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Structural dynamics of muscle protein phosphorylation

Brett A. Colson, Simon J. Gruber, and David D. Thomas*

University of Minnesota, Biochemistry, Molecular Biology and Biophysics, Minneapolis, MN 55455

Abstract

We have used site-directed spectroscopic probes to detect structural changes, motions, and interactions due to phosphorylation of proteins involved in the regulation of muscle contraction and relaxation. Protein crystal structures provide static snapshots that provide clues to the conformations that are sampled dynamically by proteins in the cellular environment. Our site-directed spectroscopic experiments, combined with computational simulations, extend these studies into functional assemblies in solution, and reveal details of protein regions that are too dynamic or disordered for crystallographic approaches. Here, we discuss phosphorylation-mediated structural transitions in the smooth muscle myosin regulatory light chain (RLC), the striated muscle accessory protein myosin binding protein-C (MyBP-C), and the cardiac membrane Ca^{2+} pump modulator phospholamban (PLB). In each of these systems, phosphorylation near the N terminus of the regulatory protein relieves an inhibitory interaction between the phosphoprotein and its regulatory target. Several additional unifying themes emerge from our studies: (a) The effect of phosphorylation is not to change the affinity of the phosphoprotein for its regulated binding partner, but to change the structure of the bound complex without dissociation. (b) Phosphorylation induces transitions between order and dynamic disorder. (c) Structural states are only loosely coupled to phosphorylation; i.e., complete phosphorylation induces dramatic functional effects with only a partial shift in the equilibrium between ordered and disordered structural states. These studies, which offer atomic-resolution insight into the structural and functional dynamics of these phosphoproteins, were inspired in part by the ground-breaking work in this field by Michael and Kate Barany.

Keywords

Muscle Regulation; Spectroscopy; Structural Dynamics; Phospholamban; Regulatory Light Chain; Myosin Binding Protein-C

A. REGULATION OF MUSCLE ATPASE ACTIVITY BY PHOSPHO-PROTEINS

Michael Barany's pioneering work in cardiac, skeletal, and smooth muscle types laid a foundation for many aspects of today's structural and functional studies of muscle and cardiovascular physiology. In his spirit, we extend the scope of his (and Kate Barany's) 1981 invited review of "Protein Phosphorylation in Cardiac and Vascular Smooth Muscle" in *Am J Physiol.* (Barany and Barany 1981) by providing new insights into the phosphorylation-mediated structural transitions that occur in muscle phosphoproteins. We highlight several of the proteins cited in that review and describe recent studies using site-directed spectroscopic probes to understand the structures and motions of phosphoproteins that drive the regulation of contraction and relaxation. We focus on the regulation of smooth muscle contraction by phosphorylation of the myosin regulatory light chain (RLC), and

*Corresponding Author: ddt@umn.edu, 612-625-0957.

regulation of cardiac muscle contraction and relaxation by phosphorylation of phospholamban (PLB), troponin I (TnI), and myosin binding protein-C (MyBP-C).

“Early in the history of ATP, it was recognized that its terminal phosphate group participates in transfer reactions.” –M. Barany, 1981 (Barany and Barany 1981)

Phosphorylation of enzymes and regulatory proteins has been a major research focus in elucidating mechanisms that regulate cellular processes. As postulated by Barany nearly forty years ago (Barany et al. 1975), structural changes in muscle protein domains and subdomains are the driving forces behind myosin’s ATP-dependent production of force and SERCA’s ATP-dependent pumping of Ca^{2+} , which in turn regulates myosin. Polymerization of actin from its globular to filamentous form catalyzes the conversion of ATP to ADP, coupled to a structural transition in actin (Barany et al. 1956). These proteins progress through an ensemble of structural states, amplifying localized structural changes to movements of entire domains. While our understanding of myosin and SERCA function and their ATPase mechanisms has advanced greatly, much less is known about how catalytic domains are coupled allosterically to regulatory proteins and to the functional assemblies that participate in contraction and relaxation.

1. Biochemistry of Serine Phosphorylation

In the systems considered in this article, each regulatory protein exerts inhibitory influence on a muscle ATPase system. This inhibition is relieved by phosphorylation of one or more specific serine residues, an event catalyzed by a protein kinase under adrenergic control. ATP participates in the covalent transfer reaction by donating its phosphate to the serine hydroxyl groups, and the protein’s newly acquired phosphomonoester bond remains stable until the reaction is reversed by a phosphoprotein phosphatase. The phosphorylation of serine adds a bulky negative charge, which can affect the protein’s structure and interactions both due to direct electrostatic effects and to more complex perturbations of structure and stability (Hamelberg et al. 2005; Groban et al. 2006; Espinoza-Fonseca et al. 2007, 2008).

2. Kinase-dependent Physiological Responses of Muscle

“In the contractile apparatus, the phosphorylation of myosin light chain seems to be an integral part of the contraction cycle of arterial smooth muscle. However, in the heart only small changes in the phosphate content of light chain were shown between diastolic and systolic states.” – M. Barany, 1981 (Barany and Barany 1981)

In smooth muscle, the primary physiological interest for kinase activity is in the Ca^{2+} -calmodulin-dependent myosin light chain kinase (MLCK), because it catalyzes RLC phosphorylation to activate myosin (Walsh et al. 1982). Before this was known, Michael and Kate Barany proposed that activation of smooth muscle required phosphorylation of myosin regulatory light chain (RLC). Using ^{32}P -labelled intact carotid arteries, they showed that contractions caused by norepinephrine and K^{+} were correlated with an increase in RLC phosphorylation (Barron et al. 1979), and RLC phosphorylation is reversed upon relaxation (Barron et al. 1980). Today, further insight into the role of MLCK in different muscle tissues has been provided by the study of animal models with tissue-specific ablation of MLCK (He et al.; Stull et al.; Ding et al.; Zhang et al.).

Phosphorylation of RLC plays a modulatory role in striated muscle, because regulation of striated muscle contraction occurs on the thin filament by Ca^{2+} binding to troponin C (TnC). In the heart, RLC phosphorylation is relatively constant between diastole and systole. However, on nearly a beat-to-beat basis, much greater variation can occur in cardiac phosphoproteins with cAMP-dependent protein kinase A (PKA) phosphorylation. β -adrenergic stimulus in response to changing circulatory demands, such as the fight-or-flight response, leads to widespread PKA-catalyzed phosphorylation of L-type Ca^{2+} channels in

the membrane, phospholamban (PLB) in the sarcoplasmic reticulum (SR), troponin I (cTnI) on the thin filament, and myosin binding protein-C (cMyBP-C) and titin on the thick filament.

This review summarizes our current knowledge about these phosphoproteins and their possible role in muscle function. More specifically, we will address how spectroscopic studies of muscle protein structural dynamics underlie the mechanisms by which phosphoproteins influence the contractile apparatus.

B. SITE-DIRECTED SPECTROSCOPIC STUDIES OF MUSCLE CONTRACTION AND RELAXATION

1. Myosin Regulatory Light Chain Phosphorylation Controls Smooth Muscle Contraction

“These data support the idea that activation and subsequent contraction of muscle are correlated with structural changes in the myosin molecule.” -M. Barany (Barany et al. 1975)

Smooth muscle contraction is regulated by phosphorylation of myosin RLC at Ser 19. EM data suggests that when RLC is unphosphorylated, the two-headed myosin adopts an asymmetric auto-inhibited conformation in which one head (free to bind actin) interferes with the other (blocked) head by sterically hindering its access to actin through head-head interactions near the free head's converter/light chain domain (Sellers and Knight 2007). Upon MLCK-catalyzed phosphorylation of RLC, inhibition of the free head by the blocked head is relieved and myosin is free to interact with actin and generate force.

The mechanism by which RLC phosphorylation activates myosin allosterically is unknown, in large part because Ser 19 is not included in any crystal structure of the RLC (Wendt et al. 2001) or resolved in EM studies of thick filaments (Alamo et al. 2008). In order to determine the structure of the RLC PD, site-directed spectroscopy (electron paramagnetic resonance, EPR; and fluorescence resonance energy transfer, FRET), along with complementary molecular dynamics (MD) simulations, has been used to measure distances, domain helical order, and dynamic protein disorder within the RLC.

Site-directed spectroscopy and MD simulations allow for a cycling feedback scheme of information wherein MD simulations can refine molecular models and make predictions to be tested experimentally. Both approaches are capable of having controlled physiological conditions, measuring distances with atomic resolution, and resolving multiple structural states. While each approach has its limitations, employing both approaches to decipher molecular mechanisms is exceptionally powerful: (a) Site-directed spectroscopy offers ensemble information for protein complexes but is limited in each experiment by the specific sites probed. (b) MD simulations supplement, verify, and enhance spectroscopic studies in providing single-molecule information and generating a detailed all-atom model.

b. Site-directed studies of RLC phosphorylation domain (PD)—The phosphorylation domain (PD) at the N terminus of the RLC was first defined by Nelson et al., (PNAS 2005)(Nelson et al. 2005) who made single-Cys mutants of chicken gizzard RLC throughout the PD (residues 1–24). Mutant RLC was spin labeled and biochemically exchanged into chicken gizzard heavy meromyosin (HMM, the fully functional two-headed soluble construct of myosin), and EPR was performed to measure mobility and solvent accessibility of the RLC PD. Upon phosphorylation of Ser 19, it was determined that the PD undergoes a dynamic disorder-to-order transition, increasing helical order while also increasing dynamics and solvent accessibility. Following the transition to this

phosphorylated PD structure, the two heads of smooth myosin move apart and become active for interaction with actin.

Does this same dynamic disorder-to-order transition occur in MD simulations of the PD? Espinoza-Fonseca et al., (Espinoza-Fonseca et al. 2007, 2008) concluded that the PD is also more helical when phosphorylated compared to the unphosphorylated helix in silico when 50 ns simulations were run. Thus, MD simulations confirm the disorder-to-order transition induced by phosphorylation and provide further details with atomic resolution. MD simulation predicts that phosphorylation produces a salt bridge that stabilizes the PD helix (Fig. 2A). The distance between the phospho-Ser 19 oxygen and the amino protons on its predicted salt bridge partner Arg 16 changes with phosphorylation. In the unphosphorylated PD, this distance is variable and ranges between 0.6 and 1.6 nm; the phosphorylated PD can also attain these variable distances, but favors a much shorter and very stable conformation at a distance of 0.3 nm, in agreement with a salt bridge between neighboring helical turns. Thus, the disorder-to-order transition in the RLC PD which drives contraction is driven by formation of this salt bridge between R16 and pS19. This conclusion from EPR spectroscopy and MD simulation was then tested, refined, and essentially visualized by using time-resolved FRET (TR-FRET).

c. Open and Closed State Structural Transitions of the RLC Phosphorylation Domain Trigger Smooth Muscle Myosin Activation—

Just as pioneering as the studies of RLC PD structural dynamics described above (Nelson et al. 2005, Espinoza-Fonseca, 2007 #64; Espinoza-Fonseca et al. 2008), Kast et al., (Kast et al. 2010) again used the powerful combination of site-directed spectroscopy and MD simulations, but this time with the use of time-resolved (TR) FRET assay. Nanosecond time resolution, following a laser pulse, provides much greater information regarding structure and dynamics than can be provided by steady-state FRET. TR-FRET can resolve multiple structural states, essentially as in single-molecule FRET, and these structural populations can be quantitated to provide direct insight into thermodynamics. Each structural state is defined by a Gaussian distribution of distances between the two probes, which indicates both the predominant structure and its internal disorder (Kast et al. 2010). TR-FRET decays of the RLC are best analyzed as two structural states (Kast et al. 2010).

In the mutant RLCs used in this study, one Cys probe was placed at position 129 in the C-lobe of the RLC and the other probe near the N terminus of the PD at position 2, in order to measure distances and dynamic disorder between the PD and RLC core (which was also verified in separate RLC mutants with probes at PD residues K3 and A7).

Following fluorescent donor-acceptor pair labeling, the RLCs were exchanged into smooth muscle myosin S1 isolated from chicken gizzard, and then TR-FRET was performed. Upon phosphorylation, the majority population of RLC structural states switches by a population shift from closed-to-open favored RLC structural states (Fig. 2B). This shift in molar ratio of structural states activates myosin's distant catalytic domain in a manner not yet understood.

It was demonstrated that the RLC resides in two structural states (open with a long distance and closed with a short distance) and that phosphorylation modulates the closed-to-open transition the RLC. This was confirmed by MD simulation, which provided further atomic structural detail. These studies showed that in all three FRET probe pairs (i.e., M129C paired with either S2C, K3C, or A7C): (a) Two populations (structural states) existed with distances of about 3 nm for the closed state distance and 5 nm for the open state. (b) Both of these open and closed state distances occur in unphosphorylated and phosphorylated RLC. (c) Phosphorylation increases the population of the open state. Thus, phosphorylation shifts the equilibrium toward the open structural state by 20–25 mol %, and this was confirmed by

MD simulations. Together, these studies demonstrate that chemical and structural states are loosely coupled. It is rather striking that complete phosphorylation causes a structural shift in just a small population of RLC PD's, but this is enough to completely activate smooth muscle.

d. Salt Bridges Critically Stabilize RLC Structural States—MD simulations also provided predictions of a structural model that remains to be tested by ongoing and future studies of RLC dynamics (Kast et al. 2010). MD simulations predicted that, after phosphorylation, an *intradomain* salt bridge (within the PD) stabilizes the open state between R16 and pS19. Thus, removal of charge from one of these residues, for example in an *Ala-for-Arg* mutation to R16A, should disrupt the open state salt bridge. Favored in the unphosphorylated RLC, MD simulations predict that an *interdomain* salt bridge stabilizes the closed state between D100 of the C-lobe and R4 of the PD. Thus removal of charge from one of these residues, for example, by an *Ala-for-Arg* mutation to R4A should disrupt the closed-state salt bridge. We predict that the closed-to-open transition is driven by an intradomain salt bridge between R16 and pS19 and that the open-to-closed transition is driven by an interdomain salt bridge between basic residues of the C-lobe (E99, D100) and acidic residues of the PD (K3, R4, K6, and K8). Another direction of future studies is to determine these same distances and molar fractions of structural states of unphosphorylated and phosphorylated RLC exchanged into two-headed HMM. We expect similar structural states and relationships as with single-headed S1, but perhaps due to steric constraints of the head-head interactions of the auto-inhibited HMM (Wendt et al. 2001; Nelson et al. 2005) or interactions between neighboring RLCs (Wahlstrom et al. 2003), slightly shorter distances may result due to confinement in the unphosphorylated RLC. Unphosphorylated myosin is predicted to be compact, with its two heads asymmetric with neighboring RLCs in very close proximity (<4 nm) (Wendt et al. 2001; Li and Ikebe 2003; Alamo et al. 2008). The close proximity of heads is further supported by EPR studies that show the unphosphorylated PD has low solvent exposure (Nelson et al. 2005), and photo-crosslinking studies that show the PD of one RLC can crosslink of the C-lobe of the other RLC (Wu et al. 1999).

e. Deciphering Interdomain Structural Connections in Allostery of Myosin Activation—Another remaining question is to determine the mechanism by which RLC phosphorylation at pSer19 triggers separation of the myosin heads for ATPase activation. Future efforts using TR-FRET, EPR, and MD simulations will undoubtedly provide the framework to map this allosteric transmission pathway from the RLC to ELC to catalytic domain to actin. Furthermore, interactions between neighboring RLCs, between RLC and ELC, as well as RLC-S2 interactions may all be important to this mechanism. Less is known about the structure of phosphorylated myosin, but EM structures were interpreted to suggest an open state. However, the high solvent accessibility of the PD, and the inability for this domain to crosslink with the partner RLC, indicate a disordered myosin where the two heads are no longer interacting. Since the RLC reflects structural differences between the biochemical states, RLC-RLC interactions, and particularly the closed and open structural states within the RLC may be critical to defining myosin structure. Recent work suggests specific interactions between the interacting surfaces of the RLC and ELC, and appears to be important for proper activation (Ni et al. 2012). Of course the presence of nucleotide and regulated actin will ultimately provide the most physiological visualization of the inherent mechanism of smooth muscle myosin activation at the atomic level.

2. Phospholamban (PLB) Phosphorylation Controls Ca²⁺ Pump Reuptake to Cardiac SR

“The potential role of phospholamban and Ca²⁺ pump ATPase system appears to be remarkable.” – M. Barany, 1981 (Barany and Barany 1981)

PLB is a single-pass transmembrane protein expressed in cardiac muscle that regulates SERCA by reducing its apparent calcium affinity ($1/K_{Ca}$) without inhibiting its maximal rate at high $[Ca^{2+}]$. PLB has three phosphorylation sites at S10, S16, and T17 that are phosphorylated by PKC, PKA, and CamKII, respectively (Kranias and Hajjar 2012). Phosphorylation at either S16 or T17 reduces PLB inhibitory potency; the PKA site is thought to be more important physiologically because it is more clearly under adrenergic control (Calaghan et al. 1998).

a. PKA-mediated Ca^{2+} Regulation in the Cardiac SR Membrane—At the level of the sarcoplasmic reticulum (SR), myocardial contractility is primarily regulated by cAMP-dependent activation of Ca^{2+} transport back into the SR by SERCA. Phosphorylated PLB remains bound to SERCA but does not lower the enzyme's Ca-affinity, leading to increased Ca^{2+} load in the SR, improved relaxation during diastole, and greater contractility in systole. The PLB:SERCA ratio in heart has been reported in the literature for several animals and varies from 1–5 (Calaghan et al. 1998; Ferrington et al. 2002). Recent efforts to quantitatively measure S16 phosphorylation in pig cardiac muscle showed that while the PLB:SERCA ratio is variable, the ratio of unphosphorylated PLB to SERCA is unchanged between animals (~2.3:1), indicating that S16 phosphorylation is tightly controlled to tune cardiac function (Ablorh et al. 2012).

Both primary phosphorylation sites, S16 and T17, are at the end of the cytoplasmic helix next to a loop connecting it to the transmembrane helix (Fig. 3A). The loop is dynamic and PLB exists in at least two structural states, tense (*T*) state in which the cytoplasmic helix is highly ordered and interacts with the membrane surface, and a relaxed (*R*) state in which the cytoplasmic helix becomes disordered and extends away from the membrane surface (Karim et al. 2004). PLB phosphorylation shifts the equilibrium of these two states in favor of the more disordered *R* state (Fig. 3A). Thus phosphorylation induces an order-to-disorder transition in the cytoplasmic domain of PLB. At first glance, this appears opposite to the RLC, in which a disorder-to-order transition is induced (Fig. 2), but the two mechanisms share a common feature – MD simulations show that the phosphorylated state is stabilized by an intramolecular salt bridge that includes the phosphorylated serine (Paterlini and Thomas 2005). The difference is that this salt bridge destabilizes the helix in PLB (Fig. 3A), while it stabilizes the helix in RLC (Fig. 2). The importance of tight control of SERCA phosphorylation in the heart is evidenced by a cardiomyopathic mutation in PLB, an arginine-to-cysteine substitution at position 9, that is pathogenic probably because it cannot be phosphorylated by PKA (Ha et al. 2011).

b. PLB Dynamics Allosterically Regulates SERCA2a Activation—The mechanism by which PLB regulates SERCA has been the subject of much debate for several decades. Some studies by cross-linking have been interpreted to suggest that relief of SERCA inhibition, by either high $[Ca^{2+}]$ or PLB phosphorylation, requires dissociation of PLB from SERCA (Chen et al. 2012; Akin and Jones 2012). However, several lines of evidence, including FRET and co-immunoprecipitation, show clearly that PLB remains bound to SERCA even when phosphorylated by PKA (Asahi et al. 2000; Li et al. 2004a) or when $[Ca^{2+}]$ is micromolar (Mueller et al. 2004). Thus taken together, all of the existing evidence is consistent with an allosteric regulation model, in which phosphorylated PLB binds to SERCA in a distinct, non-inhibitory mode. This model is supported by recent work from in which charged lipids were used to attract or repel the cytoplasmic domain of PLB from the membrane, thus stabilizing the *T* or *R* state, respectively (Li et al. 2012). As predicted, positively charged lipids were found to repel the positively charged cytoplasmic domain, promoting the non-inhibitory *R* state of PLB and activating SERCA at physiological $[Ca^{2+}]$, while negatively charged lipids had the opposite effects. Studies in 2D crystals are consistent with this order-to-disorder transition upon PLB phosphorylation, but this change

was not seen in a mutant that remains inhibitory even when phosphorylated, illustrating the coupling of function and structural dynamics (Glaves et al. 2011).

c. Phosphomimetic Therapy Approach in Live Cells—Reduced muscle and membrane protein phosphorylation is typically associated with disease and aging in human tissue, and this is the case with PLB phosphorylation and heart failure. SERCA activity has been found to decline in heart failure, and numerous efforts are underway to develop therapies based on SERCA activation (Kranias and Hajjar 2012). Several of these therapeutic approaches focused on relieving the inhibition of SERCA by PLB, such as small molecules that disrupt the SERCA-PLB interaction, introduction of pseudophosphorylated or phosphomimetic PLB via gene therapy, and inhibition of protein phosphatase I, the enzyme that dephosphorylates PLB at S16 (Kranias and Hajjar 2012). These efforts reflect the centrality of PLB as a regulator of SERCA and cardiac function.

We are currently pursuing spectroscopy-based approaches to develop treatments for heart failure that capitalize on our understanding of the SERCA-PLB regulatory mechanism. We have measured FRET between SERCA and PLB both in reconstituted membranes (Mueller et al. 2004) and in living cells (Robia et al. 2007) and use these assays to resolve changes in binding and structure (Fig. 3B&C). The FRET assays have been used successfully to screen for small molecules (potential drugs) that disrupt PLB binding in its inhibitory mode, activating SERCA allosterically either by stabilizing the R state or by completely dissociating the two proteins (Cornea et al. 2012) (Fig. 3C). Previous work showed that PLB dynamics, and therefore inhibitory potency, can be made to mimic phosphorylation by making mutations in the PLB cytoplasmic helix that destabilize the loop and promote the R state (Li et al. 2005; Ha et al. 2007). We propose to introduce one or more of these PLB mutants (PLB_M) via gene therapy to activate SERCA. These mutants must be less inhibitory than wild-type PLB but must also compete effectively with it if they are to activate SERCA in native tissue. In our first study, we used purified proteins labeled with fluorescent dyes in reconstituted membranes, to show by FRET that several PLB_M meet these criteria (Lockamy et al. 2011). We then used fluorescent fusion proteins to measure FRET in living HEK cells to quantify the interaction between CFP-SERCA and YFP-PLB (Fig. 3C), and introduced PLB_M into stable cell lines coexpressing the two fusion proteins to quantify competition (Gruber et al. 2012). We found several PLB_M, some of which are virtually non-inhibitory, that still retain the ability to bind SERCA in the native membrane and even displace wild-type PLB. The most potent PLB_M are being tested using adeno-associated viral vectors in animal models of heart failure, to develop gene therapy.

3. PKA-mediated Phosphorylation of Myofilament Proteins in Striated Muscle

“...the two proteins are phosphorylated in the heart by the same kinase. Indeed, phosphorylation of [cTnI] and [cMyBP-C] occurred in parallel in the epinephrine-treated heart.” – M. Barany, 1981 (Barany and Barany 1981)

a. Structural studies of PKA Phosphorylation of Muscle Proteins in Cardiac Relaxation—On the thin filament, PKA phosphorylation of Ser 23 and 24 of cTnI's N terminal extension becomes more compact and leads to a global change in the cTnI structure that alters the regulatory site of cTnC (Chandra et al. 1997). Thus transmission of the phosphorylation effect on cTnI to cTnC contributes to the reduced myofilament Ca²⁺ sensitivity and contributes to an earlier relaxation when Ca²⁺ dissociates from cTnC earlier than when unphosphorylated (Zhang et al. 1995; Tobacman 1996). In addition to phosphorylation, more recent studies have also characterized functional and structural changes associated with cardiotonic drugs, mutation, changes with pH, and point mutations (Li et al. 2008; Li et al. 2004b; Day et al. 2007).

On the thick filament, phosphorylation of cMyBP-C reduces its binding affinity for myosin S2 in solution (Gruen et al. 1999) and in muscle fibers (Kunst et al. 2000) and may (Shaffer et al. 2009) or may not (Rybakova et al.) alter its affinity for actin in solution. In any case, phosphorylation of cMyBP-C (Weisberg and Winegrad 1996; Colson et al. 2008) in cardiac muscle fibers reduces its structural constraint on myosin, allowing cross-bridges to be in closer proximity to actin, and thereby speeds contraction and relaxation kinetics through cooperative means. This mechanism may be similar to that of RLC phosphorylation in regulation of cross-bridge structure (Levine et al. 1995; Levine et al. 1996).

It remains unknown how the structure of the inherently disordered phosphorylation motif (Howarth et al.) changes MyBP-C structure in the neighboring domains and, consequently, its interactions with myosin and/or actin. Site-directed probes within and between cMyBP-C and its binding partners are needed to elucidate structural mechanisms by which cMyBP-C modulates contractility. Distance measurements and structural dynamics of the interacting proteins determined by spectroscopic approaches are currently being investigated for cMyBP-C's interaction with myosin S2 and actin. Future optical and EPR spectroscopy studies will investigate effects of mutations in cMyBP-C, which are the most common cause of familial hypertrophic cardiomyopathy (HCM).

b. Cardiac MyBP-C Spectroscopy—The same optical probe methodologies used to determine actin twisting motions in the microsecond timescale, that are imperative for: myosin contraction (Prochniewicz et al. 2004), cytoskeletal integrity (Prochniewicz et al. 2009; Lin et al. 2011), and cooperative properties of actin-binding proteins (Prochniewicz et al. 2005; Lin et al. 2012), have been employed to study the interaction of MyBP-C with actin. Time-resolved phosphorescence anisotropy (TPA) measures the amplitude and rate of actin filament twisting through uniform labeling with erythrosine iodoacetamide (ErIA) attached to actin at C374 (Fig. 4A, B). Here, MyBP-C restricts the amplitude of actin twisting by 20–25° when unphosphorylated. Upon treatment with PKA, the phosphorylated cMyBP-C remains bound to actin (Rybakova et al.), but no longer restricts actin amplitude, and instead, slows the *rate* of twisting (Fig. 4C). Thus, it appears that PKA phosphorylation switches the manner in which cMyBP-C interacts in its complex with actin from a *restrictive* to a *permissive* interaction with respect to actin dynamics.

While these effects on actin dynamics could play a role in the known functional role of cMyBP-C on the actin-myosin interaction, further TPA studies of cMyBP-C domain fragments with labeled F-actin show that there is much more to this story. The C terminal half of MyBP-C, which is presumably anchored to the thick filament backbone, was most effective to recapitulate the restrictive effects on actin twisting amplitude of full-length cMyBP-C, while effects of N-terminal domains were weak or undetectable. Weak interactions with actin could be useful to allow for myosin cross-bridge binding. Interestingly, phosphorylation of the fragments also suggested that the motif could transmit a structural change in the complex toward the C terminus of cMyBP-C. Thus, phosphorylation at the motif could transmit structural changes along its domain organization toward myosin and/or actin (Fig. 4 D). Future studies using dipolar electron-electron resonance (DEER) spectroscopy (Lin et al. 2011) with spin labels on cMyBP-C itself will resolve how the motif and linker structures and dynamic disorder change with myosin S2 or actin binding and phosphorylation. TR-FRET studies will provide important distance measurements between binding partners.

Similarly, PLB's complex with the Ca²⁺-ATPase pump SERCA is also mediated by PKA phosphorylation and does not involve complex dissociation, but just a subtle structural change, to enhance pump function and relaxation in myocardial inotropy and lusitropy, as discussed above. Along these lines, the complex of cMyBP-C with its myofilament binding

partner(s) (myosin and/or actin) may also involve subtle conformational changes upon PKA phosphorylation that underlie enhanced speeds of contraction and relaxation, without complete dissociation of the interacting proteins. Interestingly, cMyBP-C and RLC phosphorylation induce similar changes in myofilament structure (Colson et al. 2010) and may even interact with each other (Ratti et al.). Thus, in addition to S2 and actin, studies using probes on the RLC, especially in muscle fibers, may also shed light on the mechanism by which cMyBP-C influences contraction. Similar to PLB and cTnI, control of cMyBP-C phosphorylation status and structure may be a useful target of therapy for heart failure (El-Armouche et al. 2007).

C. CONCLUSION- STRUCTURAL DYNAMICS OF PHOSPHORYLATION-MEDIATED REGULATION OF MUSCLE ATPASES

“The in vivo phosphorylation of several proteins in cardiovascular muscles is well established. These phosphoproteins are localized in each of the major fractions of the muscle cell: myofibrils, sarcoplasmic reticulum, sarcolemma, mitochondria, and cytoplasm.” – M. Barany, 1981 (Barany and Barany 1981)

Much of our current understanding with regard to the phosphorylation-mediated regulation and modulation of muscle and membrane ATPases stems from the groundbreaking studies by Michael Barany. From his discovery in the 1950's that actin itself, and not an external enzyme, catalyzed the ATP to ADP transformation of the protein (Barany et al. 1956), to his discovery in the 1960's of the relationship between speed of muscle contraction and the actin-activated ATPase activity of the myosin molecules in fast and slow twitch muscle (Barany 1967); or from his ideas in the 1970's that conformational changes in myosin provide the driving force for muscle contraction (Barany et al. 1975) to demonstrating in the 1980's that myosin light chain phosphorylation is a prerequisite for smooth muscle contraction (Barron et al. 1979; Barron et al. 1980), Michael's pioneering work is in many ways influencing the direction and scope of today's biophysicists in muscle and cardiovascular research, including NMR studies of living muscle. This is most certainly the case for the current and future spectroscopic studies of muscle and membrane proteins to detect the microsecond timescale motions and transitions in protein conformations that drive muscle contraction and relaxation. Moving forward from static crystal structures toward an understanding of the dynamic nature of proteins along the course of their regulatory action is defined by a loose coupling between the biochemical states of protein phosphorylation and the structural states of protein conformation. Such mechanistic action likely involves transitions to new minima of energy landscapes, mediated by transitions in dynamic disorder and order of muscle phosphoproteins. In RLC, PLB, cMyBP-C, and cTnI, phosphorylation-mediated shifts in dynamic protein disorder is localized near the N terminus and leads to global changes in the protein and its assemblies to regulate and modulation muscle contraction and relaxation.

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Fig. 1. Phosphorylation of phosphoproteins, and structural changes detected in with site-directed spectroscopic probes, that underlie changes in muscle function. Green: kinase activation. “+P” indicates phosphorylation. Blue: effect of phosphorylation on the shift in equilibrium between two observed structural states in the phosphoprotein. Pink: details of those structural transitions. Orange: effect on muscle function.

A Secondary Structural States

B Tertiary Structural States

Fig. 2.

Transition in structural dynamics of myosin regulatory light chain (RLC) phosphorylation domain (PD). Two structural states exist for each biochemical state (i.e., phosphorylation). Horizontal arrows indicate the favored structure in equilibrium, which changes upon phosphorylation (+P). Conclusions from EPR studies (Nelson et al. 2005) and MD simulations (Espinoza-Fonseca et al. 2007, 2008) show an increase in helical ordering of the PD and increase in solvent accessibility with phosphorylation. MD simulations predict a salt bridge between R16 and pS19 that stabilizes the open state of the PD. *The region of the PD shown in panel A is highlighted by the green box in panel B.* (B) TR-FRET studies and MD simulations on the entire regulatory domain (Kast et al. 2010) reveal loose coupling of

biochemical and structural states of the PD: two structural states (closed and open) exist in both biochemical states (unphosphorylated and phosphorylated) and phosphorylation shifts the molar fraction to favor the open state.

Fig. 3.

(A) Phospholamban (PLB) is in dynamic equilibrium between a tense (T) state and a relaxed (R) state. Phosphorylation shifts the equilibrium in favor of R to activate SERCA allosterically. (B) Fluorescent fusion proteins, CFP-SERCA and YFP-PLB, used in FRET experiments in living cells, to test models for phosphorylation-dependent activation of SERCA. (C) Same FRET system used to screen for gene therapy (green non-inhibitory mutant PLB_M displaces wild-type PLB) or drug therapy (purple drug shifts PLB to R state) (Gruber et al. 2012).

Fig. 4.

(A) μs actin filament twisting motions. (B) Polarized emission for phosphorescent dye laser excitation of actin. (C) TPA decay reveals actin twisting motion, with respect to amplitude and rate. cMyBP-C-actin complex does not dissociate with phosphorylation but relieves restrictive effect on actin dynamics. (D) cMyBP-C domain organization, highlighting the PKA phosphorylation sites (*).