Conditions are as in Figure 24, except that T = 37 °C.

Lanes 1, 3, 5, 7 and 9 contain non-mutant tropomyosin. Lanes 2, 4, 6, 8, and 10 contain Gly24Ala. Lanes 1 and 2 contain non-digested protein and the subsequent lanes are from samples taken at 5, 10, 20 and 30 min digestion times.

minutes digestion at 25 °C, the non-mutant tropomyosin is completely cleaved (lane 9), whereas the Gly24Ala tropomyosin is mostly intact (lane 10). The same trend is evident for digestion at 37 °C (Figure 26).

Figures 27, 28 and 29 respectively show the Omp-T digestion patterns of Gly27Ala and non-mutant tropomyosin at 10, 25 and 37 °C. Similar to the Gly24Ala mutant, the non-mutant tropomyosin is more susceptible to cleavage by Omp-T at all three temperatures. Following one hour of incubation at 10 °C (Figure 27), approximately 50% of the non-mutant tropomyosin had been cleaved (lane 3) whereas none of the Gly27Ala had been cleaved (lane 4). Figures 28 and 29 show that after 30 minutes, the non-mutant tropomyosin was 100% cleaved (lanes 9) whereas the Gly27Ala mutants still had some intact tropomyosin present (lanes 10).

The results of these digestions suggest that the mutations at residues 24 and 27 have affected the stability of the N-terminal end of tropomyosin. These results show that the stability of a protein can be affected by a mutation that is 20 and 17 residues away. The glycine appears to make the N-terminus of the protein more flexible (compared to alanine and glutamine in the same position) allowing Omp-T greater accesss to the peptide bond between residues 6 and 7.

Further digestions were carried out to determine if the rate of cleavage varied between the Gly24Ala and the Gly27Ala mutants and to see if having both mutations in the protein had a different effect. Figures 30 (A and B) compare the rate of digestion by

1 2 3 4 5 6 7 8 9 10

Figure 27: Comparison of the Omp-T digestion of non-mutant and Gly27Ala tropomyosin at 10 °C

Conditions are as in Figure 24.

Lanes 1, 3, 5, 7 and 9 contain non-mutant tropomyosin. Lanes 2, 4, 6, 8, and 10 contain Gly27Ala. Lanes 1 and 2 contain non-digested protein and the subsequent lanes are from samples taken at 1, 2, 3 and 4 hour digestion times.

1 2 3 4 5 6 7 8 9 10

Figure 28: Comparison of the Omp-T digestion of non-mutant and Gly27Ala tropomyosin at 25 °C

Conditions are as in Figure 24, except that T = 25 °C.

Lanes 1, 3, 5, 7 and 9 contain non-mutant tropomyosin. Lanes 2, 4, 6, 8, and 10 contain Gly27Ala. Lanes 1 and 2 contain non-digested protein and the subsequent lanes are from samples taken at 5, 10, 20 and 30 min digestion times.

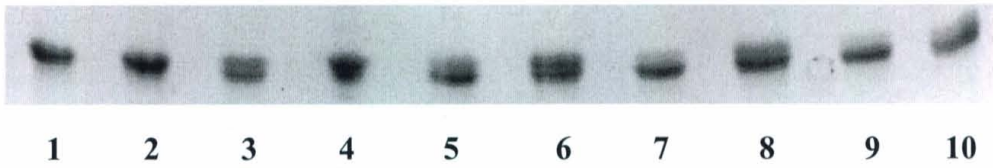


Figure 29: Comparison of the Omp-T digestion of non-mutant and Gly27Ala tropomyosin at 37 °C

Conditions are as in Figure 24, except that T = 37 °C.

Lanes 1, 3, 5, 7 and 9 contain non-mutant tropomyosin. Lanes 2, 4, 6, 8, and 10 contain Gly27Ala. Lanes 1 and 2 contain non-digested protein and the subsequent lanes are from samples taken at 5, 10, 20 and 30 min digestion times.

1 2 3 4 5 6 7 8 9

A

1 2 3 4 5 6

B

Figure 30 A & B: Comparison of the susceptibility of the three glycine mutant tropomyosins to Omp-T digestion at 25 °C

Conditions as in Figure 24 except that T = 25 °C and three tropomyosins were digested side-by-side.

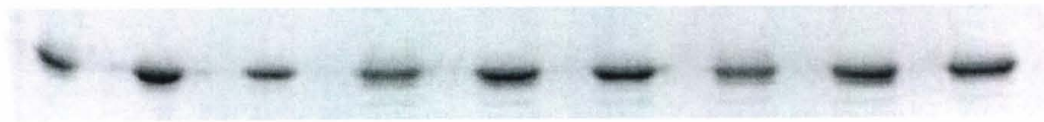
A. Lanes 1, 4 and 7 contain Gly24Ala tropomyosin. Lanes 2, 5, and 8 contain Gly27Ala. Lanes 3, 6 and 9 contain Gly24Ala/Gly27Ala. Lanes 1, 2 and 3 contain non-digested protein and the subsequent lanes are from samples taken at 15 and 30 min digestion times.

B. Lanes 1 and 4 contain Gly24Ala tropomyosin. Lanes 2 and 5 contain Gly27Ala. Lanes 3 and 6 contain Gly24Ala/Gly27Ala. Lanes 1, 2 and 3 contain samples taken at 45 min digestion time and lanes 4, 5 and 6 were from 60 min digestion time.

Omp-T between Gly24Ala, Gly27Ala and Gly24Ala/Gly27Ala at 25 °C. As can be seen in Figure 30B, after 60 minutes, the Gly24Ala/Gly27Ala mutant (lane 6) has only approximately 50% cleavage whereas the Gly24Ala (lane 4) and Gly27Ala (lane 5) both have a significant more amount of cleavage. There did appear to be a difference in the rate of digestions between Gly24Ala and Gly27Ala. Figure 30A shows that after 15 min of incubation, Gly27Ala (lane 5) had approximately 50% of intact protein remaining, whereas Gly24Ala (lane 4) had more intact protein remaining.

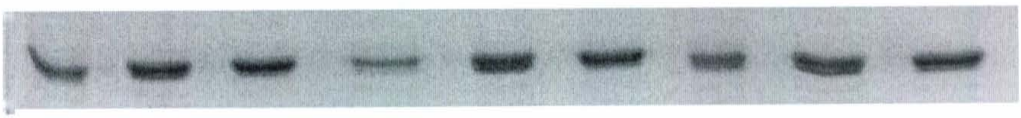
Figures 31 (A and B) shows the Omp-T digestion patterns of Gly24Ala, Gly27Ala and Gly24Ala/Gly27Ala at 10 °C. The results are similar to those obtained by digestion at 25 °C. Following 30 min of digestion (Figure 31A), digestion had started on the Gly24Ala (lane 4) and Gly27Ala mutants (lane 5), whereas the Gly24Ala/Gly27Ala (lane 6) is still completely intact. There also appeared to be a difference in the rate of digestion between Gly24Ala and Gly27Ala. Figure 31B shows that after four hours of digestion, the Gly27Ala mutant (lane 8) had ~ 50% cleavage compared to the Gly24Ala (lane 7) which showed less digestion.

From these digestions it can be surmised that the rate of digestion is: (in decreasing susceptibility to Omp-T) non-mutant > Gly27Ala > Gly24Ala > Gly24Ala/Gly27Ala. It was anticipated that the non-mutant tropomyosin would have the faster rate of digestion because there are glycines (as opposed to alanine or glutamine) at both residues 24 and 27. The glycines causes the protein to be more flexible (and therefore more conformationally unstable) because they allow it to adopt more phi and psi



1 2 3 4 5 6 7 8 9

A



1 2 3 4 5 6 7 8 9

B

Figure 31 A & B: Comparison of the susceptibility of the three glycine mutant tropomyosins to Omp-T digestion at 10 °C

Conditions as in Figure 24 except that T = 25 °C and three tropomyosins were digested side-by-side.

A. Lanes 1, 4 and 7 contain Gly24Ala tropomyosin. Lanes 2, 5, and 8 contain Gly27Ala. Lanes 3, 6 and 9 contain Gly24Ala/Gly27Ala. Lanes 1, 2 and 3 contain non-digested protein and the subsequent lanes are from samples taken at 30 and 60 min digestion times.

B. Lanes 1, 4 and 7 contain Gly24Ala tropomyosin. Lanes 2, 5, and 8 contain Gly27Ala. Lanes 3, 6 and 9 contain Gly24Ala/Gly27Ala. Samples were taken at 120 min (lanes 1, 2 and 3), 180 min (lanes 4, 5 and 6) and 240 min (lanes 7, 8 and 9).

angles. As well, Serrano *et al.* (1992) showed that alanine stabilizes the glycine compared to glycine.

The results of the Omp-T study suggest that a substitution at positions 24 or 27 affects the stability of the protein at the far N-terminus end of the protein at all temperatures. It is also shown that the substitutions at positions 24 and 27 do not have an equal affect on the stability of the far N-terminus. Having a glycine at position 24 causes increased instability at the far N- terminus compared to having a glycine at position 27, and a glycine at both residues causes greater instability.

Chapter 4

Conclusions and Future Directions

4.1 Conclusions

The majority of research on amino acid contribution to the stability of a protein has been carried out on globular proteins (Gianese *et al.*, 2001; Hayley *et al.*, 2011). This thesis focused on the protein tropomyosin in order to gain an understanding of the role that some amino acids play in the cold-adaptation of rod-shaped proteins. The specific goal was to examine the effect that three amino acids have on the conformational stability of salmon tropomyosin. It was hypothesized that the threonine at position 179 increases the flexibility of the protein because the presence of a polar hydroxyl group in the (largely hydrophobic) core would reduce stability of the coiled-coil. It was thought that the glycines at positions 24 and 27 would increase flexibility due to the fact that the hydrogen R group allows the main chain to adopt a greater range of phi-psi angles. Three single mutations (Gly24Ala, Gly27Ala and Thr179Ala) and one double mutation (Gly24Ala/Gly27Ala) of Atlantic salmon fast muscle tropomyosin were made by site directed mutagenesis. Salt and pI precipitation were used to enrich the protein followed by Q-sepharose Fast Flow and hydroxyapatite chromatography. Protein enrichment was verified electrophoretically on a 12% SDS polyacrylamide gel (Figure 11).

Circular dichroism was used to study the global conformational stability of the protein. Omp-T and chymotrypsin digestions were employed to examine the conformational stability of the protein at localized regions. When protein unfolding is

monitored by circular dichroism, there is no indication that there is a difference in the stability of Gly24Ala, Gly27Ala and non-mutated tropomyosin because all three had similar melting temperatures (36.9, 37.3 and 37.0 °C, respectively) (Figure 18 and Table 2). However, the results of the Omp-T digestion experiments demonstrated that there was a local difference, specifically within the amino-terminal region. At three temperatures (10, 25 and 37 °C), non-mutated tropomyosin was digested more readily compared to Gly24Ala and Gly27Ala (Figures 25-30). These results suggest that replacing a glycine with an alanine causes an increase in the flexibility at this end of the molecule. This finding confirms those from a previous study by Serrano *et al.* (1992) which showed that having a glycine instead of an alanine in α-helices leads to an increase in protein flexibility. Nevzorov *et al.* (2011) reported similar results when they replaced Gly126 of tropomyosin with an alanine.

The Gly24Ala/Gly27Ala mutant had a higher melting temperature (38.1 °C) compared to the single glycine mutants and the non-mutant tropomyosin (Figures 17 and 19 and Table 2). As well, the double glycine mutant was less susceptible to Omp-T digestion compared to the single mutants (Figure 30 and 31). These results infer that the presence of both glycines has additive influence on the flexibility of the N-terminus compared to a single glycine. It also showed that, compared to a single glycine, the double glycines noticeably affect the stability of a significant stretch of the molecule, such that the difference can be detected by circular dichroism.

In rabbit skeletal tropomyosin the amino acids at positions 24 and 27 are glutamine and alanine respectively. It can be hypothesized that the glycines are

advantageous to the protein at a temperature of 10 °C, which is near the salmon's natural temperature, because they allow for more flexibility in the protein. Without the glycines, such as the case with rabbit tropomyosin, the protein would be rigid at such a low temperature. Excessive rigidity can be predicted to adversely affect the function of tropomyosin via its interactions with other proteins at the N-terminal end, including itself. Research that has been done on globular proteins proposes that psychrophiles have an increased number of glycines compared to mesophiles and thermophiles and that the glycines have a greater tendency to appear in clusters (Feller *et al.,* 1997). The evidence presented in this thesis indicates that rod shaped psychrophilic proteins also incorporate glycine clusters in order to create a more flexible and less stable protein. Glycine residues increase the flexibility of the protein because it allows for more phi and psi angles so that the back bone is not as constrained as it would be with other amino acids (Creighton 1993).

Omp-T digestion reveals that the glycine at position 24 had a somewhat greater influence on the N-terminal flexibility of the protein compared to the one at position 27. It also shows that one mutation can affect the stability of the protein at least 21 residues away from the original mutation. The results of the Omp-T digestions suggest the influence that the glycines have on the stability of the protein increased in the order: Gly27 > Gly24 > Gly24/Gly27.

From the Omp-T digestions and thermal induced unfolding experiments it can be deduced that glycines 24 and 27 affect the N-terminal flexibility of the protein but their individual contributions are not sufficient as to be detected by circular dichroism.

However, their additive effects do bring about a change in the stability of the complete protein as indicated by the thermal unfolding experiments.

It is unusual to have a threonine in a core position of tropomyosin because the polar side chain has a tendency to destabilize the helix. An alanine is at position 179 in rabbit skeletal tropomyosin. The Thr179Ala mutant had the greatest increase in melting temperature compared to the non-mutant tropomyosin. Replacing the threonine with an alanine at position 179 resulted in an increase in the melting temperature from 37.0 °C to 40.7 °C (Figure 20 and Table 2). Chymotrypsin digestion patterns between Thr179Ala and the non-mutant tropomyosin shows that the non-mutant sample was more susceptible to proteolysis at sites other than the initial cleavage site (Figures 21-23). Both the heat induced unfolding and chymotrypsin digestion experiments show that replacing the threonine at position 179 with an alanine leads to an increase in protein stability and a decrease in protein flexibility. This result is in agreement with a study performed by Tripet *et al.* (2000) where they show that an alanine in a *'d'* position of a coiled-coil peptide is more stable than a threonine in the same position. It is likely that the salmon tropomyosin, which naturally occurs at marine temperatures of below 10 °C, has incorporated a threonine at this position in the helix in order to increase the flexibility of the protein.

A mutation from threonine to alanine increased the melting temperature by 3.5 °C (from the non-mutant tropomyosin), whereas the single glycine to alanine mutations did not significantly increase the melting temperature and a double glycine to alanine mutation increased the melting temperature by 1 °C (which is on the border line of

detection with the Jasco 810 spectropolarimeter). The results of the heat induced unfolding experiments suggest that the presence of a threonine at position 179 has a greater influence on the global stability of the molecule compared to glycines at positions 24 and/or 27. This can be rationalized on the basis of Thr179 occupying a core '*d*' position in the coiled-coil, whereas the Gly24 and Gly27 occur at '*c*' and '*f*' positions respectively. Further, residue 179 occurs in a region of the molecule that, in the case of the mammalian protein, is intrinsically unstable (Lehrer, 1978; Betcher-Lange and Lehrer, 1978) and contains disease mutations (Thierfelder et al., 1994).

Edman based sequencing of a fragment of the non-mutant recombinant tropomyosin produced by limited chymotrypsin digestion revealed that the initial cleavage site was between Leu11 and Lys12. This site is different from the initial cleavage site of wild type rabbit α-tropomyosin which initially cleaves on the C-terminal side of Leu169 (Pato *et al.*, 1981a). The difference between the initial sites of cleavage may be due to the fact that the recombinant tropomyosin that was used for the experiment was not N-acetylated because that has been shown to increase the stability of the N-terminal (Hitchcock-DeGregori and Heald, 1987; Greenfield *et al.*, 1994; Frye *et al.* 2010). It is also possible that the difference in cleavage sites is due to a decrease in stability caused by the two glycines (residues 24 and 27) that are present in the salmon tropomyosin but not the rabbit. The results of the chymotrypsin digest prove that the presence of a threonine at position 179 causes the protein to be more susceptible to chymotrypsin digestion at sites removed from the amino terminus. This fact is illustrated in Figure 21 where the digestion pattern of the non-mutant tropomyosin is compared to

that of the Thr179Ala mutant. The effect that the threonine has on the protein's stability is more noticeable at 37 °C compared to 10 and 25 °C, although the reasons being are not yet clear. It seems that Thr179 does not affect the N-terminal stability of the protein because the initial rate of cleavage between Leu11 and Lys12 appears to be the same for the non-mutant and Thr179Ala.

It can be concluded from the results of the heat induced unfolding, Omp-T digestions and chymotrypsin digestion experiments that the glycines at positions 24 and 27 and the threonine at position 179 all contribute to the stability of salmon skeletal tropomyosin. The heat induced unfolding experiments suggest that the threonine has a greater impact on the overall stability and flexibility of the protein in contrast with the two glycines. Omp-T digestions show that both of the glycines contribute to the flexibility of the N-terminal but they do not contribute equally. The stability of the four mutants and non-mutant tropomyosins decreased in the order:
Thr179Ala>Gly24Ala/Gly27Ala>Gly27Ala>Gly24Ala>non-mutant.

4.2 Future Directions

The research presented in this thesis can be expanded upon in order to understand the amino acids that are important in maintaining flexibility in rod-shaped psychrophilic proteins.

- Chymotrypsin digestion can be performed to determine whether the difference in initial cleavage site of rabbit tropomyosin and salmon tropomyosin is due to the lack of acetylation of the recombinant salmon tropomyosin or due to the presence of two glycines at the N-terminus. The rate of proteolysis at the initial cleavage site would have to be compared amongst the wild-type, non-mutant recombinant and double glycine mutant tropomyosins. If they are the same for the double mutant and the non-mutant but different for the wild type, then the cleavage site is due to the lack of acetylation of the recombinant proteins. However if they are the same for non-mutant and wild type proteins but different for the double mutant, then the cleavage difference is caused by the glycines.

- Omp-T digestion of the Thr179Ala mutant would conclusively determine if the threonine at position 179 has any influence on the stability of the N-terminal. A difference in the digestion patterns between this mutant and the non-mutant tropomyosin would confirm that Thr179 contributes to the N-terminal stability. If there was dissimilarity, an Omp-T digestion between that mutant and the three

glycine mutants would give an indication as to how much of a contribution it makes to the protein's N-terminal stability.

- An obvious future avenue of investigation would be to investigate the effect of the mutations on the interaction of tropomyosin with other thin filament proteins. For example, experiments performed by Singh and Hitchcock-DeGregori (2006) indicate that there are amino acids between residues 165-188 that are essential for actin binding and it would be interesting to determine if residue 179 was one these residues. The interaction between tropomyosin and actin can be studied by sedimentation in an ultracentrifuge. –However, in this instance a requirement would be to express the protein in a eukaryotic cell line so that the tropomyosin is acetylated, because as stated early in this thesis unacteylated tropomyosin binds weakly to actin (Hitchcock-DeGregori and Heald 1987).

- The core of the troponin complex (Takeda *et al.*, 2003; Vinogradova *et al.*, 2005), comprising the I and C subunits together with the T2 portion of T, attaches to tropomyosin approximately two-thirds of the way along the molecule (Pirani *et al.*, 2006) in the vicinity of cysteine-190 (summarised in Stewart and McLachlan 1976 and Heeley *et al.*, 1987). Support for such an arrangement includes the results of chemical cross-linking (Chong and Hodges 1982) and the sensitivity of a fluorescent probe attached at this cysteine to troponin-T fragment T2 (Morris and Lehrer 1984). Thus, residue 179 again appears to lie in a critical region. Tropomyosin-troponin binding is conveniently assayed using Sepharose affinity

media to which one of the proteins has been covalently attached (Pearlstone and Smillie 1982). For example, tropomyosin (either mutant or non-mutant) could be chromatographed in a column containing immobilized whole troponin or troponin-T by linearly increasing the concentration of sodium chloride. Then, the affinities of the various tropomyosins can be assessed by comparing the conductivities of the chromatography fractions having maximum UV absorption.

- A mutation from a lysine (rabbit) to a threonine (salmon) at residue 77 ('*g*') results in the loss of an ion pair in the salmon tropomyosin compared to the rabbit. Heat-induced unfolding experiments and proteolytic digestion (Omp-T and chymotrypsin) can be used to determine if this mutation has any effect on the stability and flexibility of the protein. Site-directed mutagenesis can be used to mutate threonine in salmon to a lysine.

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