The structural role of the unique myosin-binding motif (m-domain) of cardiac myosin-binding protein-C remains unclear. Functionally, the m-domain is thought to directly interact with myosin, whereas phosphorylation of the m-domain has been shown to modulate interactions between myosin and actin. Here we utilized NMR to analyze the structure and dynamics of the m-domain in solution. Our studies reveal that the m-domain is composed of two subdomains, a largely disordered N-terminal portion containing a more ordered and folded C-terminal subdomain. A PARTIALLY FOLDED DOMAIN

Background: Cardiac myosin-binding protein-C is a sarcomeric assembly protein necessary for the regulation of sarcomere structure and function.

Results: The cMyBP-C motif is composed of two subdomains, a largely disordered N-terminal portion and a more ordered C-terminal subdomain.

Conclusion: The C-terminal subdomain is capable of forming a three-helix bundle.

Significance: The three-helix bundle may provide a platform for actin binding.

Cardiac myosin-binding protein-C (cMyBP-C) is a sarcomeric assembly protein, which is necessary for the regulation of sarcomere structure and function. It constitutes 2% of the total myofibrillar protein (1). Exclusively found in the vertebrate heart, cMyBP-C is encoded by a distinct gene (MYBPC3) (2, 3) and is localized in the inner two-thirds of the A band of the sarcomere. The m-domain weakly interacts with the S2 fragment of myosin (9, 10), and it is strongly anchored to light meromyosin via the C10 domain (11) and titin via C8–C10 domains (12). Importantly, the cardiac isoform differs from the other two isoforms in that it contains an extra N-terminal domain (C0), a Pro-Ala-rich linker connecting the C0 and C1 domains, a unique structural motif termed “m-domain” for myosin-binding motif located between the C1 and C2 domains, and an insertion of ∼30 amino acids in the C5 domain. The Pro-Ala-rich linker...
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sequence has been shown to contain a potential actin binding sequence, and species-specific differences in the Pro-Ala linker have been postulated to play a role in modulating heart rate in different species (13). Recent data provide evidence for in vitro interaction between cMyBP-C and F-actin via the C0 domain (14), C1-m-C2 domains (15–19), and the C5 domain (20). In addition, the m-domain is unique in that it contains three serine residues (Ser-273, Ser-282, and Ser-302) that are differentially phosphorylated by the enzymes PKA, PKC, PKD, ribosomal S6 kinase, and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (21). Phosphorylation of the m-domain may modulate interactions of the C1-m-C2 region with the myosin S2 region and actin. cMyBP-C is extensively phosphorylated under basal conditions (22). However, the level of cMyBP-C phosphorylation decreases in animal models during injury-reperfusion injury (22), pathologic hypertrophy (22), and myocardial stunning (23, 24), atrial fibrillation (25), and heart failure (26). In vitro studies demonstrated that phosphorylated cMyBP-C displays a weakened interaction with the S2 region of myosin and that this state promotes myosin-actin interaction, activating cross-bridge cycling rates (9). Taken together, these studies support the idea that cMyBP-C modulates sarcomere assembly and stabilizes thick and thin filaments via its N-terminal region. However, little is known about the structural mechanisms by which the N-terminal region of cMyBP-C modulates cardiac contractility in different vertebrate species.

A complete understanding of cMyBP-C arrangement in the sarcomere is essential in determining the roles that post-translational modification plays in modulating cMyBP-C interaction with myosin and/or actin. Two structural models are currently being discussed in the literature: the trimeric collar model and the rod model. The collar-like model proposes that three cMyBP-C molecules interact with each other at the C5–C10 domains and form a collar-like structure around the core of the thick filament (27). The rod model proposes that the C-terminal region of cMyBP-C interacts along the axis of the thick filament with the N-terminal region, extending perpendicularly toward the thin filament (28). Although there are conflicting data for each of the models, all invoke interaction with the thick and thin filament systems, suggesting the necessity of further studies on cMyBP-C to determine the structure and arrangement of the N-terminal region, its interaction partners, and the role of phosphorylation.

Small angle x-ray scattering of the N-terminal region in combination with the NMR structures of the individual C0, C1, and C2 domains suggests an elongated rodlike particle of sufficient length to span the interfilament cross-bridge distance (29). Bioinformatics analyses and scattering data for C1-m-C2 suggest that the structure of the m-domain is compact and consistent with an immunoglobulin-like fold (29). These studies further show that interdomain interfaces between C0, C1, m-domain, and C2 have a degree of rigidity, whereas the Pro/Ala linker connecting the C0 and C1 domains provides structural flexibility at the extreme N terminus (30). In addition, atomic force microscopic analysis predicts that the m-domain is likely to be disordered, increasing the flexibility between the C1 and C2 domains (31). Small angle neutron scattering with contrast variation in the presence of actin demonstrated a specific interaction between the N-terminal region of cMyBP-C and actin (32). The best fit model predicts that C0-C1 lie at the interface between two adjacent actin molecules with C0 lying close to the DNase I binding loop and C1 lying close to the actin filament near subdomain 1 (32). In this configuration, the m-domain and C2 project away from the actin filament partially as a consequence of steric hindrance. Taken together, the available structural data suggest that the N-terminal region of cMyBP-C interacts with both myosin and actin, providing a means for cMyBP-C to modulate thick and thin filament interactions. However, the molecular details of these interactions and the structural consequences of m-domain phosphorylation on the interactions of cMyBP-C with the thick and thin filaments remain unknown. In the present study, we utilized NMR to analyze the structure and dynamics of the m-domain in solution. Surprisingly, we found the N-terminal subdomain to be largely disordered and the C-terminal subdomain containing three helices to be capable of forming a three-helix bundle, potentially providing a platform for interactions with actin.

EXPERIMENTAL PROCEDURES

m-domain Plasmid Construction—DNA coding for amino acids 255–357 of mouse cMyBP-C (UniProt accession number 070468), representing the m-domain, was amplified by PCR using C0C2-coding DNA as a template. The forward and reverse cMyBP-C m-domain primers were 5’-ATGCCG-CATATGCAAGCCCATTTGCTTGGA3’ and 5’-ATG-CATGCGGCCGCTCACTTCTTTTCATCCTG-3’. The PCR product was then cloned into pET 28a(+) using the Ndel and NotI restriction enzymes and subsequently transformed in DH5α cells immediately following successful ligation. Plasmid minipreps were done, and the authenticity of the m-domain insert was confirmed by DNA sequencing.

Protein Expression and Purification—Uniform 13C/15N isotope-labeled protein representing the cMyBP-C m-domain was expressed following transformation into BL21(DE3) host cells (Novagen) using M9 medium supplemented with 1.0 g/liter 15NH\(_4\)Cl and 2 g/liter [13C]glucose. Cells were grown to an A\(_{600}\) of 1.0 and induced with 0.1 mM final concentration of isopropyl-1-thio-β-D-galactopyranoside for recombinant protein expression. Cells were harvested by centrifugation after 4 h of induction and stored at −80°C until further use. Frozen cells were thawed, resuspended in lysis buffer (50 mM NaH\(_2\)PO\(_4\) pH 8.0, 200 mM NaCl, 5 mM imidazole, 1 mM PMSF, 1 mM β-mercaptoethanol) containing 0.1 mg/ml lysozyme, and subsequently lysed by sonication. Following centrifugation, the clarified supernatant was loaded on a nickel-nitrilotriacetic acid column equilibrated with lysis buffer. The column was washed, and the m-domain was eluted with a 200-mL linear gradient of 5–300 mM imidazole. Fractions containing m-domain were pooled, concentrated, and loaded onto a Superdex 75 gel filtration column. The m-domain eluted as a single peak and was determined to be homogenous by SDS gel electrophoresis followed by Coomassie Brilliant Blue staining. Protein concentration was estimated by Bradford analysis. The purified m-domain was concentrated by ultrafiltration and exchanged into NMR buffer (20 mM Tris-\(d_4\), pH 6.8, 50 mM NaCl, 2 mM tris(2-car-
boøxyethylphosphine, 1 mM EDTA, 10% 2H2O) to a final concentration of ~1 mM.

**NMR Experiments and Structure Calculation**—Backbone and side-chain resonances for [13C, 15N]cMyBP-C(255–357) were assigned using the following suite of NMR experiments: HNCaCµ, HNCO, HN(CA)CO, CµCµ(CO)NH, HµHs, (CO)NH, and C(CO)NH (Table 1). 15N-{1H}TOCSY (50-ms mixing time), 15N-edited NOESY-HSQC (100- ms mixing times), and 15N-edited HSQC-NOESY-HSQC (100-, 150-, and 200-ms mixing times) were also used (Table 1). 15N-[1H]NOE values were collected at 800 MHz as described previously (33). Chemical shift assignments for the m-domain were deposited in the BioMagResBank the under the accession number 17867. The secondary structure of the m-domain was predicted using BioMagResBank the under the accession number 17867. The structure ensemble representing the effective structure. The structure ensemble representing the ensemble. This produced an ensemble of 20 structures with 5000 structures in Rosetta. The energy of these Rosetta structures was visualized using Coomassie Brilliant Blue. Lane 1, molecular mass standards; Lane 2, 15 μg of [13C, 15N]m-domain protein.

**RESULTS AND DISCUSSION**

Characterization of m-domain Secondary Structure—We have cloned, expressed, and purified the m-domain, corresponding to residues 255–357 (human 257–361), of mouse cMyBP-C in an effort to probe the structure, dynamics, and consequences of phosphorylation on the N terminus of this multidomain protein. A 20% SDS-polyacrylamide gel of the m-domain was used to evaluate the purity of the protein before initiating structural studies. The m-domain was visualized using Coomassie Brilliant Blue. Lane 1, molecular mass standards; Lane 2, 15 μg of [13C, 15N]m-domain protein.

To further explore the structure and stability of the m-domain, isotope-labeled [13C, 15N]m-domain was prepared for analysis by heteronuclear multidimensional NMR. Backbone and side-chain NMR resonances were assigned for all 104 residues using standard triple resonance assignment strategies. Consistent with CD spectra, dispersion in the 1H-15N HSQC spectrum was significantly lower than that observed for a well structured protein (Fig. 2). To evaluate the dynamic properties of the m-domain, 15N-[1H]NOE values at 800 MHz were collected. The 15N-[1H]NOE data suggest that the protein can be divided into two regions, a well folded C-terminal portion having an average 15N-[1H]NOE value of 0.78 and an N-terminal portion having 15N-[1H]NOE values less then 0.6, suggesting a much more flexible structure (Fig. 3). Residues 309–314 appear...
to form a short linker between the less ordered N-terminal portion and the structured C-terminal portion (Fig. 3).

Backbone and $^{13}$C$_\beta$ chemical shifts were used to evaluate m-domain secondary structure. Secondary chemical shifts, deviations in the observed chemical shifts from their random coil values, can be used to calculate the CSI, an accurate predictor of fully formed $\alpha$- and $\beta$-structure (37, 38). Backbone chemical shifts for the C$_\beta$, C$_\alpha$, and H$_\alpha$ resonances were used to determine CSI values for each m-domain residue (Table 2). The consensus CSI predicted five potential helical regions in the m-domain. Residues showing helical propensities were 265–268, 293–295, 314–322, 329–336, and 340–348 (Table 2). No regions of $\beta$-structure were detected. Backbone chemical shifts were also analyzed using a residue-specific secondary structure propensity (SSP) score (39). This approach has been highly successful in comparing secondary structure propensities in both intrinsically disordered and folded proteins. Residue-specific SSP scores of 1 or –1 reflect fully formed $\alpha$- and $\beta$-structure, respectively. Scores between 0 and 1 are representative of the fraction of $\alpha$-structure, whereas scores between 0 and –1 are
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representative of the fraction of $\beta$-structure. Thus, SSP scores qualitatively correspond to the secondary structural propensity at that residue. The SSP scores calculated for m-domain residues are shown in Fig. 4. Five regions with varying secondary structural propensities were identified. Residues 263–267 for helix 1 and residues 292–298 for helix 2 were found to have average structural propensities of 27 and 36%, respectively (Table 2). Lower structural propensities for these helices correspond to reduced $^{15}\text{N}[^{1}\text{H}]\text{NOE}$ values observed for the N-terminal region of the m-domain (Fig. 3). Residues 317–322, 324–329, and 341–351, enclosing helices 3, 4, and 5, exhibited $^{15}\text{N}[^{1}\text{H}]\text{NOE}$ values above 0.7, reflecting more stably formed secondary structures in the C-terminal region of the m-domain (Fig. 3). Table 2 summarizes secondary structure elements for the m-domain based on our experimental backbone and $^{13}\text{C}_\beta$ chemical shift assignments.

Computational Analysis of m-domain—NMR analysis of the m-domain demonstrated the presence of three regions of helical structure in the C-terminal portion, whereas the N-terminal portion exhibited significantly increased mobility and only two short regions characterized with a preference for occupying helical regions of $\phi/\psi$ space. Previously, Phyre homology modeling of C1-m-C2 modules suggested that the m-domain could be structurally related to an Ig fold (29). The authors noted that Phyre modeling of the m-domain alone failed to produce a useful model (29). We performed Phyre$^2$ analysis on the m-domain to compare the secondary structural results obtained by NMR with the Phyre$^2$ prediction (40). Although Phyre$^2$ also failed to produce a reliable homology model, five helical regions largely consistent with those determined by NMR analysis were predicted. Secondary structure predictions were also performed using the SABLE, Psipred, and GOR V servers. All servers yielded five regions having a propensity for helical structure consistent with the NMR analysis (Table 3). SABLE, Psipred, and Phyre servers predicted helices 3–5 with high confidence and helices 1 and 2 at lower confidence levels (Table 3). The lower confidence in prediction of helices 1 and 2 may indicate regions of less stable structure capable of undergoing conformational transitions. This region contains three known phosphorylation sites at Ser-273/282/302. Serine residue 273 is a target for phosphorylation by both PKA and PKC (26).
TABLE 3
Bioinformatics analyses of secondary structural elements in m-domain of cMyBP-C
Serine residues known to be phosphorylated are shown in red, seq. sequence.

| m-domain seq | SABLE | SS confidence | Psipred | Psipred conf | Phyre consensus | Phyre cons_prob | GOR V pred | Disopred |
|--------------|-------|---------------|---------|-------------|----------------|----------------|------------|---------|
| MEAIGSDLRSSAFFRTSLAGAAGTSDSHEDAGTDLFK | CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC...
the L348P mutation on the stability of the three-helix bundle can be evaluated from a stability analysis performed with FoldX 3.0b5 (48). After repairing the low energy CS-Rosetta structure in FoldX and applying the BuildModel method to mutate Leu-348 to Pro, a stability analysis was performed. This revealed a \(-9.5\) kcal/mol decrease in the favorable free energy of folding for the L348P mutation. In contrast, FoldX predicted a \(-1.6\) kcal/mol decrease in the favorable free energy of folding for the V338D mutation located in the more mobile \(\alpha1-\alpha2\) loop. The remaining two hypertrophic cardiomyopathy missense mutations, R236Q and E334K (mouse sequence), are more surface-exposed and outside of the hydrophobic core (Fig. 5C).

Interestingly, the three-helix bundle fold is often found in actin-binding proteins. The headpiece domain from villin, a 35-residue actin-binding protein, is among the most studied three-helix bundle proteins. Sequence comparison of the villin headpiece with the C-terminal portion of the m-domain identifies a short sequence motif LK(R/K)XK in the third helix of both proteins. This motif is conserved between species in the m-domain of cMyBP-C proteins. Studies have shown this motif to be present in a number of actin-capping and -binding proteins and to bind actin (49–52). Taken together, our structural studies suggest that the C-terminal region of the m-domain may function as an actin-binding protein. Supporting this hypothesis, functional studies showed that the m-domain increased the \(Ca^{2+}\) sensitivity of tension and increased rates of tension redevelopment (53) and that C1-m binds F-actin in a saturable manner (15). Phosphorylation of the m-domain was shown to decrease the actin binding affinity (15). Both neutron scattering (32) and electron microscopy (16) are consistent with the binding of the N-terminal region of cMyBP-C (C0-C1-m-C2-C3) to actin.

Chemical shift mapping provides a powerful technique to monitor changes in protein structure and to map protein-protein interfaces. Amide proton and nitrogen chemical shift mapping was used to qualitatively monitor conformational changes in the m-domain induced by the presence of surrounding Ig domains, C1 and C2. Although complete amide resonance assignments are available for the isolated m-domain, only partial amide resonance assignments are available for C1-m and C0-C1-m-C2-C3. Amide resonance assignments for C-subdomain residues in the m-domain were mapped onto TROSY spectra of both C1-m and C0-C1-m-C2-C3. No significant chemical shift changes were detected for resolved m-domain resonances mapped onto the TROSY spectrum of C1-m. However, modest chemical shift differences were observed when resolved m-domain resonances were mapped onto the TROSY spectrum of C0-C1-m-C2-C3. The absence of chemical shift dispersion for resonances in the N-subdomain hindered chemical shift mapping of these resonances in C1-m and C0-C1-m-C2-C3. Although preliminary, chemical shift mapping suggests that addition of the C1 domain at the N terminus and the C2 domain at the C terminus of the m-domain does not greatly influence the conformational flexibility of the N-subdomain or the stability of the three-helix bundle in the C-subdomain. Modest chemical shift differences observed in the m-domain
comparison with C0-C1-m-C2-C3 suggest protein-protein interactions between the C-subdomain and neighboring Ig modules.

In summary, we have shown that the N-terminal portion of the m-domain, containing phosphorylation sites Ser-273, Ser-282, and Ser-302, is conformationally flexible, containing two transiently formed helices that span residues 263–267 for helix 1 and residues 292–298 for helix 2. Bioinformatics analyses predict that introduction of negative charge at Ser-273, mimicking phosphorylation, will extend and stabilize helix 1. In contrast, 15N{1H}NOE and chemical shift analyses show that the C-terminal portion of the m-domain folds into a stable three-helix bundle. A known actin-binding motif, LK(R/K)X, is positioned in the third helix (α3), similar to that found in villin and related proteins. These results suggest that the m-domain in cMyBP-C may alter actomyosin interactions in the heart through interactions with actin. Current studies are aimed at the role of the N-terminal portion of the m-domain in modulating actomyosin interactions via protein phosphorylation.

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