1 Introduction

There are three types of muscle in the body, skeletal, cardiac and smooth muscle. Skeletal muscle deals with most everyday movements. It is a striated muscle, meaning that the structure of the muscle is composed of fibres. In order to contract these muscles these fibres must contract and thicken. Cardiac muscle is another form of striated muscle found in the walls of the heart, this also features striations, and like skeletal muscles is multinucleated. Smooth muscle, generally surrounding organs such as the stomach or trachea, is mono nucleated and therefore does not feature striations.

Troponin is a protein found only in striated muscle types, it acts in regulating the contraction of these muscles. The fibres ubiquitous types of muscle are encased in a cell wall which contains the sarcoplasmic reticulum and myofibrils. These myofibrils contain even smaller fibres known as thick and thin filaments. The former contains the molecular motor myosin which provides the force and movement in a muscle. These myosin molecules act as a motor in the presence of ATP (fuel for the process) and the thin filament, actin. When the myosin motors act on the actin these two filaments are pulled past each other and the muscle contracts and thickens, just as is seen when moving your arm. This action is controlled by calcium ions which are released from the sarcoplasmic reticulum when the brain triggers it. Troponin is the intermediary structure that controls whether the myosin binds to the actin. The basic structure of muscle is shown in figure 1.

Troponin is a family of three proteins that act together in inhibiting the binding of myosin to actin strands. This family compromises three troponin structures T, C and I. These form a complex with an inhibitory part (I) that binds to the actin, blocking the active binding site that the myosin molecule needs. The T structure binds to tropomyosin (a long protein which also blocks the myosin binding by coiling with the actin double helix on top of its binding sites). Troponin C (TnC) joins the two sides of the complex and, using its EF hand structure performs a conformal change in the molecules moving the tropomyosin and the I structure away from myosin’s binding site. This conformal change is initiated via a binding of Ca\textsuperscript{2+} onto the TnC structure[2]. This action is illustrated in figure 2. Without this inhibitor complex, muscles would not function as the contraction of the sarcomere would be constant and ATP would be continually used.

2 Action in the Cell

Ca\textsuperscript{2+} binding to an EF hand structure is found in many cellular processes and here controls the in-
Figure 2: Diagram showing basic structure of the troponin complex and the binding of miosin after the Ca$^{2+}$ induced conformal change [3]

Interaction between myosin and actin. This trigger is relayed to the tropomyosin via the rest of the troponin complex. Knowing a biological item's structure is vitally important in understanding its function. The second half of this report will concentrate on a single method of working out structure with particular reference to the TnC molecule.

Actin strands form pseudodouble helical filaments with around 13 monomers per turn of the helix. Tropomyosin coils contact seven actin monomers as well as contacting two other tropomyosin strands, one at each end [5]. Figure 4 shows the (silver) bindings to other tropomyosin molecules giving a continuous strand of tropomyosin along the actin binding sites. Each tropomyosin strand is associated with one troponin complex. The figure shows the tropomyosin dimer. This coiling of the dimer is tissue specific, with this example being pig and humans possessing other forms [6]. Each tropomyosin strand connects to the TnT and this TnT molecule is bound to the TnC. TnI binds to both TnC and TnT and so lies across both of them and the tropomyosin. These bonds between proteins give the complex its full structure (illustrated in figure 3) this is a relatively recent development, although the structures of the individual proteins have been studied extensively [7].

Myosin forms cross bridges with actin in a cyclic manner with the hydrolysis of ATP inducing the conformal change in the myosin molecule. As the myosin molecule is hydrolysed ATP is changed to ADP and a phosphate is released. This creates a weak binding potential between the actin and myosin head. After this binding is achieved a strong binding can then be formed via the removal of a phosphate. In this switch between weak and strong binding states the myosin head changes angle relative to the surface of the actin and this performs the power-stroke. ADP now leaves the head and a new ATP molecule binds with the removal of the head. This cycle is dependent on the myosin being able to find a suitable binding site on the actin filaments [8]. As mentioned above the tropomyosin blocks these binding sites and thus tropolin regulates muscle contraction. In fact tropomyosin instead of strictly blocking the actin sites it lies in such a way as to prevent the myosin transition between weak and strong bindings and so prevents the power-stroke.

When Ca$^{2+}$ is present TnC accepts the ion and it undergoes a bending [10]. This allows the troponin complex to move the tropomyosin strand away from...
the actin binding sites, allowing the strong binding to take place (The inhibition of a strong interaction is a classic sign of a quickly responding process).

The calcium acceptor sites (a COOH and an NH$_2$ group) on the TnC protein take the calcium ions in different ways. When Ca$^{2+}$ is introduced to the system the Mg$^{2+}$ in the C terminal swaps for Ca$^{2+}$ and the empty N terminal is filled by the calcium. This occurs with even micro-molar calcium concentrations. When these substitutions occur the TnC protein undergoes a conformal change and folds. The addition of calcium also changes other bond strengths in the complex so that the whole complex acts together to expose the actin sites from beneath the tropomyosin strand and allow the contraction of the muscle.

3 Structure

As the structure of the entire complex is elaborate, TnC will be focused on as it is the structure that most clearly defines the action of the troponin complex. TnC is an EF hand structure, these hand structures have a helix-loop-helix form and use a COOH group to bind the cations to the oxygen atoms. In analysing the structure crystallography (figure 5) shows two globular regions connected via an α helix this data infers that the protein is a long rigid structure. More recent NMR data refutes this showing a malleable central region that allows the protein to fold in half (figure 6). Troponin C is able to exist in both a long (NMR studies show it is in fact more open than the X-ray structures suggest[11]) and a very compact structure. It is the movement between these two states that creates the conformal change needed to move the tropomyosin.

3.1 TnC interactions with TnI

In the conformal change experienced by TnC the other parts of the complex must also move in order to remove the tropomyosin from the actin binding sites. In the case of TnI it binds to both tropomyosin and TnC, its binding affinities are changed by the addition of Ca$^{2+}$. The interaction between the TnC and TnI molecules are paramount in understanding the function of the troponin complex. When Ca$^{2+}$ is added to the system the two proteins bind very strongly (force rises by ≈ 2 orders of magnitude) this is due to the action of three extra binding sites under these conditions[14]. As the TnC molecule under goes its movement the binding of TnI to actin is also weakened so the movement of tropomyosin is favoured. Thus the conformal change is passed through the complex.
Figure 6: Structure of (Ca\textsuperscript{2+} saturated) Troponin C found via NMR spectroscopy showing two globular regions that are able to move relative to each other due to the partial melting of the central $\alpha$ helix [13]

3.2 TnT and Tropomyosin action in the complex

TnT occurs in the body in various isoforms (128 in the case of rats[15]) each performing the same task and having very similar structural aspects. Troponin T is the primary tropomyosin binding subunit of the complex each isoform has common structural themes, a COOH terminal, a NH\textsubscript{2} terminal and in the case of cardiac troponin a central protein segment. How the C and N terminals are spliced to the structure is the reason for the number of isoforms present. The COOH globular binding region binds with the central part of a tropomyosin strand, also interacting with TnC via TnI and the actin strand[16]. The N terminal of TnT interacts with the C terminal of the tropomyosin strand (this is where the tropomyosin strands join so it is in-fact interacting with two tropomyosin strands). Using electron microscopy it can be seen that TnT has a comma like structure wrapping around part of the TnI. TnT also interacts with tropomyosin via Ca\textsuperscript{2+} dependant bonds, one site can form a triple coiled coil with tropomyosin that also binds to the TnC part of the complex, this is stabilised via hydrophobic interactions. The other is another binding site that binds the tropomyosin close to the other. Both of these bindings are calcium dependant due to the change in electrostatics. TnT’s function in the complex is therefore primarily to fix the troponin complex to the tropomyosin strand but it also provides a greater inhibition of the strong binding of myosin heads to the filament because of its bonding action to other parts of the troponin complex.

4 Structural Analysis Techniques

Protein structure can be found in a variety of ways. Isolating various structural components by deconstructing the protein can tell biologists a certain amount about a proteins structure in relation to its function but to get a better idea of the whole structure of a sample physical methods have to be used. The main three are electron microscopy, X-ray crystallography and Solution NMR [17]

4.1 X-ray Crystallography

Early experiments in determining the structure of TnC used X-ray crystallography. The basic theory of this technique is based upon the diffraction of photons from a crystal lattice acting as a diffraction grating. When the diffracted photons are collected a 3D picture of the electron densities (via amplitude and phase measurement) inside the crystal is built up and from this structure positions of atoms and bonds can be formulated. By changing the angle of incidence of the radiation, different planes of the crystal can be analysed. A total crystal structure inferred is via these different 2D projections. Crystallography is the most common way of imaging proteins but does not allow for movable, active structures. In this case these structures must be flash frozen in order to stabilise their structure into a single conformation and so information about the conformational change is lost. This is true of globular proteins such as TnC[18]. Thus in order to investigate these proteins a new technique must be used that allows work in a solution.
4.2 NMR Spectroscopy

Nuclear Magnetic Resonance (NMR) Spectroscopy is a technique that analysis dynamic structures in solution it is therefore the best method for analysing TnC. This method not only allows the characterisation of a structure but also allows the detection of movement in the proteins. This is in contrast to other methods of detecting motion in proteins as they rely upon a labelling agent and so do not map the motion of the entire structure. NMR uses the magnetic moment of a nucleus to infer the atoms type and what energy state it is in.

4.2.1 Classical description

All nuclei with an even number of nucleons will have no intrinsic spin whereas their isotopes or other elements will have a spin of either an integer or a half integer depending upon their number of protons and neutrons. Although NMR with integer spins is possible (usually restricted to $^2\text{H}$ and $^4\text{N}$[19]) it is more usual to use half-integer spin nuclei (as these have only two energy states). Organic molecules usually contain this type of isotope (i.e. $^1\text{H}$, $^{13}\text{C}$ and $^{31}\text{P}$ among many more) and so NMR is a suitable technique for analysing these biological structures.

Spin is quantised and each spin state is given a spin quantum number $I$. A magnetic dipole is created by the addition of individual spins and orbital angular momentum these are summed to give a value for total angular momentum ($J$). As is known from electromagnetism a circling charge creates a magnetic field along its axis of rotation.

We will be looking at half integer spin nuclei and their $z$ component of spin which is quantised to $\pm I$. thus there are two states for the particle to exist in ($\pm \frac{1}{2}$) with no magnetic field applied these states exist in their ground state at the same energy level. When applying a $B$ field the two energy states split due to the magnetic moments being either parallel ($\pm \frac{1}{2}$ at lower energy state) or anti parallel ($-\frac{1}{2}$ in a higher energy state) when viewed along the $z$ axis defined by the applied field. The gap between these states is proportional to the $B$ field applied this is illustrated in figure 7.

As each energy gap will be different this allows the identification of many different atoms in the substance. The atoms are flipped using a monochromatic radiation source in order to excite the $+\frac{1}{2}$ spin into a $-\frac{1}{2}$ state when the incident radiation is of the right frequency for this gap. High magnetic fields (over 1T) are used in NMR spectroscopy by creating these high fields the energy gap is made large but is still a very small value of energy. The energy gap created here is usually defined in terms of the excitation frequency of radiation. The energy gaps seen in NMR thus are of order 10 to 1000MHz. There are two main ways to perform NMR spectroscopy, by slowly scanning the incident radiation through a range of frequencies and detecting the absorption of the photons via a receiving coil (known as continuous wave spectroscopy). Alternatively a pulsed signal that excites many transitions can be used. Continuous wave spectroscopy is restricted to one dimensional analysis and is relatively insensitive thus statistical analysis must be used to clean the signal, thus each scan has a time associated with it in which the spins are allowed to fall into their original states in order to "reset" the sample for the next run meaning the continuous wave method is time intensive. Due to these problems pulsed NMR is mainly used in investigating protein structures. This classical description of NMR is valid for one dimensional problems but for imaging a 3D structure a full quantum mechanical approach must be taken in order to fully understand the action of the process. This is beyond the scope.

Figure 7: Figure showing the splitting of energy states of $\pm \frac{1}{2}$ spin particles due to the application of a $B$ field (image taken from [20])
of this report, see [21] chapters 4-5 for details. This description does not stress that more than a simple spectrograph can be produced via NMR. Each particle in a structure if the same isotope will not necessarily have the same energy gap due to shielding from other particles. Electronic shielding perturbs the energy state of the particle in a structure and so generally reduces the energy gap by screening the magnetic field at the nucleus. Due to these effects NMR can be used to give information on electronic densities over the structure.

4.2.2 Pulsed NMR

Pulsed NMR consists of a short radio frequency pulse (gaussian distribution) followed by the detection of the emitted signal. The spins are excited as before and these excited states produce an oscillating magnetic field, detectable in the receiving coil [21]. In this case the same coil can be used to receive the signal as excite the particles as the pulse is not active during signal collection. The received current is measured as a function of time and a fast Fourier transform used to construct a frequency spectrum showing peaks at valid energy gaps.

This is best understood by considering the spins in a rotating reference frame (the same frequency as the precession of the spins about the z axis) therefore there appears to be no static magnetic field. Now by applying a $\mathbf{B}$ field along the x axis of the rotating frame, now the applied field is static and during a certain time frame the spins will rotate through an angle to line up with this field [22]. The pulse is now timed to give a 90° tilt to the spins onto the x,y plane. In order to achieve these changes the pulse frequency must be equal to the Larmor frequency of the precession in a single applied field thus the energy gap is found. The application of this pulse aligns precessing spins and so gives a coherent state for all the particles, as the spins precession are now coherent a current can be induced in the receiving coil [23].

4.2.3 Protein Imaging

Protein imaging in practice compromises several steps. Firstly the sample must be prepared, for an accurate structure to be determined the protein must be highly purified so as not to introduce other structures to the analysis. The highly purified protein is often imaged in a buffer solution but the importance of not saturating the $\text{H}_2\text{O}$ is not to be ignored as this can eliminate certain parts of the spectrum[24]. Following preparation resonances must be assigned, this is often achieved using previous NMR data so as to correlate the found energy shifts to the correct structures. Once the shifts have been assigned the experiment can be taken in full, the details of this are shown in figure 8.

This figure illustrates how experiential constraints are used to gradually build up a picture of the molecule and testing against models of the pro-
tein is done to check the results at every level [25]. Most usually used are inter proton distances and bond orientations. Also constraints such as peptide torsional angles from chemical shifts and the orientation of the complete structure in the magnetic field are used.

5 Conclusion

The constituents of the troponin complex have been discussed with reference to their structure and how this effects their function. The TnC protein has been focused upon as it is this protein that induces the conformal change and therefore promotes the function of the complex. The difficulties of using crystallography to analyse this protein were highlighted and a discussion of a more appropriate technique, NMR, was made in reference to the physics of the technique and its application.

References


