Cryo-EM structure of the inhibited (10S) form of Myosin II

1. SPECIFIC AIMS

Inherited hypertrophic cardiomyopathy (HCM) is a genetic cardiovascular disorder characterized by abnormal thickening of the left ventricular walls. Genetic mutations have been identified in the sarcomeric proteins β-cardiac myosin and cardiac myosin binding protein-C *(MyBP-C) [1]* which are responsible for causing the disease [2]. A significant proportion (30-35%) of the mutations are located in the myosin heavy chains. It has been hypothesized that these mutations affect the motor function of cardiac myosin, which is responsible for ventricular contraction [3].

The main goal of this project was to determine the high-resolution structure of myosin II, known to form filaments. The interface between the two heads and between the heads and the tail is known to be the hotspot for HCM mutations, and disruptions to these interfaces have been suggested to lead to HCM. My post-doctoral lab is a pioneer in the myosin field, so they had a sufficient understanding of the molecule. This provides an ideal background and feasibility demonstration for my proposal to conduct similar studies on cardiac muscle myosin.

The specific aim was "**To determine the atomic/near-atomic structure of myosin in its inhibited form by Cryo-EM**"

SIGNIFICANCE AND INNOVATION

Many of the cardiac myosin heavy chain mutations leading to HCM occur in the interfaces between the heads and the tail of the IHM [4, 5]. It has been suggested that these mutations disrupt the interactions in these interfaces, releasing the heads from their relaxed state and leading to hypercontractility and incomplete diastole, which characterize the disease. These interactions are known so far only at 20 Å resolution, based on hybrid structures, so hypotheses invoking the IHM must be speculative. This combined study will provide a structural basis for understanding myosin-based HCM. Neither of these structures has yet been studied by cryo-EM, a revolutionary technique capable of generating atomic and near-atomic resolution [6]. I took advantage of state-of-the-art cryo-EM imaging and image processing techniques to obtain reconstruction of the single molecule as well as filaments. The myosin molecules' highly flexible nature and relatively weak intra-molecular interactions have been roadblocks to studying these molecules in the past. However, I successfully optimized buffer conditions for studying smooth muscle myosin by using brief crosslinking of the molecules and will use this expertise in this proposal. The non-covalent forces that stabilize the IHM state are weaker interactions that can be disrupted easily during EM sample preparation.

2. Research strategy

Background and rationale

Myosin is a hexameric protein composed of two heavy chains, two essential light chains (ELCs) and two regulatory light chains (RLCs) [7]. The heavy chains form two globular regions (heads) in their N-terminal halves and combine to form an α-helical coiled-coiled tail in their C-terminal halves. In muscle, myosin molecules polymerize to form thick filaments, which pull on actin to produce contraction. Electron microscopy (EM) of thick filaments in my sponsor's laboratory showed that in the relaxed state, the myosin heads interact with each other and the first part of the tail, forming a structure known as the interacting heads motif (IHM) [8]. This motif is found throughout animal evolution and is considered fundamental to muscle relaxation [9]. The interactions contribute to the relaxed state of cardiac muscle (diastole) by sequestering heads from actin.

The intramolecular interfaces formed in the IHM have been proposed to be essential for normal cardiac function. They are also hotspots for mutations that cause HCM [4, 5]. It has been suggested that such mutations might lead to the hypercontractility that characterizes HCM by disrupting IHM interactions, thus making more heads available to interact with actin. To properly define these interactions and fully understand how and whether mutations in the IHM interfaces could disrupt them, a high-resolution IHM structure in cardiac muscle is required. In the past, some crystal structures were solved for single myosin heads, but none were found for the two-headed structure that forms the IHM [10]. Our current understanding is therefore based on thick filament studies of invertebrates (cryo-EM [8]) and vertebrates (negative staining EM [11]) and is limited to 20-40 Å resolution, which is much lower than required to resolve atomic detail. Our research group has just reported a breakthrough high-resolution structure of the IHM in smooth muscle myosin by cryo-EM (Nature, 2020, volume 588, pages521–525), - the first study to reveal the entire myosin molecule at near-atomic (4.3 Å) resolution (Fig. 1).

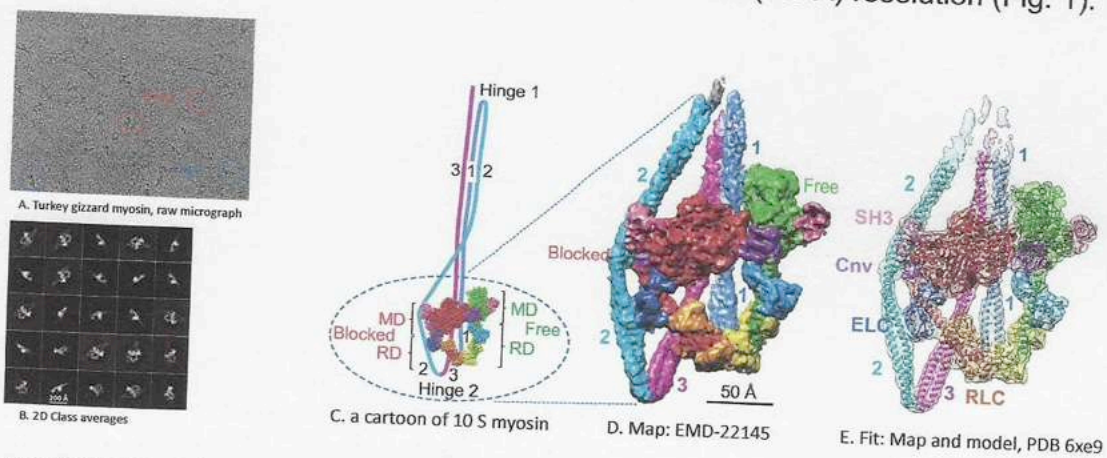

Fig 1. Key findings A. A raw micrograph showing different view of the particles, B. Class averages showing the details at 2D structure of the 10S form, C- E. details of the 3D map and model showing the molecular details and interactions.

The structure reveals many new interactions between the two heads and between the heads and the tail responsible for shutting off activity in the molecule. It also suggests a model for activation and provides a rationale for understanding the distribution of disease mutations in the molecule.

Thus, we now know the crucial interactions responsible for the IHM in smooth muscle myosin. However, *cardiac myosin has a different amino acid sequence, is biochemically different, and the IHM formed is not the same*. I aim to solve the cryo-EM structure of human cardiac myosin, which will fill the gap where no cardiac myosin structure is available until now. In our Nature paper, I performed crucial experiments, including finding perfect conditions to preserve the complex in the shutdown state; I have high confidence that I can solve the structure of the human cardiac myosin. I also carried out all the key structure refinement steps required to validate the PDB structure (PDB 6xe9) we obtained. This places me in an excellent position for a similar analysis of cardiac myosin reconstruction, where my continued training will enable me to build on this initial experience. These studies of single cardiac myosin molecules (**Aim 1**) will provide an understanding of the IHM structure at its simplest, in the absence of interaction with other thick filament components. Knowing the IHM structure in atomic detail will enable us to understand the structural implications of HCM myosin mutations and provide a basis for structure-based drug discovery. Also, it will aid other researchers to explore mutations in the interface and observe the effects. I will then use this information to compare the structural changes occurring due to the mutation (E525K) (**Aim 2**). Most importantly, solving the cardiac IHM structure, with its clear relevance to normal cardiac function and HCM disease, supports a core mission of the AHA in its fight against heart disease.

APPROACH

The goal of this study was to solve the structure of cardiac myosin in IHM conformation at near-atomic resolution and to determine the crucial interactions responsible to stabilize IHM. The strategy includes initial EM observation of the myosin constructs under different buffer and EM grid conditions to establish optimum conditions for stabilizing the IHM conformation. Next, I performed the grid-plunging in liquid ethane to achieve thin ice and a uniform particle distribution on the grid with different blot forces and times to obtain good ice. Specimens prepared under optimal conditions were then be imaged by cryo-EM. The images were processed using programs like Relion or CryoSparc, which resulted in a high-resolution map. Then the map density was used to create a molecular model, and the resulting model will be deposited in the Protein Data Bank (PDB). The structure was analyzed thoroughly, revealing the residues and the interactions involved to stabilize the IHM conformation. The residues involved in the disease were identified, and the information shall be used for other research groups and structure-based drug design to find potential drugs.

FUTURE ASPECTS

The atomic structure of the myosin was examined to locate the HCM genetic mutations [5] and their interactions with other residues in the IHM structure. This will provide insights into the likely impact of the mutations on IHM stability. For example, residues involved in charge attraction ionic interactions, whose charge is either neutralized or reversed, will be expected to weaken or disrupt the IHM. The mutation studies can be performed

whereby by mutating the interacting amino acid residues, we can determine the most fatal mutation and the kind of structural changes caused in the HCM.

KEY OUTCOMES

This near-atomic structure of myosin in its shut-down state was the first structure to reveal the interaction between the head and the tail, which is commonly known as the interacting head motif (IHM). Several key interactions were reported for the first time.
The most important insight was to report the activation mechanism of myosin II. Myosin changes its conformation from inactive to active to balance the load of actin interaction during muscle contraction.
Depending on the muscle load, how many heads will remain in an active/inactive state will be decided. We proposed a mechanism of myosin activation by regulatory light chain (RLC) phosphorylation by myosin light chain kinase (MLCK).
The cardiomyopathy mutation hotspots (myosin mesa) were seen in our data. Hence, it became useful as a reference for finding HCM drugs.

REFERENCES

1. Maron, B.J. (2002). Hypertrophic cardiomyopathy: a systematic review. JAMA *287*, 1308-1320.
2. Ho, C.Y., Charron, P., Richard, P., Girolami, F., Van Spaendonck-Zwarts, K.Y., and Pinto, Y. (2015). Genetic advances in sarcomeric cardiomyopathies: state of the art. Cardiovasc. Res *105*, 397-408.
3. Spudich, J.A. (2014). Hypertrophic and dilated cardiomyopathy: four decades of basic research on muscle lead to potential therapeutic approaches to these devastating genetic diseases. Biophys. J *106*, 1236-1249.
4. Nag, S., Trivedi, D.V., Sarkar, S.S., Adhikari, A.S., Sunitha, M.S., Sutton, S., Ruppel, K.M., and Spudich, J.A. (2017). The myosin mesa and the basis of hypercontractility caused by hypertrophic cardiomyopathy mutations. Nat Struct Mol Biol *24*, 525-533.
5. Alamo, L., Ware, J.S., Pinto, A., Gillilan, R.E., Seidman, J.G., Seidman, C.E., and Padron, R. (2017). Effects of myosin variants on interacting-heads motif explain distinct hypertrophic and dilated cardiomyopathy phenotypes. Elife *6*.
6. Egelman, E.H. (2016). The Current Revolution in Cryo-EM. Biophys J *110*, 1008-1012.
7. Geeves, M.A., and Holmes, K.C. (1999). Structural mechanism of muscle contraction. Annu. Rev. Biochem *68*, 687-728.
8. Woodhead, J.L., Zhao, F.Q., Craig, R., Egelman, E.H., Alamo, L., and Padron, R. (2005). Atomic model of a myosin filament in the relaxed state. Nature *436*, 1195-1199.
9. Lee, K.H., Sulbaran, G., Yang, S., Mun, J.Y., Alamo, L., Pinto, A., Sato, O., Ikebe, M., Liu, X., Korn, E.D., et al. (2018). Interacting-heads motif has been conserved as a mechanism of myosin II inhibition since before the origin of animals. Proc Natl Acad Sci U S A *115*, E1991-E2000.

10. Dominguez, R., Freyzon, Y., Trybus, K.M., and Cohen, C. (1998). Crystal structure of a vertebrate smooth muscle myosin motor domain and its complex with the essential light chain: visualization of the pre- power stroke state. Cell *94*, 559-571.
11. Zoghbi, M.E., Woodhead, J.L., Moss, R.L., and Craig, R. (2008). Three-dimensional structure of vertebrate cardiac muscle myosin filaments. Proc Natl Acad Sci U S A *105*, 2386-2390.
12. Hooijman, P., Stewart, M.A., and Cooke, R. (2011). A new state of cardiac myosin with very slow ATP turnover: a potential cardioprotective mechanism in the heart. Biophys. J *100*, 1969-1976.
13. Yang, S., Lee, K.H., Woodhead, J.L., Sato, O., Ikebe, M., and Craig, R. (2019). The central role of the tail in switching off 10S myosin II activity. J Gen Physiol *151*, 1081-1093.
14. Zivanov, J., Nakane, T., Forsberg, B.O., Kimanius, D., Hagen, W.J., Lindahl, E., and Scheres, S.H. (2018). New tools for automated high-resolution cryo-EM structure determination in RELION-3. Elife *7*.
15. Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E. (2004). UCSF Chimera--a visualization system for exploratory research and analysis. J. Comput. Chem *25*, 1605-1612.
16. Afonine, P.V., Poon, B.K., Read, R.J., Sobolev, O.V., Terwilliger, T.C., Urzhumtsev, A., and Adams, P.D. (2018). Real-space refinement in PHENIX for cryo-EM and crystallography. Acta Crystallogr D Struct Biol *74*, 531-544.
17. Emsley, P., Lohkamp, B., Scott, W.G., and Cowtan, K. (2010). Features and development of Coot. Acta Crystallogr D Biol Crystallogr *66*, 486-501.