Site-directed spectroscopy of cardiac myosin-binding protein C reveals effects of phosphorylation on protein structural dynamics

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We have used the site-directed spectroscopies of time-resolved fluorescence resonance energy transfer (TR-FRET) and double electron–electron resonance (DEER), combined with complementary molecular dynamics (MD) simulations, to resolve the structure and dynamics of cardiac myosin-binding protein C (cMyBP-C), focusing on the N-terminal region. The results have implications for the role of this protein in myocardial contraction, with particular relevance to β-adrenergic signaling, heart failure, and hypertrophic cardiomyopathy. N-terminal cMyBP-C domains C0–C2 contain binding regions for potential interactions with both thick and thin filaments. Phosphorylation by PKA in the MyBP-C motif regulates these binding interactions. Our spectroscopic assays detect distances between pairs of site-directed probes on cMyBP-C. We engineered intramolecular pairs of labeling sites within cMyBP-C to measure, with high resolution, the distance and disorder in the protein’s flexible regions using TR-FRET and DEER. Phosphorylation reduced the level of molecular disorder and the distribution of CO2 intramolecular distances became more compact, with probes flanking either the motif between C1 and C2 or the Pro/Ala-rich linker (PAL) between CO and C1. Further insight was obtained from microsecond MD simulations, which revealed a large structural change in the disordered motif region in which phosphorylation unMASKS the surface of a series of residues on a stable α-helix within the motif with high potential as a protein–protein interaction site. These experimental and computational findings elucidate structural transitions in the flexible and dynamic portions of cMyBP-C, providing previously unidentified molecular insight into the modulatory role of this protein in cardiac muscle contractility.


definitions

Phosphorylation of cardiac myosin-binding protein C (cMyBP-C) accelerates contraction kinetics in skinned myocardium and plays a critical role in modulating the strength and kinetics of force development and relaxation in the heart via β-adrenergic stimulus (1–3). cMyBP-C is uniquely situated in the sarcomere to influence contractility and dynamic interactions between myosin and actin, anchored at its C-terminal end to the myosin tail that forms the thick filament (4–6) and available to interact at its N-terminal end with both the myosin neck region subfragment-2 (S2) (2, 4, 7) and actin (8–11). In vitro analyses have shown that the interactions of cMyBP-C with myosin–S2 and the regulated actin filament are subject to modulatory tuning with respect to affinity, stoichiometry, and function (12). Thus, with varied cardiovascular load or adrenergic tone, cMyBP-C regulates the probability of interaction between myosin and actin to help govern crossbridge binding to actin and the activation of the thin filament (13). Similar to the molecular building blocks of titin (14), cMyBP-C is composed of eight Ig I and three fibronectin type III domains (Fig. 1A) and is phosphorylated by PKA, as well as by Ca2+/calmodulin-dependent kinase II and other kinases, at a cluster of three serine residues in the MyBP-C motif (also called the “M-domain”). The cardiac isoform, as compared with the two skeletal isoforms, has an additional Ig domain, C0, at its N terminus, which is linked to the neighboring C1 domain by a Pro/Ala-rich linker (PAL) (Fig. 1).

The N-terminal region of cMyBP-C [domains C0–C2 (C0C2)] plays a crucial role in modulating contractile kinetics in a phosphorylation-dependent manner. The MyBP-C motif regulatory domain, situated between domains C1 and C2, is an intrinsically disordered motif composed of ~100 residues, approximately the size of an Ig domain, that contains the cardiac PKA phosphorylation sites (2). Unlike the rest of this primarily disordered domain, a segment at the motif’s C-terminal end is a well-ordered triple-helix bundle (Fig. 1B) (15–17). We hypothesize that phosphorylation-dependent structural changes in this region are critical for the function of cMyBP-C. The PAL, positioned between domains C0 and C1 (Fig. 1), contains a high content of Pro and Ala, which probably cause this segment to be flexible, extended, and capable of influencing the interactions of its flanking domains with actin and myosin. The MyBP-C motif and PAL regions within C0C2 contain apparent binding sites for myosin-S2 and actin (Fig. 1A) and play important regulatory roles, but their intrinsic structural disorder has made them largely inaccessible to conventional techniques of determining protein structure, such as crystallography and NMR.

Therefore, in the present study, we used site-directed spectroscopic and computational techniques that are capable of resolving structural transitions, even in the presence of substantial disorder, to define the phosphorylation-dependent structural transitions of the N-terminal portion of cMyBP-C, i.e., C0C2 (Fig. 1). We have shown previously that cMyBP-C alters the torsional dynamics of actin and that this restriction of the actin filament’s twisting motion would not be possible if the domain had not undergone a structural change.

Significance

Contractility in cardiac muscle is finely tuned in response to changing circulatory demands, and phosphorylation of cardiac myosin-binding protein C (cMyBP-C) plays an important role in this regulation of function. The molecular mechanism of this regulation is not well understood because the N-terminal domain [domains C0–C2 (C0C2)] containing the phosphorylatable region of cMyBP-C is highly dynamic and disordered, thus inaccessible to standard structural techniques. Therefore, we used site-directed spectroscopic probes (fluorescence and magnetic resonance) and computational simulations that are optimized for analyzing dynamic disorder. We found that phosphorylation makes C0C2 more compact and less disordered in the linker region and phosphorylation motif, and a new binding site simultaneously emerges. Our results provide insight into muscle contraction with implications for heart failure.


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PKA treatment did not affect the intra-C1 domain probes in C0C2. Such as X-ray crystallography or electron microscopy. As expected, exists within a single Ig-domain (Fig. 2 and SI Appendix, Table S2) (19, 20). In the FRET and DEER measurements, the major peak mean distance between C1 probes was determined to be 2.55 nm, very near the 2.3-nm distance predicted from the crystal structure (19). Thus, our fluorescent and spin probes can reliably determine the molecular distances and dynamics in C0C2 (Fig. 2 and SI Appendix, Table S2). Using continuous wave EPR, we found that there are no contributions from distances shorter than the DEER detection range (<2 nm) (SI Appendix, Fig. S1 and Table S1). Our spectroscopic distance measurements within C1 reveal significant disorder (~0.5 nm FWHM) in the distribution of distances that exists within a single Ig-domain (Fig. 2 and SI Appendix, Table S2). This disorder probably arises from varying conformations in solution, which could not be resolved by static protein structural approaches such as X-ray crystallography or electron microscopy. As expected, PKA treatment did not affect the intra-C1 domain probes in C0C2.

Results

Validation of Distance Measurement Methodology Using cMyBP-C. We first aimed to validate our spectroscopic methods of measuring interprobe distances in cMyBP-C by comparing our results with data from crystal and NMR structures of the C1 domain (Fig. 2 and SI Appendix, Table S2) (19, 20). In the FRET and DEER measurements, the major peak mean distance between C1 probes was determined to be 2.55 nm, very near the 2.3-nm distance predicted from the crystal structure (19). Thus, our fluorescent and spin probes can reliably determine the molecular distances and dynamics in C0C2 (Fig. 2 and SI Appendix, Table S2). Using continuous wave EPR, we found that there are no contributions from distances shorter than the DEER detection range (<2 nm) (SI Appendix, Fig. S1 and Table S1). Our spectroscopic distance measurements within C1 reveal significant disorder (~0.5 nm FWHM) in the distribution of distances that exists within a single Ig-domain (Fig. 2 and SI Appendix, Table S2). This disorder probably arises from varying conformations in solution, which could not be resolved by static protein structural approaches such as X-ray crystallography or electron microscopy. As expected, PKA treatment did not affect the intra-C1 domain probes in C0C2.

AllostERIC EFFECT OF MOTIF PHOSPHORYLATION IN ALTERING STRUCTURAL STATES OF THE PAL. We examined the flexible portion of cMyBP-C at its linker domain, which connects C0 to C1 in the cardiac isoform of MyBP-C (and ends the skeletal isoforms that lack the C0 domain) (Fig. 1). The probe sites in the C0 and C1 domains flanking the PAL (S18C.C248 distances are shown in Fig. 3) determined by both FRET and DEER exhibit markedly increased disorder relative to the other two regions we studied here (C1 probes in Fig. 2 and the motif-flanking probes in Fig. 4, discussed below). This finding is consistent with previous structural studies showing that the C0-C1 region is extended and flexible (16, 21). In both unphosphorylated and phosphorylated COC2, the distribution of the linker probes reveals two distances in each phosphorylation state: a short distance that is more ordered (closed conformation) and a long distance that is more disordered (open conformation). The main effect of PKA phosphorylation is to increase the mole fraction of the closed conformation. The observation that a structural change occurs in the PAL region, making it very compact upon phosphorylation of the MyBP-C motif region, suggests that allostERIC changes propagate from the motif across the C1 domain to the PAL (Fig. 1). This model is consistent with in vitro observations that contacts N-terminal of the motif region (i.e., C1, the PAL, and/or C0) are capable of influencing function (10, 15, 21).

Probes Flanking the MyBP-C Motif Reveal Phosphorylation-Dependent Changes in Disorder. PKA treatment of N-terminal cMyBP-C with probes flanking the motif substantially affects both FRET and DEER decays for the labeled COC2 at S169C and C248. (C) High-resolution structure of the Ig-like C1 domain (20), showing the location of the labeling sites (red) used to measure intradomain distance controls. (D) Distance distributions from the data in A and B. The best fit was to a two-Gaussian model.

![Fig. 1](image-url)

**(A)** Organization of cardiac MyBP-C from the N terminus to the C terminus (C0–C10). Regions proposed to interact with myofilament-binding partners are indicated below the schematic. (B) Proposed structure of COC2 (enclosed in the green box in A) (modified from refs. 9, 16, 20, 42), showing the Ig-like domains and the intervening PAL (*) and MyBP-C motif domains (**). Site pairs used to measure interdomain distances across the PAL and motif are labeled in red.
population shift toward the more compact structural state (Fig. 3), whereas phosphorylation in MyBP-C motif leads to a drastic reduction in tertiary molecular disorder (Fig. 4 and SI Appendix, Fig. S5). These findings are consistent with the model that COC2 is a very dynamic region of cMyBP-C with high potential for numerous sarcomeric binding partners. These results also are consistent with findings that the MyBP-C motif is intrinsically disordered (15, 16) and that phosphorylation elicits a structural change to a more compact tertiary protein structure (22). To confirm further the nature of the motif’s intrinsic disorder and the specific structural changes associated with the unphosphorylated and phosphorylated states, we next harnessed recent advances in MD simulations (23, 24) and applied these approaches to cMyBP-C.

**MD Simulations Reveal Unique Structural Details of Regulation.** To complement our spectroscopic studies and obtain models with atomistic detail, we used microsecond MD to investigate the structural changes associated with PKA phosphorylation across the motif. We performed two sets of 2-μs-long MD simulations of the MyBP-C motif (residues His255–Lys356): unphosphorylated and phosphorylated at serines 273, 282, and 302. A complete description of the construction of the motif and MD methods is supplied in the SI Appendix, SI Text. This in silico approach has been useful in previous studies of muscle protein structural dynamics to complement studies of muscle contraction (23) and of other intrinsically disordered proteins (24). Here, we performed two independent MD simulations to determine the localized effects of cMyBP-C phosphorylation (Figs. 5 and 6).

Analysis of the secondary structure revealed that the N-terminal segment of the motif (the N-motif region, His255–Glu315) is unstructured on the microsecond time scale (Figs. 5 and 6). Although a transient helix forms near the phosphorylation site Ser282, this disordered region remains unstructured upon phosphorylation (Figs. 5 and 6).

We also found that the triple α-helix bundle (the C-motif region) remains folded in the trajectories of both the unphosphorylated and phosphorylated MyBP-C motif (Figs. 5 and 6). Moreover, phosphorylation of the N terminus has no effect on the folding or structural stability of the α-helix bundle, or on nearby residues, as also depicted in time-dependent plots of the motif secondary structure (SI Appendix, Fig. S8). Despite this lack of change in the secondary structure of the motif, we observed that phosphorylation of the three PKA sites in the MyBP-C motif, which are known to impact function, induced a large rotation of the disordered N-motif region away from residues Q335–R342 in the ordered C-motif region (i.e., the triple-helix bundle). We found that the region Q335–R342 is buried by the N-terminal segment and that phosphorylation-induced rotation of the N terminus unmask this segment (Figs. 5A and 6A). Remarkably, the region Q335–R342 is predicted to be a protein–protein interaction site by the consensus neural network method of protein–protein interaction site prediction (cons-PTISP) (25).

We next measured the changes in distance upon in silico phosphorylation by selecting two pairs of sites within the motif, with one site in each pair in the disordered N-motif region (L291 or F293) and the other site in the ordered C-motif region (E328 or R331). In both cases, these sites were in very close proximity (∼1 nm apart) when unphosphorylated, and phosphorylation induced a large structural change, with the sites separated by ∼3 nm by the rotation of the N-motif away from the C-motif (Fig. 5B and C). These results suggest that a local unfolding or extension within the motif, promoted by phosphorylation (Fig. 5), allows the concomitant collapsing or compacting of the overall COC2 region of cMyBP-C (Figs. 3 and 4).

Further analysis of electrostatic charge shows that the helix region that emerges in MD simulations upon phosphorylation comprises a clustered series of eight residues that are likely to be protein–protein interaction sites (Figs. 5 and 6 and SI Appendix, Table S3), and that are accessible for binding only in the phosphorylated state. cMyBP-C is likely to be highly phosphorylated in healthy mouse and human hearts as compared with failing hearts (26). Previous atomic force microscopy studies have suggested that structural changes occur within the motif upon phosphorylation (22).

**Discussion**

We used time-resolved FRET and DEER spectroscopies in complementary studies to resolve protein distance distributions, molecular disorder, and the mole fractions of cMyBP-C structural states across its dynamic N-terminal regions. The COC2 portion of the cardiac accessory protein cMyBP-C is critical for modulating contractility and mediating diastolic function (27, 28). We aimed to define the relationship between phosphorylation-mediated enhancement of contractility and structural dynamics to understand better the mechanistically relevant changes that occur upon phosphorylation. This approach is ideal for resolving the details of the dynamic COC2 regions—such as the proline/alanine-rich linker PAL or the MyBP-C motif (sometimes called the M-domain) containing the cardiac MyBP-C phosphoserines (Fig. 1)—which are important for modulating contractile function but for which defined structural information determined by other biophysical approaches is lacking. We further complemented our FRET and DEER studies of COC2 phosphorylation with all-atom MD simulations of the MyBP-C motif and probed the structural changes in this intrinsically disordered motif upon in silico phosphorylation; this approach revealed additional mechanistic molecular detail relevant to the enhancement of cardiac muscle contractility by cMyBP-C phosphorylation.
N-Terminal cMyBP-C Becomes More Compact and Less Disordered upon Phosphorylation. Using spectroscopy, we found that C0C2 becomes much more compact and less disordered upon PKA phosphorylation (Figs. 3 and 4), and using MD simulation we found that phosphorylation simultaneously leads to an altered binding site in the motif that is not present in unphosphorylated C0C2 (Fig. 5). Because we did not detect increased structural content (e.g., newly formed $\alpha$-helices or $\beta$-sheets) within the phosphorylated motif in our MD simulations, an alternative explanation for the narrowed distance distribution in our spectroscopic studies (Fig. 4) is that cMyBP-C phosphorylation involves the collapsing of N-terminal domains as the disordered motif structure rearranges intradomain contacts upon phosphorylation. In both unphosphorylated and phosphorylated conditions, FRET and DEER indicate that cMyBP-C domains are distributed over two distinct structural states for each set of probe pairs in the motif and the PAL. These two conformations persist in each phosphorylation state, but phosphorylation changes their distribution by altering either the level of disorder or the mole fraction occupied by each distance center. Here we refer to the shorter distance as the “closed structural state” and the longer distance as the “open state.” Strikingly, phosphorylation influences the structural dynamics of both the motif and the PAL by making these N-terminal regions much more compact and less disordered. Specifically, these distance distributions are modified by phosphorylation in unique ways. In the MyBP-C motif region, the level of structural disorder is markedly decreased and/or the motif collapses, because the width of the Gaussian distribution was reduced. In the PAL, the relative populations of the two states shifted from the open structural state toward the closed conformation (Fig. 3). Intradomain spectroscopic probes showed that the C1 domain exhibits some degree of folding-related dynamics, as is typical of Ig domains in muscle and similar to those found in titin (29).

Phosphorylation Unmasks the Charged Binding Strut of the Triple-Helix Bundle. Our MD simulations coupled with a consensus prediction program (25) suggest that phosphorylation within the motif domain uncovers a series of residues in the ordered C-motif bundle (within the MyBP-C motif) that forms a binding site for another protein. It is likely that the N-motif region shields this region from binding when cMyBP-C is unphosphorylated and that the N-motif rotates away to expose this C-motif helix region upon phosphorylation of the cMyBP-C motif. We found that the triple $\alpha$-helix bundle remains folded upon phosphorylation (Fig. 5), that is, to say, we did not detect evidence of helix bundle destabilization or rearrangement (SI Appendix, Fig. S8), in contrast to a previously proposed hypothetical model (17). As our spectroscopic results suggested, we found that when this predicted protein–protein interaction site (the $\alpha$-helical binding strut) is uncovered upon phosphorylation (Fig. 5), the overall order (stability) of C0C2, which includes the motif, is increased (Fig. 4). This increased stability could enhance the probability of binding this newly exposed region of the motif. It remains unknown which myofilament-binding interactions would be present during either low or high phosphorylation and how

![Figure 5](image5.png)

Fig. 5. (A) Microsecond-long MD simulations were performed to determine the effect of phosphorylation on the MyBP-C motif (residues 259–353). The accessibility of the three $\alpha$-helix bundles changes as the disordered region of the motif rotates away from the helices (blue to red conformation). (B) Distance measurements were simulated between three-helix bundle residues (E328 or R331) and disordered region residues (L291 or F293) with (red) and without (black) PKA phosphorylation. (C) Trajectories of the distances between these pairs with or without phosphorylation.

![Figure 6](image6.png)

Fig. 6. (A) A phosphorylated MyBP-C motif (residues 259–353) protein–protein binding site is predicted to be at residues 335–342 using cons-PPISP, in which a score of 0 is improbable and 1 is highly probable. See SI Appendix, SI Results and Table S3 for residue-specific values. (B) MD simulations suggest that this region is accessible for binding only when phosphorylated: A series of eight residues (yellow) is uncovered as the disordered N-motif (gray) shifts from the unphosphorylated (Upper) to the phosphorylated (Lower) conformation.
the altered binding modifies the contractile function of the intact sarcomere. However, this region certainly would be available for binding interactions in phosphorylated cMyBP-C. Thus, this single α-helical binding strut is especially relevant to both physiological and pathophysiological states, because the level of phosphorylation is typically high in healthy donor hearts and low in failing myocardium (30). Further, how [Ca2+]i would affect MyBP-C motif structural dynamics is not known, because PKA influences contractility at submaximal but not maximal levels of Ca2+ activation (31).

**Allosteric and Propagating Effects of cMyBP-C Phosphorylation on Actomyosin.** How does motif phosphorylation influence contractility and alter N-terminal cMyBP-C binding with its myofilament binding partners in the cardiac sarcomere? The effects of phosphorylation on all three probe sets within MyBP-C’s N-terminal domains on cMyBP-C’s structural dynamics could have profound physiological and pathologic significance. Our major finding is that PKA phosphorylation leads to a more compact and ordered cMyBP-C N terminus with robust structural changes in both the motif and PAL regions. The changes observed—the reduced level of disorder in MyBP-C motif and the allosteric change in the molecular fraction favoring the closed structural state of the PAL upon phosphorylation—may be crucial for optimal function of the myosin-actin interaction in the sarcomere. Whether these transitions occur in myocardium has not been determined, but such an allosteric change in the PAL upon phosphorylation of the motif linker could allow the propagation of alterations in binding interactions of the full-length cMyBP-C molecule or could amplify the influence of neighboring domains, such as the motif region, in cMyBP-C’s regulation of cardiac contractility. It is even possible that the C0 domain interacts directly with the motif under certain conditions, such as after phosphorylation, although this idea has not yet been tested. The necessity of the C0 domain extending beyond the linker only in cardiac muscle also remains unclear, but these results suggest it may be important in phosphorylation, given the allosteric structural change. It has been shown previously that the COC2 end of cMyBP-C interacts with actin and myosin S2, with similar changes detected in $R_{\text{max}}$ and $K_{\text{d}}$, respectively (2, 10). These changes in dynamics and exposed binding sites probably play a significant role in the mechanism by which cMyBP-C modulates cardiac contractility via modified myofilament interactions. We conclude that changes in the dynamic motif and PAL of COC2 are important in modulating contractility, conceivably by switching the accessibility of exposed binding sites and the level of protein disorder and dynamics to alter cMyBP-C binding and/or binding partners in the myofilament.

**Tuning Contractility via cMyBP-C Phosphorylation: From Residues to Myocardium.** What is the specific change in motif structure that occurs with the addition of phosphate groups to cMyBP-C’s PKA sites? Our structural measurements suggest that eight residues on the α-helix of the triple-helix bundle that are buried when the motif is unphosphorylated become exposed, and overall motif dynamics become more stable, upon phosphorylation. Because cMyBP-C is likely to be highly phosphorylated under resting physiological conditions in healthy humans and studied mouse models of human heart disease, this uncovered helix may be an important binding region critical to normal cardiac function (26, 28, 32, 33) and would certainly be the dominant cMyBP-C site during β-adrenergic stimulation with high phosphorylation levels, as occurs with the fight-or-flight acute stress response. Furthermore, because cMyBP-C phosphorylation decreases in patients with heart failure and hypertrophic cardiomyopathy (30, 32, 34), the loss of this exposed binding site could have markedly detrimental effects on the contractility response in failing hearts.

**Conclusion**

We used TR-FRET, DEER, and all-atom MD simulations to characterize the structural dynamics of the flexible and disordered regions of the cMyBP-C N-terminal fragment COC2 and assayed the structural changes associated with PKA phosphorylation. We detected structural changes in the probability distribution of conformations sampled by cMyBP-C with phosphorylation via probes placed on the C1 and C2 domains to flank the MyBP-C motif region or probes placed on the C0 and C1 domains to flank the flexible linker. The effect on FWHM to reduce disorder and increase compactness in the motif was evident. The allosteric structural change in which the molecular fraction of conformations across the PAL linker region become more compact upon phosphorylation of the motif was a more surprising result. Phosphorylation of MyBP-C produces states of cMyBP-C dynamics that are likely to impact myosin cross-bridge kinetics, allowing the tuning of the sarcomere’s response to myocardial inotropy and other physiological factors, primarily through compaction of the N terminus and exposure of phosphorylation-specific binding sites. With this knowledge of the tertiary structural dynamics, we further investigated the changes in secondary structure associated with phosphorylation. The MD simulations of motif led us to the model in which cMyBP-C phosphorylation tunes cardiac muscle contractility by altering the N-terminal surfaces available for binding in the sarcomere. Our combined results reveal that phosphorylation induces specific structural changes, leading to a well-ordered and compact cMyBP-C N terminus, including the exposure of a region of the motif that is likely to be ideal for myofilament interactions.

**Methods**

Methods for protein preparations, labeling, thiophosphorylation, mass spectrometry, and detailed descriptions of spectroscopic waveforms analyses and modeling are given in SI Appendix, SI Text.

**TR-FRET.** The protein was labeled with IAEDANS (1,5-IAEDANS, 5-[(2-iodoacetyl)amino]ethyl)amino)napththalene-1-sulfonic acid; molecular probes) and/or FmAl (fluorescein-5-maleimide; molecular probes) for TR-FRET experiments. IAEDANS-FmAl COC2 donor-only and donor-acceptor FRET samples were excited using a passively Q-switched microchip YAG laser (NanoUV-532; JDS Uniphase) at 355 nm with a pulse repetition frequency of 10 kHz. The high-energy (1 μJ per pulse) and narrow (~1 ns FWHM) laser pulses are highly uniform in shape and intensity. Emitted photons were passed through a polarizer set at a magic angle of 54.7°, followed by an interference band-pass filter (Semrock 600/15 nm and 470/20 nm), and detected using a photomultiplier tube (Hamamatsu H9540-40B; Hamamatsu, Japan) and digitizer (Agilent, CA, DC252; time resolution, 0.125 ns). All FRET experiments were performed at 25 °C in cMyBP-C buffer (SI Appendix, Fig. S4) with 1 μM labeled COC2 with or without PKA treatment. TR-FRET waveforms were analyzed globally as described previously (35) and as described in SI Appendix, SI Methods. Data were best-fit to three exponential fittings (SI Appendix, Fig. S3) and a two-Gaussian distance distribution (SI Appendix, Fig. S5) characterized by a center distance and a FWHM (SI Appendix, Fig. S5).

**EPR Spectroscopy.** We performed DEER on COC2 samples doubly labeled with Maleimide spin label [MSL: N-(1-oxyl-2,2,5,5-tetramethyl pyrrolidinyl)maleimide; Toronto Research Chemicals]. Samples were prepared for spectroscopy by dialyzing 150 μM spin-labeled COC2 into cMyBP-C buffer with or without PKA treatment, followed by the addition of 10% (vol/vol) glycerol for cryoprotection. Samples were loaded into quartz capillaries (1.1-mm i.d., 1.6-mm o.d., 20-μL sample volume) (Wilmad Lab Glass), flash-frozen in liquid nitrogen, and stored at −80 °C until use. A Bruker E580 spectrometer was used operating at Q-band (34 GHz) with an ENS107 resonator, using a four-pulse DEER protocol (36). The π/2 pulse width was 12 ns, and the electron double resonance (ELDOR) pulse width was 24 ns. The ELDOR frequency (pump position) was assigned to the maximum of the nitrogen absorption spectrum, with acceptor position (chosen by selection of the static magnetic field) being placed at a 24 Gauss higher magnetic field strength on the field sweep absorption spectrum. Temperature was maintained at 65 K during
acquisition, which lasted 4–24 h. The background-corrected DEER decay was analyzed using the Tikhonov regularization method provided in the software DeerAnalysis2013.2.0 (27) to determine the distribution of distances encoded in the spectrum, followed by Gaussian analysis of the distribution assuming a model of discrete conformational states (38). In all cases the dominant distance distributions reported by Tikhonov regularization were well fit by two Gaussians (SI Appendix, Figs. S6 and S7).

MD Simulations. A starting structure of the M-domain of mouse cMyBP-C, corresponding to residues 255–353 with four additional residues from C1 and C2 on each end of the motif to simulate all-atomic trajectories for residues 255–357 (SI Appendix, Fig. S8), was generated previously using NMR (16). The structure of the MyBP-C fragment was solvated using TIP3P water molecules with a minimum margin of 2 nm between the protein and the edges of the periodic box. Na+ and Cl− ions were added to the system to neutralize the charge of the system and to produce an ion concentration of ~150 mM. The details of the preparation of the motif structure for modeling are given in SI Appendix, SI Text. We performed MD simulations using the program NAMD 2.9 (39). Periodic boundary conditions (40), particle mesh Ewald summation (41), and a nonbonded cutoff of 0.9 nm and a 2-fs time step were used. The isothermal-isobaric ensemble was maintained with a Langevin thermostat (310 K) and a Langevin piston barostat (1 atm). The system was first subjected to energy minimization for 1,000 steps, followed by a warming up period of 200 ps. This procedure was followed by equilibration for 10 ns with backbone atoms harmonically restrained using a force constant of 100.0 kcal mol−1 nm−2. Unstrained production MD simulations of the unphosphorylated and phosphorylated MyBP-C motif were continued for 2 μs each. Binding-site predictions (Fig. 6 and SI Appendix, Table S3) were based on a computerized cons-PPISP (25).

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4. C2o ne a c he n do ft h em o t i ft os i mulate all-atomic trajectories for residues 259–353 with four additional residues from C1 and C2 on each end of the motif to simulate all-atomic trajectories for residues 255–357 (SI Appendix, Fig. S8), was generated previously using NMR (16). The structure of the MyBP-C fragment was solvated using TIP3P water molecules with a minimum margin of 2 nm between the protein and the edges of the periodic box. Na+ and Cl− ions were added to the system to neutralize the charge of the system and to produce an ion concentration of ~150 mM. The details of the preparation of the motif structure for modeling are given in SI Appendix, SI Text. We performed MD simulations using the program NAMD 2.9 (39). Periodic boundary conditions (40), particle mesh Ewald summation (41), and a nonbonded cutoff of 0.9 nm and a 2-fs time step were used. The isothermal-isobaric ensemble was maintained with a Langevin thermostat (310 K) and a Langevin piston barostat (1 atm). The system was first subjected to energy minimization for 1,000 steps, followed by a warming up period of 200 ps. This procedure was followed by equilibration for 10 ns with backbone atoms harmonically restrained using a force constant of 100.0 kcal mol−1 nm−2. Unstrained production MD simulations of the unphosphorylated and phosphorylated MyBP-C motif were continued for 2 μs each. Binding-site predictions (Fig. 6 and SI Appendix, Table S3) were based on a computerized cons-PPISP (25).
SUPPORTING INFORMATION APPENDIX

SI Results. cMyBP-C site-directed mutagenesis, labeling and phosphorylation. We determined that the C0C2 fragment of cMyBP-C (WT C0-C2) contains a single Cys (C248) that is readily labeled by maleimide-group fluorescent dyes and spin labels, whereas the other 4 Cys in C0C2 are not readily labeled under our labeling conditions. Therefore, we introduced a second reactive Cys to replace a surface accessible Ser residue either in the C0, C1, or C2 domain (S18C, S169C, or S440C) in the three varieties of double-Cys mutants used in this study. Site-directed mutagenesis was confirmed by DNA sequence analysis. C0C2 was expressed in BL-21 cells and purified by His-affinity nickel resin and further purified by size exclusion chromatography (>95% purity). For FRET experiments, the extent of labeling was confirmed by UV spectrophotometry, colorimetric protein assay, and mass spectrometry. These values were used to constrain the fits of TR-FRET analysis. The molar ratio of IAEDANS labeling (donor) [1,5-IAEDANS, 5-(((2-Iodoacetyl)amino)ethyl)amino]Naphthalene-1-Sulfonic Acid] (Molecular Probes) per Cys labeling site in double-Cys C0C2 was determined to be 9-22% and 72-89% for FMal labeling (acceptor) [Fluorescein-5-maleimide] (Molecular Probes) per Cys labeling site. The extent of MSL labeling [N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide] (Toronto Research Chemicals) was to near completion, as determined by spin-counting using a Bruker E500 spectrometer at X-band (9.5 GHz) with an SHQ cavity at a microwave power where there was no saturation (0.03 mW). The molar ratio of spin labels per labeling site was determined to be 0.96 ± 0.04.

Short distance examination within C1 domain of C0C2 (S169C.C248) using CW-EPR. Continuous-wave (CW) dipolar EPR (most sensitive to spin-spin distances <2.5 nm) was used to measure between two maleimide spin labels (MSL-MSL). Detected distances were estimated to be just beyond the instrument sensitivity (i.e., 2.6-2.7 nm distances are weakly detectable), confirming that DEER spectroscopy is most suitable for studying our set of intra- and inter-domain probe pairs that we engineered in C0C2. Indeed, a similar distance ~2.6 nm was detected using both CW-EPR and DEER. However, with DEER having sensitivity beyond 2.5 nm, with excellent resolution for both distance and disorder measurements between 2 and 6 nm, the statistical confidence of the FWHM is much better for DEER as compared to CW-EPR for these probe sites (Tables S1 and S2). For example, using CW-EPR analysis of the spin labeled C0C2 (S169C.C248) within C1 (Fig. S1), we observed a small but significant difference in the line-width of ~0.6G, which correlates to a measured distance of ~2.6 nm and fairly high disorder ~2 nm, due to the long distance sensitivity limit for CW-EPR (optimal for distances <2.5 nm); using DEER (and FRET), this FWHM is more realistic for an Ig-like domain at 0.3-0.6 nm. Thus, DEER is the dipolar EPR method of choice for studying structure and dynamics in our set of doubly-labeled C0C2 (within the C1 domain, across PAL, or across MyBP-C motif).

DEER and FRET analyses comparison. Intra-C1 domain and motif-flanking C0C2 distances distributions using DEER and FRET were determined to be very similar in comparison and nearly superimposable (main text Fig. 2C and Fig. S2), whereas PAL-flanking measurements yielded qualitatively similar broad distributions to suggest significant molecular disorder for the open conformation (FWHM ~2 nm), consistent with other structural studies [1]. Both DEER and FRET analyses for C0C2 mutants indicate that the data is best fit to a sum of two Gaussian distance distributions. For FRET analysis, the donor-only lifetimes were best fit to three exponentials (Fig. S3) and the donor-acceptor lifetimes were best fit to two Gaussians (Fig. S4). Distance distributions for all three constructs with and without PKA treatment are summarized in Fig. S5. For DEER analysis, the decays were background-subtracted and similarly fit to one or two Gaussians (Fig. S6), with the two-Gaussian giving the best fit. In addition, the DEER analysis included the model-independent Tikhonov fit, which suggested additional minor components, as analyzed further in (Fig. S7).
**SI Methods. Protein preparation and labeling.** cMyBP-C fragment C0C2 was cloned from mouse MYBPC3 cDNA and ligated as a BamHI-XhoI fragment into the pET28a vector (Novagen). C0C2 contains 5 native cysteines (C1 contains 2 near the surface and C2 contains 3 that are buried), however only one native Cys (C248) is labeled by Cys-reactive probes under our labeling conditions. Site-directed mutagenesis (Stratagene) was performed to engineer a pair of Cys residues into the construct for thio-reactive spin label attachment at chosen sites (S18C and C248, S169C and C248, or S440C and C248). The sites for Cys substitution were chosen because they are in β-sheets regions near the protein outer-surface in crystal and/or NMR structures of individual cMyBP-C domains (2K1M for C0, 2AVG and 3CX2 for C1, 2LHU for MyBP-C motif, and 1PD6 for C2), and the predicted interprobe distances are within the overlapping sensitivity range of FRET and DEER (~2-6 nm [2]).

The double-Cys mutant constructs were transformed into the *Escherichia coli* BL21 DE3 cell line and grown at 37 °C in LB media to an absorbance of 0.6-0.8 at 600 nm. Cells were induced with 1 mM IPTG and allowed to grow for 3 h at 37 °C. Cells were then harvested by centrifugation and lysed with lysozyme for 1 hour at 4°C in 50 mM sucrose, 5 mM EDTA, 1 mM PMSF, 25 mM Tris (pH 8.0, 4°C), followed by a freeze-thaw procedure in a dry ice/isopropanol bath. The lysate was treated with 2U/L DNase I and incubated with the addition of 10 mM MgCl$_2$ for 1 hour, then centrifuged at 40,000 x g for a total 30 minutes in two washes. The supernatant was purified using subsequent His60 Superflow resin (Clontech) and a HiPrep Sephacryl S-100 high resolution size exclusion column (GE Healthcare Life Sciences). His$_6$-tagged protein was eluted with imidazole for eluting protein from His60 Superflow nickel resin, prior to size exclusion chromatography for improved C0C2 purity. Fractions containing the target protein in each column run were verified using SDS-PAGE and pooled.

Before labeling, C0C2 proteins were reduced with DTT for 60 min on ice. Excess DTT was removed using Zeba desalting columns (Pierce) in 50 mM NaCl and 50 mM Tris, pH 7.5. The protein was labeled with IAEDANS and/or FMal for TR-FRET experiments or MSL for DEER experiments. For FRET experiments, the donor dye (IAEDANS) portion was under-labeled in all samples (i.e., <30% in donor-only and donor-acceptor samples) and the acceptor dye (FMal) was used in excess to label the remaining Cys in FRET samples (i.e., in donor-acceptor samples). Excess dye was removed by dialysis in fresh buffer (50 mM NaCl, 50 mM sodium phosphate, pH 7.0, and 1 mM fresh DTT) and subsequent desalting spin columns.

Labeling efficiency was determined by UV absorbance for dye concentration and colorimetric BCA assay for protein concentration (Pierce) and by mass spectrometry (see below). For DEER and EPR experiments, the MSL reaction labeled to near completion with ~5 molar equivalents of label to Cys in C0C2, and excess MSL was removed following labeling by desalting spin columns. Electrospray mass spectrometry and EPR spin counting both showed that all samples were full spin-labeled. Experiments were performed in cMyBP-C buffer containing 100 mM NaCl, 10 mM Tris-HCl pH 7.5, 2 mM MgCl$_2$, and 0.2 mM ATP (also a suitable buffer for studies in the presence of actin). For DEER, the MSL-C0C2 samples were flash-frozen in liquid nitrogen at stored at -80 °C with 10% glycerol added as a cryoprotectant.

**In Vitro Phosphorylation of MyBP-C.** Purified cMyBP-C C0C2 was phosphorylated with the catalytic subunit of Protein kinase A (PKA, Sigma), using 0.01 units of PKA per µg of MyBP-C for 1 h at 25°C [3]. Phosphorylation status of MyBP-C was assessed by Pro-Q Diamond phosphoprotein staining followed by Sypro-Ruby protein staining (Invitrogen) or mass spectrometry. Untreated MyBP-C was unphosphorylated and was incubated for 1 h at 25 °C without PKA. To confirm that C0C2 was homogeneously unphosphorylated without PKA treatment and homogenously phosphorylated with PKA treatment, samples were additionally run on SDS-PAGE containing 50 µM Phos-tag™ acrylamide (SuperSep Phos-tag™, Wako Chemicals) to analyze for differential cMyBP-C phosphorylation. The gels were run for 120 minutes at 20 mA constant current. Gels were then fixed, stained and analyzed as has previously been done for cMyBP-C phosphorylation using this method [4, 5]. The use of Phos-tag™ allowed for resolving zero to four cMyBP-C phosphorylation sites. Phosphorylated species were not detected in untreated samples and samples treated with PKA were highly phosphorylated (Fig. S9), consistent with earlier results [5] using software for gel image processing.

**TR-FRET Analysis.** Fluorescence waveforms were acquired using a high-performance time-resolved fluorometer built in collaboration with Fluorescence Innovations, Inc., using direct waveform recording rather than the conventional method of time-correlated single-photon counting [6], which offers 10$^5$ times higher throughput at comparable performance. Waveforms were analyzed as described previously [7].
The observed donor-only waveform $F_{\text{Dobs}}(t)$ was fitted by a simulation $F_{\text{Dsim}}(t)$, consisting of a multiexponential decay $F_{\text{D}}(t)$ convolved with the instrument response function (IRF, from water light scatter),

$$F_{\text{D}}(t) = \sum_{i=1}^{n} A_i \exp(-t/\tau_{Di}), \quad \text{[S1]}$$

$$F_{\text{Dsim}}(t) = \int_{-\infty}^{+\infty} \text{IRF}(t-t') F_{\text{D}}(t') \, dt',$$

where $\tau_{Di}$ are donor-only fluorescence lifetimes. The best fit was obtained with $n = 3$ in Eq. 1. The waveform of donor-acceptor labeled myosin before convolution, $F_{\text{DA}}(t)$, was fitted assuming that the acceptor increases the decay rate due to energy transfer [8]. In the most general case, a distribution of donor-acceptor distances $\rho(r)$ was assumed:

$$F_{\text{DA}}(t) = \int_{-\infty}^{+\infty} \rho(R) \cdot \sum_{i=1}^{n} A_i \exp\left\{(-t/\tau_{Di})\left(1+\left[R_0/R_i\right]^6\right)\right\} \, dr,$$

and the observed waveform $F_{\text{D+Aobs}}(t)$ was fitted by $F_{\text{D+Asim}}(t)$:

$$F_{\text{D+A}}(t) = X_D F_{\text{D}}(t) + (1-X_D) F_{\text{DA}}(t), \quad \text{[S3]}$$

$$F_{\text{D+Asim}}(t) = \int_{-\infty}^{+\infty} \text{IRF}(t-t') \cdot F_{\text{D+A}}(t') \, dt',$$

where $X_D$ is the fraction of proteins labeled only with donor.

Several functional forms of $\rho(R)$ were tested: single discrete distance (1$R$), two discrete distances (2$R$), single Gaussian (1$G$), and two Gaussian components (2$G$): The best fits (indicated by residual plots and $\chi^2$) were consistently obtained for the 2$G$ model,

$$\rho(r) \approx \frac{1}{\sigma \sqrt{2\pi}} \exp\left\{-\frac{(r-R)^2}{2\sigma^2}\right\}, \quad \sigma = \frac{\text{FWHM}}{2\sqrt{2\ln 2}}, \quad \text{[S4]}$$

where $\sigma$ is the standard deviation and FWHM is the full width at half maximum of the distribution. Each fit yielded five independent parameters of $\rho(R)$: centers $R_1$ and $R_2$, widths $\text{FWHM}_1$ and $\text{FWHM}_2$, and mol fraction $X_1$.

**CW-EPR spectroscopy.** Spin-spin distances were also determined by dipolar continuous wave (CW)-EPR, for detecting distances from 0.5 to 2.5 nm, using a Bruker E500 spectrometer at X-band (9.5 GHz) with an SHQ cavity, as described previously [9]. Spectra were acquired at 200K with a 200G field sweep to detect spectral broadening. The modulation amplitude was decreased to 1G to minimize modulation broadening, and all spectra were acquired under non-saturating conditions (0.03mW).

**Modeling and preparation of the MyBP-C motif structure.** The NMR structure reported for the MyBP-C motif (i.e., M-domain) only contains residues Glu315-Lys349 (PDB: 2lhu). Therefore, we constructed a structural model of the full MyBP-C motif (residues His255-Lys356). We used five methods to predict structural disorder in the motif: PONDR-FIT [10], IUPred [11], DisEMBL [12], SPINE-D [13], and ESpritz [14]. In addition, we also performed secondary structure predictions using JPRED [15] and PSIPRED [16]. By using these methods, we confirmed that residues 315-350 are structurally ordered, in agreement with NMR data. Furthermore, we found that residues His255-Glu315 are intrinsically disordered, and that this segment does not contain any significant amount of secondary structure. Based on these findings, we constructed a model of the motif. First, we generated a completely extended model of the N-terminal domain (residues His255-Glu315). To generate a truly random model of the isolated N-terminus in solution, we performed a 50-ns MD simulation of this segment at 310K. The
final structure was then attached to the NMR structure of the 3-helix bundle (residues 315-350). Residues Gly350-Lys356 were modelled as an ideal α-helix at the C-terminus of the protein. We performed an explicit solvent, 100-ns MD run at 310K to optimize the final structure. The structure at the end of this simulation was used for the production MD simulations.

The final structure at t=100 ns was used as a starting structure to simulate unphosphorylated and phosphorylated MyBP-C motif. For the phosphorylated motif, phosphoserine patches were added to serines 273, 282 and 302. Both unphosphorylated and phosphorylated proteins were solvated using TIP3P water molecules with a minimum margin of 2.5 nm between the protein and the edges of the periodic box. Na⁺ and Cl⁻ ions were added to the system to neutralize the charge of the system and to produce a NaCl concentration of approximately 150 mM. CHARMM36 force field topologies were used for the protein, water and ions [17, 18]. We used this force field because it was recently shown that it describes well the structural dynamics of a disordered protein in solution [19].

**Electrospray Ionization Mass Spectrometry.** To confirm extent of labeling and phosphorylation, we used a calibrated electron-spray ionization source (ESI) to ionize the proteins and determine the associated change in mass. Labeled and unlabeled C0C2 samples at 80 μM were dialyzed in Ammonium Bicarbonate buffer (10 mM, pH 7.9 with 1 mM DTT) in order to minimize salts in the solution that may affect the protein charge. A typical sample injected into the spectrometer was 10 μL with consistent results. Mixtures of unlabeled and labeled proteins were used to assess the fraction of labeled protein and analysis of peaks was performed using the Analyst QS program.

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**Fig. S1.** Dipolar EPR of C0C2 S16C.C248 shows that there is a small but significant difference in linewidth of ~0.6G, between singly-labeled (non-interacting in black, ¹Cys EPR) C0C2 and doubly-labeled (red, blue) C0C2 with spin labels in the C1 domain. Singly- and doubly- labeled C0C2 spectra were compared in peak broadening and fit to 1 (red, ²Cys 1G Fit) and 2 (blue, ²Cys 2G Fit) Gaussians, without significant improvement of the fit, with the conclusion that distances much shorter than 2.5 nm are not present in the sample. See Table S1 for distance measurements of each fit.
**Fig. S2.** Probability distribution of C0C2 (C248.S440C) with probes flanking the MyBP-C motif, as determined by (A) TR-FRET (black) or DEER (dashes) and (B) the effect of phosphorylation by PKA treatment using TR-FRET (red) or DEER (red dashes). All were fit to a two-Gaussian distribution with centers at the mean distance of each peak. The Gaussian's full-width at half-maximum height (FWHM) relates to the level of labeled C0C2 disorder.

**Fig. S3.** Details of FRET analysis for donor-only data used in Fig. 4. (A) Time-resolved (TR) fluorescence data of donor-only C248.S440C-C0C2 (thick waveform) and Instrument Response Function (IRF, thin waveform). (B) Donor-only lifetimes of labeled C0C2 for FRET were analyzed by 1, 2, 3, and 4–exponentials in the fit (Eq S1). Residuals of each fit (data minus fit) are plotted. The 4-exponential fit is indistinguishable from the 3-exponential fit. (C) $\chi^2$ values for the fits shown in B, showing clearly that the 3-exponential fit was better than 2, and that 4 exponentials gave no further improvement. Thus the 3-exponential analysis was used.

**Fig. S4.** Details of FRET analysis for data in Fig. 4. (A) TR-fluorescence of C248.S440C-C0C2. Fluorescence waveforms of both donor (D) and donor-acceptor (D + A), and the IRF. (B) Residuals from fits for donor-acceptor data in A, assuming 1, 2, and 3 Gaussian distance distributions (Eq S2-S4), showing (data – fit)/(maximum fit value). (C) $\chi^2$ values for the fits shown in B, showing clearly that the 2-Gaussian fit was better than 1, and that 3 Gaussians gave no further improvement. Thus the 2-Gaussian analysis was used.
Fig. S5. Results of independent fits (Eqs. S2-S4, \( n = 3 \)) for unphosphorylated (black) and phosphorylated (red) C0C2, assuming two Gaussian distributions: closed state (solid) and open state (striped). Error bars are SEM (\( n = 3 \)). (A) Center distances and (B) widths of the distribution for each of the 3 intra-molecular probe pairs (in PAL, C1, or motif) to C248 in C1 are shown in the bars.
Fig. S6. Raw DEER data and analysis of data shown in Fig. 2, illustrating how different models were evaluated. (A) Time-dependent DEER waveform (echo amplitude) of doubly spin-labeled C0C2 (raw data shown in black) was background-subtracted (background shown in red) by fitting a portion of the spectrum (blue to red dotted lines at ~0.25 to 2.6 μs of the evolution time) to an exponential decay, assuming a 3D homogeneous distribution of spins. (B) Normalized DEER waveform (black) and fit (red) to Tikhonov, 1 Gaussian, or 2 Gaussians (red lines: top, middle, bottom, respectively). (C) Residuals from fits. (D) Distance distributions from fits. (E) Statistics from fits. More detailed illustration of fitting procedure is shown in Supplementary Index, Fig. S7.
Fig. S7. Spin-echo DEER waveforms of doubly spin-labeled C0-C2 were analyzed, with 2-Gaussians providing the best fit for the dominant spectral features. However, direct fitting of DEER waveforms to Gaussian models are easily trapped in unrealistic solutions due to experimental artifacts and deviations from ideal background components, therefore fits were derived initially from a model-independent Tikhonov fit then further analyzed for Gaussian populations. Shown above is the (A) fitting of the DEER decay with Gaussian fit residual (inset). Reduced Chi-squared ($\chi^2$) for the Gaussian fit was computed using the Tikhonov profile as a reference. (B) Shown in the middle panel is the Gaussian fit (shaded envelope and colored sub-populations) of the Tikhonov distribution (thin black line) having additional features not in the shaded region of the distribution. The * marked populations are deemed to be artifacts via comparison with CW dipolar experiments (short distance peak) and through the use of the background correction validation tool provided in DeerAnalysis 2013 (long distance peak). (C) Shown below is the resulting Gaussian populations (shaded region) derived from Tikhonov fit (thin black line) of DEER waveforms after artifact populations were removed.
Fig. S8. Time-dependent secondary structure changes of unphosphorylated and phosphorylated MyBP-C motif (i.e., M-domain). The location of the phosphorylation sites is shown as dashed lines in red. The keys on the left indicate the location of the unstructured N-terminus (residues His255-Glu315) and the 3-helix-bundle (residues Glu315-Lys349). Secondary structure is colored as α-helix (pink), 310-helix (blue), β-strand (yellow) turn (cyan), and coil (white).
Fig. S9. (A) Phosphorylation of C0C2 detected by phosphate affinity SDS-PAGE (containing Phos-Tag™ acrylamide; Wako Chemicals, Osaka, Japan). The same gel was stained with Pro-Q Diamond (top) or Sypro Ruby (bottom) to resolve individual phosphospecies due to PKA treatment. Untreated C0C2 (-) had no detectable phosphorylation in Pro-Q Diamond stain and migrated lower than PKA-treated samples (+) after staining with Sypro Ruby. PKA-treated C0C2 (+) was nearly homogenously phosphorylated at 3-4 phosphorylation sites, with faint detection of 1-2 phosphorylation sites. (B) Relative intensity of phosphospecies with no phosphorylation, 1-2 sites, or 3-4 sites phosphorylated (N.D. = not detectable), corrected for protein load.
TABLE S1: Fits for dipolar EPR of C0C2 S169C.C248 within the C1 domain does not exhibit strong evidence for distances with peak centers shorter than 2.5 nm.

<table>
<thead>
<tr>
<th>Gaussians</th>
<th>Unbroadened</th>
<th>X₁ (mol)</th>
<th>R₁ (nm)</th>
<th>X₂ (mol)</th>
<th>R₂ (nm)</th>
<th>χ²</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>0.95</td>
<td>2.8 ± 2.1</td>
<td>-</td>
<td>-</td>
<td>1.5x10⁻⁵</td>
</tr>
<tr>
<td>2</td>
<td>0.08</td>
<td>0.74</td>
<td>2.6 ± 0.7</td>
<td>0.18</td>
<td>1.3 ± 1.5</td>
<td>1.4x10⁻⁵</td>
</tr>
</tbody>
</table>

Dipolar EPR (i.e., CW-EPR) measurements show that there is a difference in the linewidth of ~0.6G, which is small but significant (p<0.05). These distances have fairly high disorder, >2 nm, because these distances are at the sensitivity limit for dipolar EPR, and in a much better distance range for DEER and TR-FRET spectroscopic approaches, with sensitivity in the 2-6 nm range.

TABLE S2: Distance measurements for C0C2 from S169C to C248 of the C1 domain.

<table>
<thead>
<tr>
<th>Spectroscopy</th>
<th>Mean Distance (nm)</th>
<th>Deviation from Mean (nm)</th>
<th>Mole Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMR</td>
<td>2.3</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>X-ray</td>
<td>2.3</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>TR-FRET</td>
<td>2.6</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>DEER</td>
<td>2.6</td>
<td>0.6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

C1 domain NMR and crystal structure measurements are Cα-Cα distances using computer software (PDB: 2AVG and 3CX2, respectively), whereas TR-FRET and DEER measurements are from fitted spectra of labeled-C0C2 with probes across the C1 domain.
TABLE S3: Likelihood of protein-protein interaction site locations within the MyBP-C motif

<table>
<thead>
<tr>
<th>AA</th>
<th>Residue</th>
<th>Probability</th>
<th>Prediction</th>
</tr>
</thead>
<tbody>
<tr>
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<td>N</td>
</tr>
<tr>
<td>D</td>
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</tr>
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<td>0.000</td>
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</tr>
<tr>
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<td>P</td>
</tr>
<tr>
<td>E</td>
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</tr>
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<tr>
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<tr>
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<tr>
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</table>

Protein-protein binding site predictions within MyBP-C motif (i.e., M-domain) using cons-PPISP [20]. The consensus method is based on protein structure, amino acid charge, position-specific sequence profiles, and solvent accessibilities of each residue and its spatial neighbors, based on known structures of protein complexes. P: positively predicted residue for interactions, N: negatively predicted residue for interactions, -: buried residue; 0: low probability of protein-protein interaction site, 1: high probability of protein-protein interaction site.
REFERENCES