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cAPK-phosphorylation controls the interaction of the regulatory domain of cardiac myosin binding protein C with myosin-S2 in an on-off fashion

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Abstract Myosin binding protein C is a protein of the myosin filaments of striated muscle which is expressed in isoforms specific for cardiac and skeletal muscle. The cardiac isoform is phosphorylated rapidly upon adrenergic stimulation of myocardium by cAMP-dependent protein kinase, and together with the phosphorylation of troponin-I and phospholamban contributes to the positive inotropy that results from adrenergic stimulation of the heart. Cardiac myosin binding protein C is phosphorylated by cAMP-dependent protein kinase on three sites in a myosin binding protein C specific N-terminal domain which binds to myosin-S2. This interaction with myosin close to the motor domain is likely to mediate the regulatory function of the protein. Cardiac myosin binding protein C is a common target gene of familial hypertrophic cardiomyopathy and most mutations encode N-terminal subfragments of myosin binding protein C. The understanding of the signalling interactions of the N-terminal region is therefore important for understanding the pathophysiology of myosin binding protein C associated cardiomyopathy. We demonstrate here by cosedimentation assays and isothermal titration calorimetry that the myosin-S2 binding properties of the myosin binding protein C motif are abolished by cAMP-dependent protein kinase-mediated trisphosphorylation, decreasing the S2 affinity from a K_d of ≈ 5 μM to undetectable levels. We show that the slow and fast skeletal muscle isoforms are no cAMP-dependent protein kinase substrates and that the S2 interaction of these myosin binding protein C isoforms is therefore constitutively on. The regulation of cardiac contractility by myosin binding protein C therefore appears to be a 'brake-off' mechanism that will free a specific subset of myosin heads from sterical constraints imposed by the binding to the myosin binding protein C motif.

Key words: Myosin binding protein C; Myosin regulation; Protein interaction; Phosphorylation

1. Introduction

Myosin binding protein C (MyBP-C) is a modular muscle protein of the intracellular immunoglobulin superfamily (reviewed in [1]) which is expressed in at least three isoforms, its cardiac isoform being strictly specific for heart muscle in mammals [2,3]. The C-terminal region interacts with the light meromyosin portion (LMM) of myosin as well as with titin, thus anchoring the protein to the thick filament shaft and specifying its sarcomeric localization [1]. MyBP-C is localized to the central region of the thick filament (C-zone) in regular stripes spaced 43 nm apart [4–6]. There are 11 C-zone stripes. However, in mammalian muscle, only stripes 3–11 are usually occupied by MyBP-C, stripe 3 can also be occupied by MyBP-H. The proteins of stripes 1 and 2 are unknown. Due to the 43 nm spacing, only every third level of myosin heads in the C-zone associated with a MyBP-C molecule [4] and the function of the protein may involve cooperative changes over larger distances. Furthermore, the localization of MyBP-C to the central region of the thick filament implies that its unknown regulatory functions can only affect myosin heads when the overlap of actin and myosin filaments extends into the C-zone. The presence of the protein may therefore help to sense the degree of actomyosin overlap.

Cardiac MyBP-C is phosphorylated in a dynamic way by cAMP-dependent protein kinase (cAPK), suggesting a role in the β-adrenergic regulation of muscle contraction [7–12]. Phosphorylation occurs on three sites in a MyBP-C-specific domain in the N-terminal region, the MyBP-C motif [12]. This region is highly conserved between all isoforms of MyBP-C and between species [12,13]. The high degree of sequence identity and cross-species conservation between various MyBP-C isoforms suggests that the function of the MyBP-C motif is specified by the conserved regions and that the three phosphorylation sites found experimentally in the cardiac isoform represent an additional feature. We could show recently that the MyBP-C motif binds to the proximal 126 residues of the myosin-S2 segment, close to the lever-arm domain of the myosin head [13]. This segment of myosin-S2 is almost completely identical between all sarcomeric myosin isoforms and vertebrate species [13]. The interaction with MyBP-C is hence independent of the MyBP-C or myosin isoform and the MyBP-C motifs of both cardiac as well as slow skeletal MyBP-C were demonstrated experimentally to bind to the N-terminal S2 segment with comparable affinities [13]. The interaction with S2 directs the A-band association of N-terminal cardiac MyBP-C fragments in neonatal rat cardiomyocytes [13,14]. It has been proposed that the interaction with myosin-S2 could modulate the head-tail motility of the two-headed sarcomeric myosin [13], based on the observation that antibody Fab-fragments against the same region of S2 constrain the movement of the myosin heads [15]. The control of such a constraint by phosphorylation of MyBP-C might therefore release this 'brake' and recruit myosin heads for active force production, possibly in a cooperative way along several crowns of myosin heads.

MyBP-C has been shown to be one major protein involved in the pathophysiology of familial hypertrophic cardiomyopathy [1]. However, its physiological role is still poorly understood. Most MyBP-C mutations that cause FHC are pre-

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dicted to result in N-terminal fragments of the protein [1]. Therefore, the effect of cardiac MyBP-C phosphorylation on the interaction with myosin-S2 is of great importance to understand the basis of the regulatory mechanism of this protein and to gain insight into possible pathological mechanisms in FHC.

2. Materials and methods

2.1. Buffers

Buffer A (myosin minifilaments): Tris-citrate pH 8.0 10 mM. Buffer B (cosedimentation assay): Tris-HCl pH 7.0 20 mM, NaCl 200 mM, DTE 1 mM, EDTA 1 mM. Buffer C (isothermal titration calorimetry (ITC)): MES/NaOH pH 7.0 20 mM, NaCl 50 mM, DTE 1 mM, EDTA 1 mM)

2.2. Protein expression, purification and phosphorylation

MyBP-C and β-myosin-S2 fragments were expressed soluble as described [12,13,16] using the pET expression system [17]. His_6-tagged proteins were purified on Ni^{2+} NTA columns following the manufacturer's instructions (Qiagen, Germany) and further purified by anion exchange chromatography on a monoQ column (Pharmacia, Sweden). The His_6 tag was cleaved off by recombinant TEV protease (Gibco BRL, UK). Myosin (rabbit, skeletal) was prepared according to the method of [18]. Myosin minifilaments were prepared as described [19] from myosin which was previously purified by three cycles of high- and low salt precipitation. The content of endogeneous MyBP-C after this scheme was estimated by Western blotting using a specific MyBP-C antibody [3] and by gel densitometry to be below 5%.

Preparative phosphorylation of cardiac C1C2 (c-C1C2) was carried out in a volume of 10 ml at a concentration of 1 mg/ml c-C1C2, 100 U/ml bovine brain cAPK catalytic subunit (Sigma, Germany) and 0.7 mM ATP in 20 mM HEPES pH 7.0, 5 mM $MgCl_2$, 1 mM dithiothreitol at 25°C. Samples of 5 μl were taken at regular intervals and the degree of phosphorylation was monitored by mass spectrometry. Complete tris-phosphorylation was observed after about 3 h.

2.3. Cosedimentation assay

All proteins (except myosin minifilaments) were dialyzed into buffer B and centrifuged prior to use at 4°C for 30 min at $400\,000 \times g$ using a Beckman TLA 100.1 rotor and an Optima TL ultracentrifuge. Appropriate amounts were mixed in Beckman polycarbonate centrifuge tubes (number 343776) and the volume was made up to 25 μl with buffer B. Myosin minifilaments (25 μl of 5 μM in buffer A) were added to the mixture to give a final volume of 50 μl, pH 7.3, and a NaCl concentration of 100 mM. The mixture was incubated at 4°C for 30 min and subsequently centrifuged for 20 min at $400\,000 \times g$. The supernatant was removed and the pellet was washed twice with 100 μl of a 1:1 mixture of buffer A and buffer B. The pellet was redissolved in 50 μl of 7 M urea and appropriate amounts of supernatant and pellet were analyzed by SDS-PAGE as described [20]. Gels were stained for 24 h in staining solution (1.5% w/v Coomassie brilliant blue R250, 40% ethanol, 10% acetic acid) and subsequently destained for 48 h.

2.4. ITC

All proteins were dialyzed into buffer C and centrifuged at $100\,000 \times g$ for 10 min immediately prior to use. Calorimetric experiments were carried out using a titration calorimeter from Microcal, USA, with a 250 μl injection syringe while stirring at 400 rpm. The concentrations of the myosin-S2 constructs in the syringe were generally 10–20 times higher than the C1C2 concentrations in the reaction cell. The reference cell was filled with 1 mM sodium azide. An initial injection was performed with a small volume of 0.25 times the experimental injection volume (5–10 μl). Data analysis was performed with the manufacturer's software. Experimental values for the binding constant, heat of binding and stoichiometric ratio are from deconvolution using non-linear least-squares minimization. The heats of dilution were determined in independent experiments and subtracted from the raw data prior to data analysis.

2.5. Mass spectrometry

Nano-electrospray mass spectroscopy was performed with a Finnigan LCQ mass spectrometer equipped with a micromanipulator for the correct positioning of the nanospray needle. The needles and the ion source were made following the original design of Mann et al. [21]. In short, capillaries (GC120F from Clark Instruments, Reading, UK) were pulled using the DMZ-Universal puller (Zeitz-Instrumente GmbH, München, Germany). They were coated with gold using a Polaron SC7610 Sputter Coater (VG Microtech, Uckfield, UK) and a gold target. These needles were filled with 1–4 μl of aqueous solution and centrifuged for several seconds using a PicoFuge (Tomy Capsule, Tomy Tech, Palo Alto, CA, USA). The needles were mounted on the micromanipulator and were carefully brought in contact with the surface of the mass spectrometer. Air pressure was applied to the needle until a small droplet appeared on the tip. The needle was then adjusted 2.5 mm opposite to the inlet of the heated capillary of the mass spectrometer. A potential of 900 V was applied to the needle and the spectrum was recorded. The original data (i.e. abundance of the multiple charged protein populations versus mass/charge) were deconvoluted into mass spectra (relative abundance versus mass) by means of the deconvolution software BioExplore (Finnigan). The error of the mass spectrometer was 100 ppm in the normal mass range (150–2000 mu), which was used for all experiments shown here.

3. Results

3.1. Phosphorylation by cAPK is restricted to the cardiac isoform of MyBP-C

Phosphorylation by cAPK as well as by an associated calcium-calmodulin-dependent protein kinase is well-documented for cardiac MyBP-C. We wanted to establish experimentally whether also the known skeletal isoforms from slow and fast muscle contain accessible substrate sites for cAPK which are not evident from multiple sequence comparison [12,13,22]. We therefore expressed the C1C2 fragments of human fast and slow skeletal MyBP-C (f-C1C2 and s-C1C2), which contain the phosphorylatable MyBP-C motif [12], and compared their substrate properties for cAPK phosphorylation with c-C1C2. We observed phosphorylation for the c-C1C2 fragment but not for the two skeletal isoforms (Fig. 1). Quantitative analysis of the kinetics of c-C1C2 phosphorylation was carried out by mass spectrometry under preparative conditions. After 2 min of phosphorylation, conversion to the monophosphorylated form was visible with no detectable bis- or tris-phos-

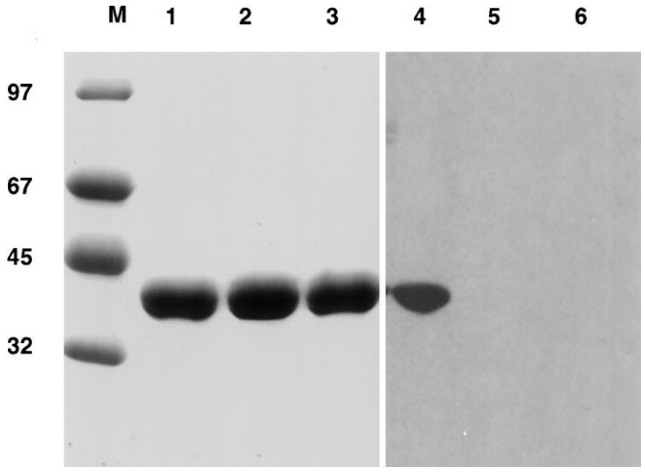
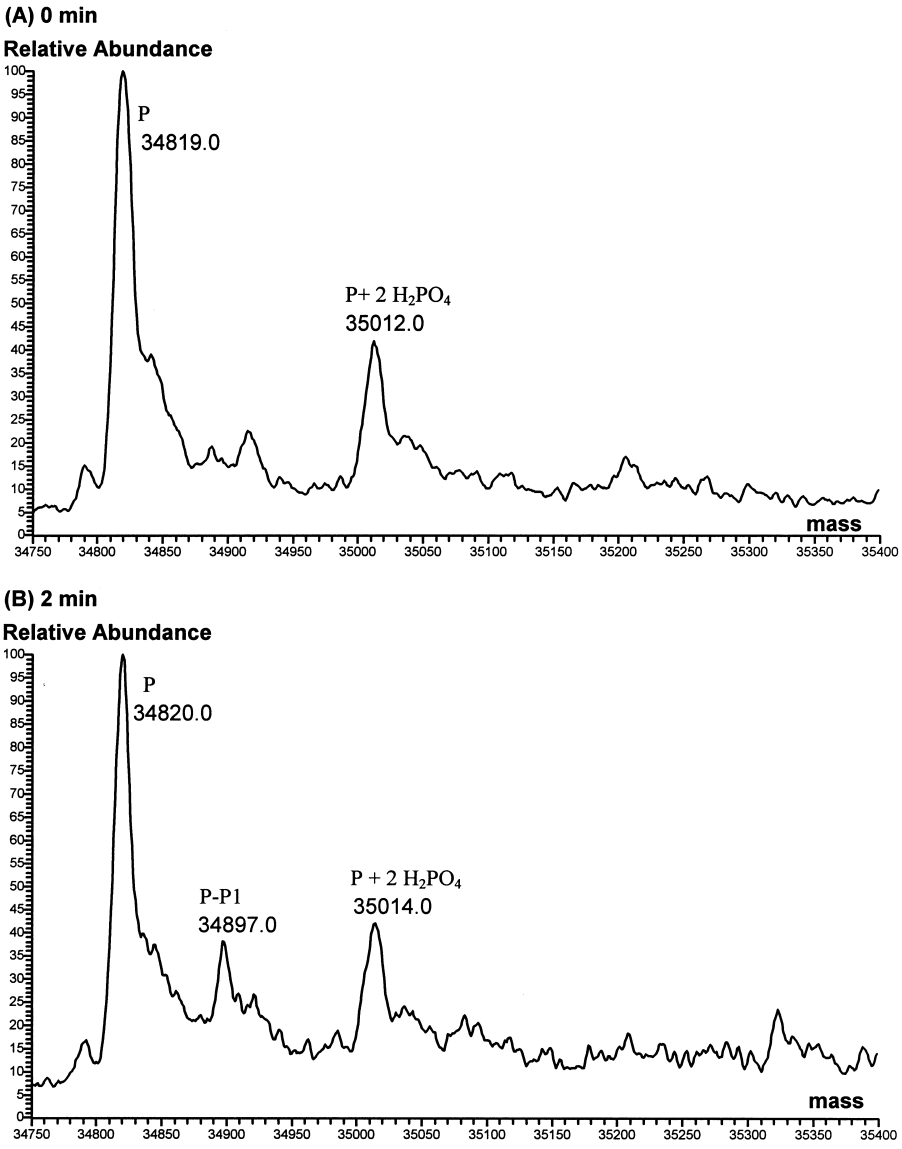


Fig. 1. Phosphorylation by cAPK results in phosphate incorporation into the cardiac MyBP-C motif (lanes 1 and 4), but not the slow (lanes 2 and 5) or fast (lanes 3 and 6) skeletal isoforms. This demonstrates that adrenergic regulation can act exclusively on the cardiac isoform. Lanes 1–3: Coomassie-stained gel; Lanes 4–6: autoradiograph of duplicate. M: marker proteins.



phorylated protein (Fig. 2A, B). After 90 min, the monophosphorylated protein was totally converted to the bis-phosphorylated (ca. 20%) and the tris-phosphorylated form (ca. 80%) (Fig. 2C). After ca. 5 h, the reaction was completed with 100% tris-phosphorylated protein. The sequential appearance of the three phospho-isoforms of MyBP-C supports the earlier notion that not all phosphorylation sites in the MyBP-C motif are equally used and that an ordered hierarchy of phosphorylation exists [12].

3.2. *The cosedimentation of the MyBP-C N-terminal region with myosin is sensitive to the phosphorylation state of MyBP-C*

We have recently established a cosedimentation assay using myosin minifilaments that detects the interaction of the MyBP-C motif with myosin-S2 [13]. In order to establish whether tris-phosphorylation of cardiac MyBP-C would influence this putative regulatory protein-protein interaction, we used unphosphorylated and the quantitatively prepared tris-phosphorylated c-C1C2 in this assay (see Section 2). The unphosphorylated c-C1C2 was found to cosediment with myosin minifilaments, signifying the binding to the myosin-S2 portion (Fig. 3). However, tris-phosphorylated c-C1C2 was found to remain in the supernatant, suggesting that the phosphorylated protein has a greatly reduced ability to interact with its myosin binding site (Fig. 3).

3.3. *The binding of cardiac MyBP-C to myosin-S2 is abolished by cAPK phosphorylation of MyBP-C*

For a detailed analysis of the effect of cardiac MyBP-C phosphorylation on myosin-S2 binding, we used ITC as a quantitative liquid phase protein interaction assay. Binding was assayed against the 126 residue S2 fragment S2-Δ which contains the MyBP-C binding site. Unphosphorylated c-C1C2 binds to S2-Δ with a K_d of ≈ 5 μM (Fig. 4A) as described previously [13]. Tris-phosphorylated c-C1C2 was prepared in the reaction monitored in Fig. 2 and further purified by Ni-NTA chromatography to remove excess cAPK and ATP after the reaction. The titration of S2-Δ with completely tris-phosphorylated c-C1C2 revealed only the heat of dilution of the protein sample but no specific binding reaction (Fig. 4B), the titration curve is therefore level. This demonstrates that the

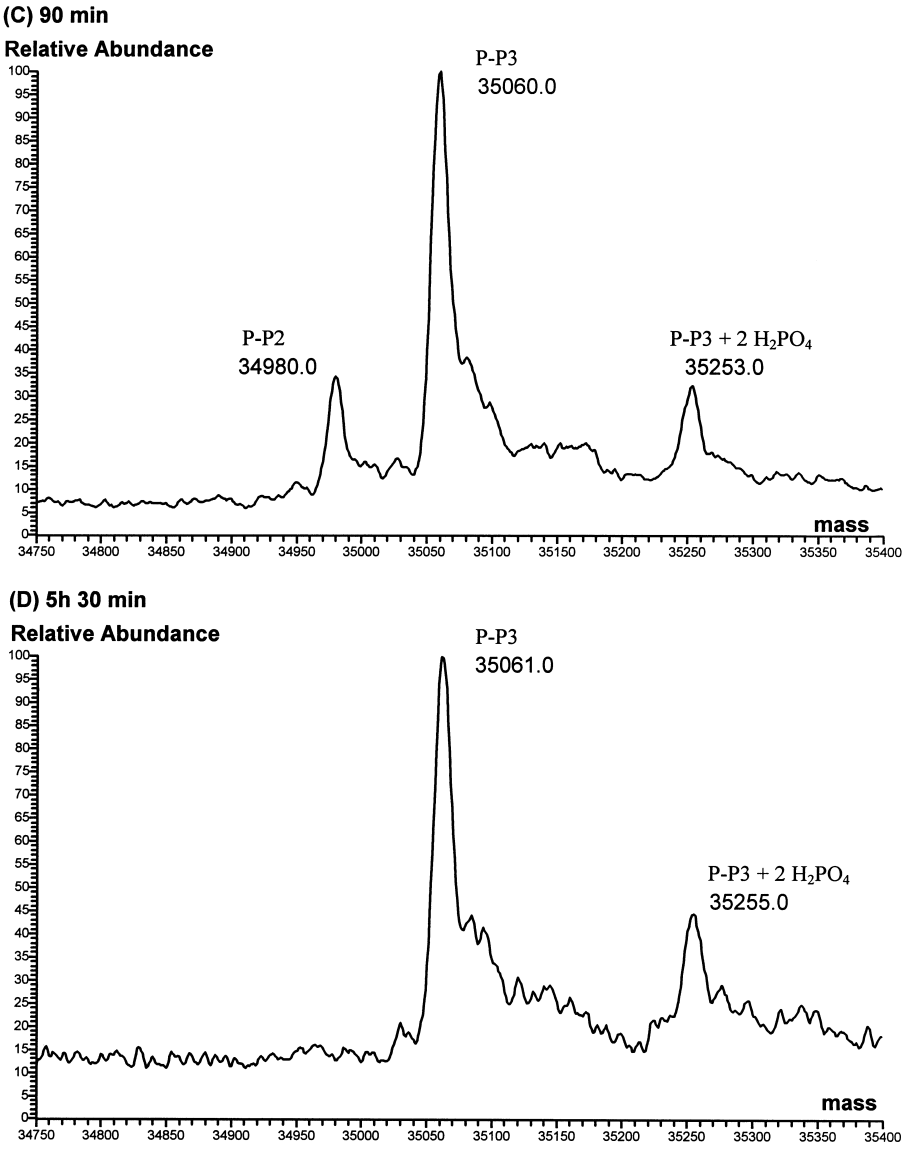


Fig. 2. Time-course of the sequential phosphorylation of c-C1C2 by cAPK. The control (reaction mixture at 0 min) shows the unphosphorylated protein (P) with a mass of 34 819 Da and several ion adducts (A). After 2 min incubation, monophosphorylated protein appears (P-P_1) (B). After 90 min, the pools of unphosphorylated and monophosphorylated protein have disappeared and both bis- and tris-phosphorylated protein (P-P_2 and P-P_3) are detected (C). After 5.5 h, the reaction led to a complete conversion to tris-phosphorylated protein P-P_3).

interaction of c-C1C2 with myosin-S2 is abolished by tris-phosphorylation or reduced to levels undetectable by ITC or cosedimentation.

4. Discussion

Regulation of protein-protein interactions by phosphorylation is a common mechanism to control cell function in eukaryotes. Both the response of assembled cellular structures as well as their assembly itself are regulated by phosphorylation of specific proteins by dedicated kinases. Sarcomeres, the contractile units of muscle, represent a complex and dynamic macromolecular assembly that responds to changes in physiological demands on the molecular level. Long-term regulation is achieved by changes in gene expression patterns [23], whereas short-term dynamic adaptations are mediated by protein phosphorylation. MyBP-C is one of three proteins rapidly phosphorylated when myocardium is stimulated by adrenergic agonists [10]. This stimulation results in an increase in contraction force and speed.

We demonstrated recently that the regulatory domain of c-MyBP-C interacts with a short segment of myosin-S2 close to the motor domain. This interaction raises the possibility that the regulation of cardiac contractility by MyBP-C could involve the mobility of the two myosin heads at their head-tail junction [13]. Recent ultrastructural analysis of phosphorylated and unphosphorylated native thick filaments suggested a swinging out of myosin heads concomitant with the phosphorylation of cardiac MyBP-C [24,25].

We demonstrate here that the interaction of the N-terminal regulatory domain of cardiac MyBP-C (MyBP-C motif) with the myosin-S2 domain is sensitive to cAPK phosphorylation of the MyBP-C motif. The interaction of both proteins, as judged from cosedimentation assays and ITC, is regulated in

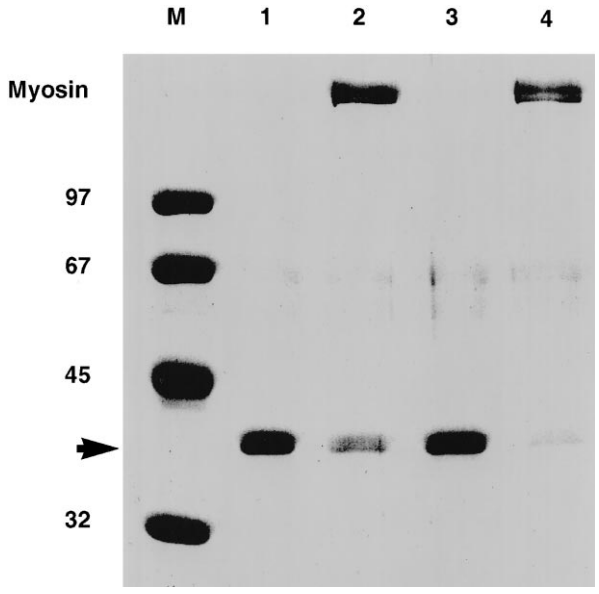


Fig. 3. Cosedimentation binding assay of unphosphorylated (lanes 1 and 2) and tris-phosphorylated c-C1C2 (lanes 3 and 4) to myosin minifilaments. Supernatants: lanes 1 and 3; pellets: lanes 2 and 4. Whereas the unphosphorylated control binds to myosin and is found in the pellet (lane 2), tris-phosphorylated c-C1C2 does not cosediment and stays in the supernatant of the reaction (lane 3). M: marker proteins.

an on-off fashion by tris-phosphorylation. It is possible, however, that in vivo phosphorylation intermediates may occur which result in a more subtle change in affinity. This on-off regulation suggests that whatever steric constraints would be imposed by MyBP-C either on the mobility of the myosin head-tail junction or the swinging out of the myosin head from the thick filament shaft, would be relieved by MyBP-C phosphorylation. This could (i) recruit heads for actin binding and hence force generation or (ii) alter the binding state and cycling rates of the two-headed myosin. Both mechanisms would be synergistic to the increased generation of active force that is achieved by the cAPK-mediated phosphorylation of sarcomeric proteins in cardiac muscle. The major phosphorylation site in cardiac MyBP-C is a cardiac-specific loop insertion in the MyBP-C motif of the sequence LAGGGRRIS [12]. Removal of this site restricts the accessibility to the two other cardiac cAPK sites. All three sites are not found in the skeletal isoform sequences. Since sequence comparison may overlook physiologically relevant phosphorylation sites, we tested the skeletal isoforms of MyBP-C experimentally for cAPK phosphorylation and found them to be no substrates. Recent work identified two phosphorylation sites for protein kinase C (PKC) in the cardiac MyBP-C motif [22]. These sites are also not found in the skeletal isoforms. Furthermore, investigations on intact heart muscle show no PKC-mediated phosphorylation of MyBP-C under physiological conditions [26,27]. Therefore, the selected interaction of MyBP-C with only few myosin heads must have a fibre-type-dependent modulating effect on contractility independent of the phosphorylation-dependent on-off switch of this interaction, which is a specific feature of cardiac muscle.

All isoforms of MyBP-C bear a specific sequence at the very N-terminus which, in the cardiac isoform, comprises a proline-alanine rich stretch and an additional Ig domain [16]. In addition, a short isoform-specific charged sequence is found in the MyBP-C motif [13]. The interactions of these regions are yet unknown. Some sequence homology to the N-terminal regions of regulatory light chains (not shown) and the spatial proximity to the myosin light chains suggest that this region might contribute to isoform-specific regulation possibly in concert with the regulatory light chain.

It is interesting to note that at least one point mutation in β-myosin-S2 which causes FHC has an identical net effect on the interaction of myosin and MyBP-C. The exchange of lysine-924 for glutamate also abolishes their interaction without affecting the dimeric coiled-coil structure of S2 [13]. Possibly,

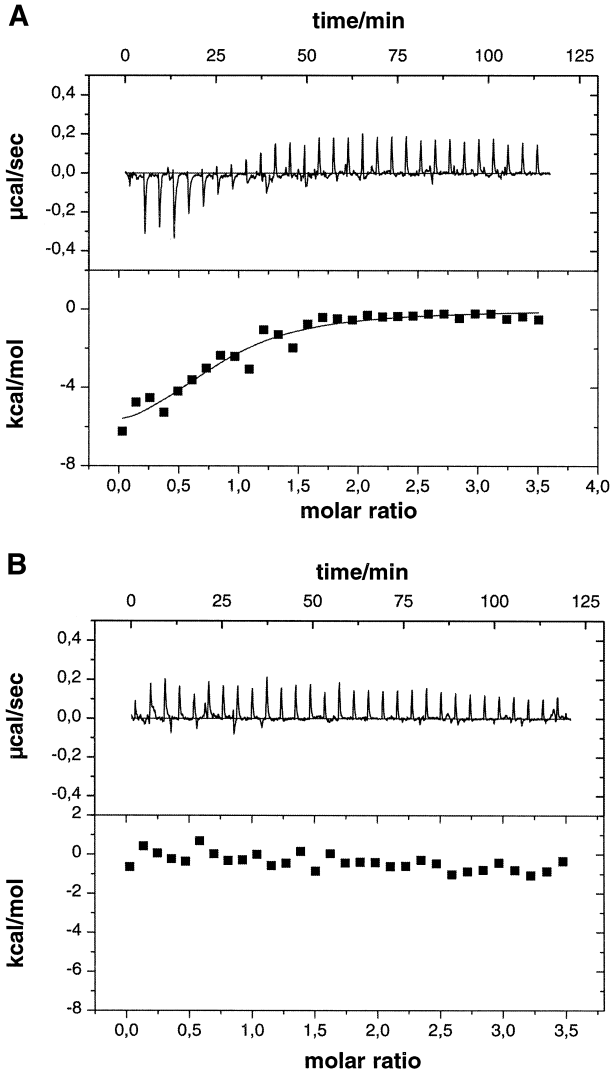


Fig. 4. The binding of c-C1C2 to myosin-S2 is regulated by phosphorylation. Isothermal calorimetry demonstrates the liquid phase interaction of S2-Δ with C1C2. S2-Δ is titrated against C1C2 in regular time intervals and the reaction heat is monitored as a change of the heating current (µcal/s, upper panels). Integration of the resulting peaks yields the titration plot (lower panel). (A) 20 µM c-C1C2 and 350 µM S2-Δ, (B) 20 µM tris-phosphorylated c-C1C2 and 350 µM S2-Δ. The experiments demonstrate the interaction of both proteins in a 1:1 complex in the unphosphorylated state and yield a dissociation constant of 5.2 µM for the interaction (A). The binding of both proteins is abolished after tris-phosphorylation of c-C1C2. only the heat of dilution is detectable (B).

therefore, some of the FHC-associated mutations in β-myosin-S2 could result in the constitutive mimicry of the phosphorylated state of MyBP-C and result in chronic dysregulation of contractility. Indeed, hypercontractility is a feature observed in several mutations causing FHC [28]. Our results provide the molecular basis for a functional investigation of the phosphorylation regulation of cardiac contractility in normal and diseased heart by the interaction of myosin and MyBP-C.

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