Relation Between Crossbridge Structure and Actomyosin ATPase Activity in Rat Heart

Andrea Weisberg, Saul Winegrad

Abstract—Cardiac myofilaments contain proteins that regulate the interaction between actin and myosin. In the thick filament, there are several proteins that may contribute to the regulation of the contraction. The myosin binding protein C, or C protein, has 4 sites that can be phosphorylated by a Ca\(^{2+}\)-calmodulin–controlled kinase, protein kinase A or protein kinase C. Using electron microscopy and optical diffraction, we examined the structure of thick filaments isolated from rat ventricles with either the \(\alpha\) or \(\beta\) isoform of myosin heavy chain (MHC) and the effect of specific phosphorylation of C protein on the structure. In thick filaments with \(\alpha\)-MHC, crossbridges were clearly visible. Phosphorylation of C protein by protein kinase A extended the crossbridges from the backbone of the filament, changed their orientation, increased the degree of order of the crossbridges, and decreased the flexibility of the crossbridges. Crossbridges in filaments with \(\beta\)-MHC were less ordered and apparently more flexible. Phosphorylation of C protein in \(\beta\)-MHC–containing filaments did not extend the crossbridges and did not alter degree of order or flexibility. The relative flexibility of the crossbridges inferred from the optical diffraction pattern correlated well with the rate of ATP hydrolysis by actomyosin. These results suggest that (1) crossbridge flexibility is an important parameter in setting the rate of crossbridge cycling, and (2) C protein–mediated control of the position and flexibility of crossbridges may regulate actomyosin ATPase activity by modifying the kinetics of crossbridge cycling. (Circ Res. 1998;83:60-72.)

Key Words: crossbridge ■ ATPase ■ C protein ■ myosin ■ phosphorylation

The performance of the heart is determined not only by the amount of force generated during a contraction but also by the velocity and extent of shortening and the efficiency of the conversion of biochemical energy to hydrodynamic work. Since it is generally believed that every cardiac myocyte contracts in each contraction of the normal heart, regulation of these properties of the contraction occurs within each myocyte. Physiological regulation of contraction can occur at 3 different locations in the contractile apparatus: the cytosolic concentration of Ca\(^{2+}\) ions activating the contractile proteins, the proteins in the thin contractile filament, and the proteins in the thick contractile filament. The concentration of cytosolic Ca\(^{2+}\) is modulated by the net effect of the Ca\(^{2+}\) conductance in the sarcolemma, the Ca\(^{2+}\) uptake and release systems in the sarcoplasmic reticulum, and the Ca\(^{2+}\)-Na\(^{+}\) exchanger in the sarcolemma. Phosphorylations of thin-filament proteins, in particular, the inhibitory subunit of troponin, modify the binding of Ca\(^{2+}\) to the thin filament and the nature of the interactions among the proteins within the thin filament.\(^2,3\) This can alter the interactions of crossbridges with their binding sites on actin in the thin filament.

Two proteins in the thick filament, RLC and myosin binding protein C (or C protein), can be phosphorylated. MLCK can phosphorylate RLC. PKC, but not cAMP-regulated PKA, can also phosphorylate RLC. The effect of the phosphorylation of RLC is to increase the amount of tension developed at submaximal levels of Ca\(^{2+}\) without changing the maximum Ca\(^{2+}\)-activated force, possibly by increasing the flexibility of the crossbridges.\(^4,5\) C protein has 4 phosphorylation sites per molecule in cardiac muscle, in contrast to the single site found in skeletal muscle.\(^6–10\) The 4 sites do not appear to behave identically. One site is preferentially phosphorylated by CAMK, which is bound to the thick filament. This kinase is a different protein from MLCK. The other 3 sites can be phosphorylated by PKA or PKC and, to some extent, by CAMK. Some evidence suggests that phosphorylation of the CAMK-regulated site is required before either PKA or PKC can phosphorylate the remaining sites.\(^10\)

There is controversy about whether phosphorylation of C protein plays any important role in regulating the properties of the contraction. Data show that C protein influences actomyosin ATPase activity and the kinetics of crossbridge cycling and that its phosphorylation modifies actomyosin ATPase activity and the rate of relaxation.\(^8,11–16\) However, the failure of the specific phosphorylation of C protein to alter the Ca\(^{2+}\) sensitivity of actomyosin ATPase activity in reconstituted contractile protein systems has been cited by some as strong evidence against a role for phosphorylation of C protein in regulating the contraction.\(^17–19\) It has been suggested that C protein is primarily important in the formation and organization of the proteins in the thick filament. Reconstituted protein systems do not, however, reproduce the normal...
Selected Abbreviations and Acronyms

CAMK = Ca$^{2+}$-calmodulin–regulated kinase
MHC = myosin heavy chain
MLCK = Ca$^{2+}$-calmodulin–regulated kinase in thick filament
PKA, PKC = protein kinase A and C
RLC = regulatory light chain of myosin

The results show that the positions of the crossbridges are different in filaments containing α- or β-MHC. C protein phosphorylation changes the structure and position of crossbridges in thick filaments with α-MHC in ways that are compatible with the alterations in crossbridge cycling already reported. The same changes in structure of crossbridges do not occur in thick filaments with >95% β-MHC. A structural change in cardiac thick filaments from phosphorylation of C protein with only α-MHC has already been reported.

The present study examines the difference in structure between isolated cardiac thick filaments with α- and β-MHC and the difference in the response of each to phosphorylation of C protein. The validity of differences in structure between different types of isolated thick filaments and isolated filaments subjected to different protocols is enhanced if the different filaments or protocols are studied simultaneously under similar conditions, eg, the same reagents, solutions, and grids coated with carbon at the same time. For this reason, some of the experiments with thick filaments containing α-MHC that were necessary for the comparison but have already been described have been repeated. Electron micrographs of filaments with higher magnification and resolution are now included for both types of filaments (α- and β-MHC), providing more information about differences in the respective crossbridges.

Materials and Methods

Preparation of Isolated Thick Filaments

Isolated thick filaments were prepared by a modification of the method of Kensler and Levine. Hearts from 19 euthyroid and 9 hypothyroid Wistar rats weighing 175 to 250 g were used. Filaments with >95% α- or β-MHC were isolated respectively from euthyroid rats and 8- to 10-week-old rats that had been thyroidecтомized at 5 weeks. Isoforms of MHC were determined by native gel electrophoresis and densitometry. The details of the procedure for preparing the isolated thick filaments have already been reported. The degree of phosphorylation of filaments with α- and with β-MHC were measured using the same procedure that has been reported in detail. Retention of C protein in the filaments with β-MHC during their isolation was determined in filaments, as already described in detail.

Optical Diffraction

Thick filaments lying on the carbon over the holes in the grids were viewed with a JEOL transmission electron microscope and photographed at >20,000 magnification. The electron micrograph negatives were diffraacted in a laser optical diffractometer (2-mW helium-neon, 632.8 nm) using a diffractometer camera that had been calibrated from diffraction patterns of micrographs of catalase crystals. The diffraction pattern from single thick filaments was produced by masking material in other areas of the electron micrograph during the period of illumination with the laser. Measurements of diffraction pattern spacings were made on large prints of the transform. The use of optical diffraction to study the structure of thick filaments and crossbridges has been well established, and the theory has been described in detail.

The relative intensities of the first-order reflections were determined by comparing their intensities with the average intensity of the reflections along the meridian between 43 and 14 nm. The procedure was chosen in order to provide an internal standard in each film in order to avoid variations from different batches of film or developing reagents. A direct comparison of the intensity of the reflection among the optical diffraction patterns gave similar results as long as the exposure and development times were the same. The intensity of

Thick-filament structure or the steric arrangement of contractile proteins in a filament lattice. It is very unlikely that the normal strain developed within the crossbridges attached to actin in the normal intact contractile filament lattice is reproduced in reconstituted systems. From both theoretical and experimental considerations, this strain is very important in the kinetics of crossbridge cycling, especially in the rate of their detachment, which is believed to be a crucial factor in determining the velocity of shortening and the efficiency of energy transduction. Velocity and efficiency are the same determining the velocity of shortening and the efficiency of their detachment, which is believed to be a crucial factor in altering components of crossbridge cycling, such as attachment and detachment from actin in the thin filament.

To test this possibility unambiguously, it is desirable to study the effect of C protein phosphorylation on the structure of the thick filament in the absence of the thin filament. Under these conditions, any effect of phosphorylation within the thin filament is eliminated. For this reason, we have studied the effects of phosphorylation of C protein on the structure of the crossbridges in negatively stained natural thick filaments isolated from normal hearts. From the electron microscope images and their optical diffraction patterns, useful information has been generated about the position of the crossbridge, its flexibility, and the relative degree of order.

Since PKA-mediated phosphorylation of cardiac myocytes has different effects on actomyosin ATPase activity depending on whether α-MHC or β-MHC is present, we have studied both types of hearts. In rat hearts with >95% β-MHC, there is no significant change in actomyosin ATPase activity associated with PKA-mediated phosphorylation of C protein and the inhibitory subunit of troponin. Phosphorylation in cells with α-MHC increases the actomyosin ATPase activity. Of the myofilament proteins that are phosphorylated, only C protein phosphorylation has a clear correlation with the direction and magnitude of the change in ATPase activity. If phosphorylation of C protein is important in regulating the kinetics of the contraction of heart muscle, one might expect to see a difference in its effect on thick filaments containing exclusively α- or β-MHC.
the reflections was measured by densitometry and, in some cases, by projection of the diffraction pattern on an array of photodetectors.

### Filament Thickness

For the measurement of the thickness of filaments, only those with clear central bare zones, a length of 1.55 to 1.65 μm, tapered ends, and visible periodicity were chosen. The distribution of crossbridges is helical, and the filament in mammalian cardiac muscle is 3-stranded. Because an electron micrograph is a 2-dimensional representation of a 3-dimensional structure, crossbridges are generally not seen directly opposite each other on either side of the filament. Measurements of the dimensions of individual filaments were made in 2 ways, each taking into account this asymmetry of crossbridge position in the micrograph. Each method gave the same result. Enlargements were made of each half filament, with a portion of the bare zone included in each print. A line was drawn along the outermost edge of the most extended crossbridges on either side of the filament away from the tapered ends, and the thickness of the filament was taken to be the distance between the 2 lines. Any effect of the small difference in the longitudinal position of the crossbridges on either side of the backbone was eliminated by this procedure. As a check, electron micrograph negatives were visualized directly through a stereomicroscope with a calibrated scale in one eyepiece, and the thickness was measured in the same way. It was important to measure the thickness of the filaments and not include the stain surrounding the filaments because the amount of stain deposited in the negative staining can vary, even in the presence of identical filaments with identical thicknesses. The values for each filament were averaged. Results were not significantly different when 2 different people who had not been informed of the protocol for the preparation of each filament made measurements on the same filaments.

### Statistics

Values are expressed as mean±1 SE. For comparisons between mean values for filament thickness, location of the center of mass of the crossbridges with respect to the axis of the thick filament, and the intensities of the reflections produced by optical diffraction, the t test was used. Differences were considered significant at P<0.05. Where multiple preplanned comparisons were made (Tables 1 and 2), the value of P is given after a correction for the multiple comparisons using the Bonferroni method.\(^*\) When the P value is <0.05 without the correction and slightly >0.05 with the correction, both values are given. The variances in the data populations in each comparison were tested for equality using the Levene test.\(^{33,34}\) The populations were tested for normality using the Lilliefors test\(^{15}\) with the SPSS Graduate Pack (SPSS Inc). The differences in variances were sufficiently small or nonexistent to support the use of the t test. The distributions of filament thickness were fit by the equations for 1 or 2 normal distributions with Origin 4.0 software (Microcal Software Inc), and the goodness of fit was measured by the \(\chi^2\) test.

### Isolated Thick Filaments

A majority of the isolated thick filaments retained their normal structure, as judged by length, shape, and periodicity (Figure 1). Crossbridges were recognized with negative staining.\(^{5,26,28-30}\) To be included in the study, each filament had to be at least 1.55 μm in length and have a central bare zone corresponding to the region normally devoid of crossbridges. The central bare zone had to equal 0.15 to 0.20 nm. Both ends of each filament had to be tapered. On each side of the central bare zone, there had to be a visible periodicity that equaled 14 nm or a simple multiple of 14 nm. Because crossbridges around the circumference of the filament are not separated by 180°, several different patterns of crossbridge repeats may be seen in the 2-dimensional electron micrograph.\(^{31}\) Several hundred filaments from 28 different hearts met all of these criteria and were included in the study. Periodicities of 14.4±0.1 and 43.1±0.1 nm were observed, which agree very well with the periodicity of the crossbridges and the helical arrangement (120° rotation every 14 nm) that have been determined by x-ray diffraction and electron microscopy of the intact filament lattice.

The absence of any significant loss of C protein or myosin light chains from the filaments with α-MHC during the isolation procedure has already been reported.\(^{24}\) During the isolation procedure, there was no loss of C protein from thick filaments isolated from hearts of animals that had been thyroidectomized at least 3 weeks earlier to convert the isoform of MHC from α to β. Treatment of β-MHC–containing thick filaments with PKA plus cAMP produced an

### Table 1. Thickness of Filaments and Position of Center of Mass of Crossbridges

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Mean Thickness, nm</th>
<th>P</th>
<th>Best Fit Modes, n</th>
<th>Center of Mass, nm</th>
<th>P</th>
<th>n^\text{1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MHC</td>
<td>32.6±0.3</td>
<td>...</td>
<td>31, 37</td>
<td>266</td>
<td>11.7±1.1</td>
<td>... 266</td>
</tr>
<tr>
<td>β-MHC–PO_4</td>
<td>35.1±0.3</td>
<td>0.003*</td>
<td>36</td>
<td>140</td>
<td>13.9±0.8</td>
<td>0.009* 140</td>
</tr>
<tr>
<td>β-MHC–PO_4</td>
<td>30.3±0.3</td>
<td>0.003*</td>
<td>25, 31</td>
<td>111</td>
<td>11.2±0.2</td>
<td>NS 213</td>
</tr>
<tr>
<td>α-MHC–PO_4</td>
<td>27.9±0.4</td>
<td>0.003*</td>
<td>27</td>
<td>53</td>
<td>10.4±0.1</td>
<td>0.021* 167</td>
</tr>
</tbody>
</table>
| PO_4 indicates C protein phosphorylation from PKA. The center of mass values are given as the distance from the axis of the filament. The number of measurements for the center of mass (n) is greater than that for the filament thickness (n) in order to increase the level of significance. Values are mean±1 SE. For comparisons between values (\(a\), \(b\), \(c\), \(d\)), theBonferroni method was used. Differences were considered significant at \(P<0.05\). Where multiple preplanned comparisons were made (Tables 1 and 2), the value of \(P\) is given after a correction for the multiple comparisons using the Bonferroni method.\(^*\) When the \(P\) value is \(<0.05\) without the correction and slightly \(>0.05\) with the correction, both values are given. The variances in the data populations in each comparison were tested for equality using the Levene test.\(^{33,34}\) The populations were tested for normality using the Lilliefors test\(^{15}\) with the SPSS Graduate Pack (SPSS Inc). The differences in variances were sufficiently small or nonexistent to support the use of the \(t\) test. The distributions of filament thickness were fit by the equations for 1 or 2 normal distributions with Origin 4.0 software (Microcal Software Inc), and the goodness of fit was measured by the \(\chi^2\) test. 

### Table 2. Relative Density of the 43-nm Reflection

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Relative Density*</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±1</td>
<td>...</td>
</tr>
<tr>
<td>PKA-treated α-MHC</td>
<td>139±2</td>
<td>0.001 vs control α-MHC</td>
</tr>
<tr>
<td>Control</td>
<td>52±2</td>
<td>0.037 vs control α-MHC</td>
</tr>
<tr>
<td>PKA-treated β-MHC</td>
<td>56±2</td>
<td>NS vs β control</td>
</tr>
</tbody>
</table>

\*Relative units.

Values are mean±1 SE.
increase in the phosphate content of the filaments of 2.4±0.6 mol/mol, a value not significantly different compared to what was observed and reported with α-MHC–containing filaments (P=0.57 for a t test comparing the 2 sets of data; data not shown).

**Filament Structure**

**α-MHC Filaments**

In the vast majority of filaments from euthyroid hearts, crossbridges were extended from the backbone of the filament in a helical arrangement with a periodicity of ≈14 nm
Some crossbridges were approximately perpendicular to the backbone, whereas others formed smaller angles in an arrowhead conformation that, in general, pointed away from the bare zone. The amount that the crossbridges appeared to extend from the backbone varied from 2 to 7 nm, apparently depending on their position relative to the plane of the electron micrograph. The overall appearance of the crossbridges was one of relative disorder. The backbone of the thick filaments, estimated from the thickness of the filament between crossbridges, was \( \approx 20\% \) thicker than the bare zone.

In a small number of filaments from euthyroid hearts (<5%), the crossbridges were not extended. The filaments had the “sawtooth” pattern produced by crossbridges lying along the backbone of the thick filament. There was no region in which the backbone was clearly visible.

In <10% of the filaments, crossbridges existed in a more regular conformation with a 14-nm repeat, but they were not as apparent as the crossbridges in the majority of filaments described above. The crossbridges had a more spherical appearance than the more rodlike crossbridges present in the majority of filaments. The overall thickness of these 2 types of filaments varied more than in the majority of filaments that had more prominent crossbridges. The average was \( >32 \) nm.

The average thickness of the filaments in the regions where the crossbridges were present was 32.6±0.3 nm (Table 1, n>200). The distribution of thicknesses among these filaments deviated from normal and was complex because of the presence of more than one pattern of crossbridges. To examine this, the values for the thicknesses of control filaments were placed in a series of bins each differing by 2 nm (Figure 2A). The complexity of the distribution cannot be ascribed to skew because of the low value for skewness (0.22). The best fit of the data with 2 overlapping normal distributions (shown in Figure 2) is also inadequate (\( \chi^2=20.04 \)). The results are consistent with the presence of \( >1 \) (and probably \( >2 \)) forms of the thick filament. Such a mixed population could be the result of the low level of phosphorylation present in C protein at different sites or at different numbers of sites within the C protein molecules. The distinction among the several possibilities is not important for the major biological conclusions of the present study. Isolated control filaments from hearts of young euthyroid rats had central bare zones with a thickness of 16.1±0.1 nm.

\( \beta \)-MHC Filaments

The appearance of thick filaments from rats that had been thyroidectomized at least 3 weeks earlier to convert the MHC isoform to the \( \beta \) isoform was quite different from that of the filaments with the \( \alpha \) isoform (Figure 1). The crossbridges lay along the backbone of the filament forming the sawtooth pattern. In some spots, the ends of the crossbridges extended by a small amount, \( \approx 2 \) nm, compared with the 2 to 7 nm found in most thick filaments with \( \alpha \)-MHC.

The average thickness of these thick filaments containing >95% \( \beta \)-MHC was 30.0±0.3 nm, which was significantly different from that of control filaments from the euthyroid rats with >95% \( \alpha \)-MHC (Table 1). The thickness of filaments with \( \beta \)-MHC appears to be bimodal and is best fit by 2 normal

gaussian distributions with means at 25 and 31 nm (\( \chi^2=0.037 \)) (Figure 2B). Most (\( \approx 85\% \)) of the filaments are included in the thicker component, which has a mean value that is very similar to that of the majority of filaments with \( \alpha \)-MHC.

Effect of Phosphorylation of C Protein

Filaments With \( \alpha \)-MHC

After phosphorylation of C protein with PKA, almost all of the filaments had the crossbridges lying along the backbone of the filament without clearly visible ends. The filaments formed a sawtooth pattern, indicating that the crossbridges were lying close to the filament backbone instead of extending away from the backbone, as in the control condition (Figure 3). The thickness of the central bare zone was not changed by phosphorylation of C protein. The average thickness of the kinase-treated filaments was increased over the control filaments to 35.1±0.1 nm (n>100, Table 1). The values for thickness were fit best by a single gaussian
distribution with a single mean of 36 nm ($\chi^2 = 7.22$), which is very similar to the higher peak value for control filaments (Figures 2 and 4). These results indicate that PKA treatment had increased the thickness of the previously thinner filaments by extending the crossbridges, but in a different way from the extension that existed in the controls (not treated with PKA). The minority of filaments that were thick without the PKA exposure were not altered. Any possible skew in the distribution is likely to be due to the presence of a small amount of unphosphorylated C protein among the PKA-treated filaments. These results indicate that the whole filament outside of the bare zone appears to open up with the phosphorylation of C protein, and the crossbridges assume a more ordered pattern (see below).

In a few cases, a thick filament was lying parallel and next to a thin filament. In the region between the thick and thin filaments, crossbridges were extended from the former to the latter with a periodicity of 43 nm (Figure 5). Between the thick and thin filaments in this region, the thickness of the thick filament excluding the crossbridge was reduced, but it remained the same when the crossbridge was included. This suggests that when the crossbridge attaches to the thin filament, the body of the thick filament becomes more tightly packed again.

**Filaments With $\beta$-MHC**

There was no meaningful change in the appearance of the crossbridge pattern after phosphorylation of C protein (Figure 6). The sawtooth pattern remained. This is in contrast to the changes produced in the thick filaments with $\alpha$-MHC after phosphorylation. The only consistent change that was detected was a small decrease in overall thickness of the filament outside the bare zone equivalent to a retraction of the crossbridges of $1.2 \pm 0.1$ nm (Figure 3 and Table 1). The bare zone itself was unchanged.

After treatment with PKA, the population of filaments was well fit by a single normal distribution with a mean value of
27 nm ($\chi^2 = 4.32$) (Figure 7). This value is similar to the value for the minor component of the control filaments from the hypothyroid rats. Any skew could have been produced by the presence of a small fraction of thicker filaments, consistent with the absence of complete phosphorylation of C protein.

The different changes in thickness of filaments with $\alpha$- and $\beta$-MHC induced by C protein phosphorylation indicated that the phosphorylation of C protein produces different changes in structure of the thick filament depending on the specific isoform of MHC.

**Optical Diffraction Patterns**

**Filaments With $\alpha$-MHC**

Electron micrographs of the individual filaments were illuminated by laser light, and the diffraction patterns were recorded. The reflections that were visible were produced by periodically arranged structures in the thick filament and fell along meridional layer lines because of the helical arrangement of structure in the filament. As the most prominent periodic structure in the filament, the crossbridges are responsible for most of the diffraction pattern. In filaments with well-ordered crossbridges (as with phosphorylated C protein; see below), strong reflections along as many as 6 layer lines were present (Figure 8). Reflections along the 43-nm layer line (first layer line) were clearly visible in the diffraction patterns of all filaments. These reflections are produced primarily by the helical arrangement of crossbridges around the thick filament. Other reflections produced by the crossbridges, such as those along the 14-nm layer line (third layer line), were generally visible on the meridian in phosphorylated filaments but were seen in only a small minority of control filaments. The 14-nm reflection is due to the periodicity of the crossbridges along the filament. It is normally less intense than the 43-nm reflection. Reflections on the meridian can be produced either by crossbridges or by the backbone of the filament. C protein present, at 43 nm along the filament, also contributes to the meridional reflections. For this reason, the characteristics of the off-meridional layer line reflections were used to extract unambiguous information about the crossbridges.

The optical diffraction patterns contain at least 2 different kinds of information relevant to the structure of the crossbridges. The intensity of the reflections along the 43-nm layer line is significantly higher in phosphorylated filaments than in control filaments. This difference is likely due to the increased periodicity of the crossbridges in the phosphorylated state.
28-, and 14-nm layer lines is influenced by the degree of order of the crossbridges: the greater the order, the greater the intensity. The degree of order, in turn, is a function of the degree of flexibility of the crossbridges, with greater flexibility producing decreased order. A change in mean orientation of the crossbridges can also alter the intensity of the reflection, but the magnitude of this change in the absence of altered flexibility should be small. The first maximum reflection along the 43-nm layer line corresponds to the first maximum of the third-order Bessel function, and its distance from the meridian is an indication of the distance of the center of mass of the crossbridges from the filament axis (the equations for the calculations are given in References 25, 26, and 37). Since the reflections exist in reciprocal space, the closer the reflection is to the meridian, the farther the center of mass of the crossbridge is from the axis of the filament. Because the deposition of stain in the negative staining process was not always uniform, symmetrical reflections were not always seen in all 4 quadrants of the diffraction pattern, but diagonal symmetry was always present and was sufficient both to ensure the significance of the reflection and to make quantitative measurements to calculate crossbridge positions.

The optical diffraction pattern produced by control filaments with α-MHC gave good patterns along the 43-nm layer line (Figure 8, top). In ≈85% of the control filaments, the position of the first maximum indicated that the center of mass of the crossbridges was 11.7±0.1 nm from the axis of the filament. The optical diffraction pattern of the filaments that had been treated with PKA plus cAMP differed from the control in important ways (Figure 8, middle). The average intensity of the first maximum reflections along the 43-nm layer line was 39±2% greater than that of the control (P=0.001), and reflections along the second to the sixth layer lines were stronger (Table 2). This indicates that after C protein phosphorylation, the crossbridges are more ordered as a result of decreased flexibility. After treatment of the filament with PKA, the first maximum along the 43-nm layer line was always closer to the meridian. The amount corresponded to the center of mass of the crossbridges 13.9±0.1 nm from the axis of the thick filament, a 2.2-nm extension of the crossbridges from the backbone of the thick filament.

In ≈15% of the control filaments, the first maximum along the 43-nm layer line was closer to the meridian than in the other 85%, located at the same position as the first maximum of the PKA-treated filaments. In these filaments, the intensity of the 43-nm reflections was different from that in the majority of control filaments. This type of minority pattern (Figure 8, bottom) occurred when the control filaments were thicker and is probably the result of a mixture of radial crossbridge positions along the thick filament with the majority extended to the same extent as in the PKA-treated filaments.
Filaments With β-MHC
There were important differences between the optical diffraction patterns of the α- and the β-MHC–containing control filaments. The intensity of the reflections along the 43-nm layer line was significantly weaker than those produced by α-MHC–containing filaments (Figure 9). The intensity of the first maximum in the β-MHC–containing filaments was 47±3% lower (Table 2). This indicated that the β-MHC–containing crossbridges were less ordered and that they were more flexible than the α-MHC–containing crossbridges. The distance of the first maximum from the meridian was not significantly different from that produced by α-MHC–containing filaments. These data indicate that the structure of the crossbridges differs with the 2 myosin isoforms but that the average position of the center of mass with respect to the axis of the filament is the same. This is consistent with the similarity in filament thickness in filaments with the 2 isoforms of MHC.

There was no significant change in intensity of the reflections along the 43-nm layer line after incubation with PKA plus cAMP, unlike the response of the α-MHC–containing filaments (Table 2). This indicates that no significant change in the flexibility of the crossbridges at the level of detection of this technique has been produced by the C protein phosphorylation. There was a small but significant movement of the first maximum away from the meridian, one that would be produced by a retraction of the center of mass of the crossbridges of 0.8 nm (Table 1).

Discussion
There are 2 major new conclusions from the results of the present study: (1) the structure of the crossbridges in the thick filaments is different with different isoforms of MHC, and (2) crossbridges with different isoforms of MHC react to phosphorylation of C protein differently. When the isoform of MHC is α, phosphorylation of C protein decreases the crossbridge flexibility and appears to change the packing of the myosin rods within the backbone of the thick filament. Phosphorylation of C protein does not alter crossbridge flexibility when the isoform of MHC is β, but it does retract the crossbridge by a small amount. Comparisons of the structure of the thick filaments with physiological measurements of velocity and actomyosin ATPase activity indicate good correlation between the relative degree of flexibility of the crossbridges and their rate of cycling, as indicated by actomyosin ATPase activity and maximum velocity of unloaded shortening (Figure 10).

C Protein
C protein is a large molecule that, according to antibody studies, is located in the thick filament every 43 nm within the central portion of each half of the A band. The periodicity of the C protein corresponds very closely to that of the helically arranged crossbridges in a 3-stranded array of myosin molecules in the thick filament. There is a myosin binding domain near the C terminus of C protein and C protein binding domains in myosin in the thick filament. There is a myosin binding domain near the C terminus of C protein and C protein binding domains in myosin in the thick filament. These results indicate that C protein is closely associated with myosin in the thick filament and binds to a functionally critical portion of the myosin molecule. From the nature of the binding, one might predict that C protein would have a significant effect on crossbridge movement and myosin packing. In fact, when myosin and C protein were coexpressed in COS cells, which normally do not have myosin II (the form in muscle) or C protein, the presence of C protein influenced the packing of myosin molecules in myosin polymers. In the presence of C protein, there were longer filaments, and their packing appeared to be tighter.

C protein is not found in all striated muscle. It is a major component of mammalian cardiac muscle and fast glycolytic muscle, but it is absent or present in only small amounts in mammalian red skeletal muscle. There are 4 phosphorylation sites in the cardiac isoform, in contrast to 1 site in the skeletal isoform. A highly specific kinase bound tightly to the thick filament phosphorylates a specific site in C protein and may influence the phosphorylation of the remaining PKA-sensitive
sites, strongly suggesting that important posttranslational changes in C protein can alter the structure of myosin and the crossbridge.

C protein contributes to the 43-nm reflections in striated muscle and may also contribute to the "forbidden reflections" found in resting striated muscle that are presumed to be due to imperfections in the order of protein arrangements in the thick filament. During contraction of some striated muscle, reflections associated with mass at the locations of C protein and the forbidden reflections markedly diminish or disappear. This change in the diffraction pattern associated with C protein or a nearby protein suggests that a change in C protein structure occurs during the contraction.

Taken collectively, these data provide a strong reason for considering that C protein and its posttranslational modifications through phosphorylation have an important role in the structure of the myosin molecules in the thick filament of cardiac muscle and changes in the structure that occur with contraction.

Structure of Thick Filament Depends on MHC Isoform and Phosphorylation

There are major differences in the thick-filament structure depending on whether the isoform of MHC is α or β. With α-MHC, the crossbridges are generally substantially extended, but when the β isoform is present, the crossbridges lie along the filament apparently oriented along the long axis of the filament. Although the crossbridges on most of the control filaments were extended and relatively disordered when α-MHC was present, there were other conformations in a minority of filaments. These conformations cover a range of crossbridge positions relative to the backbone, from lying flat in a position essentially parallel to the long axis of the filament to extension of several nanometers. It is possible that these different conformations in the non–PKA-treated filaments are related to the small amount of phosphorylation of C protein that is present, equivalent to a bit less than 1 phosphate per molecule of C protein.

Selective phosphorylation of the C protein in α-MHC-containing thick filaments isolated from rat ventricles produces 3 detectable changes in crossbridge structure: (1) an expansion of the backbone, including an outward movement of the end of the crossbridge of ~2.5 to 3 nm (using the mean values or the modes of the distribution on the histograms respectively), bringing it to what would be the surface of a thin filament in the intact filament lattice; (2) a similar movement of the center of mass of the crossbridge away from the backbone of the thick filament, and (3) an increase in the order of crossbridges. Phosphorylation of C protein in filaments with β-MHC does not change the flexibility of the crossbridges but does cause a small movement of the crossbridge toward the backbone of the filament. Since the center of mass and the end of the crossbridge move in the same direction, it is reasonable to conclude tentatively that the locus of at least part of the change in the crossbridge is the hinge region of the myosin molecule. C protein binds to myosin in subfragment 2, and α and β isoforms differ in amino acid sequence at the hinge region. The difference consists of 10 nonconservative amino acid substitutions, including 2 charged amino acids.

The phosphate could be uniformly distributed among the molecules of C protein, or if phosphorylation of C protein is cooperative, it may be nonuniform. The possibility of 2 phosphorylation-dependent structural states of crossbridges in the same filament when there are 4 phosphorylation sites on C protein suggests that some form of cooperativity among these sites may exist. The results from molecular biological studies of cardiac C protein indicate that phosphorylation of 1 site by a calmodulin-regulated kinase bound to the thick filament may facilitate the phosphorylation of the remaining sites by PKA. This property could lead to cooperative binding of phosphate by C protein. Since phosphorylation may occur first at the site preferentially responsive to CAMK, these may be the sites phosphorylated. Differences in filament structure could therefore be due to different degrees of phosphorylation of the CAMK-regulated site. To resolve this conundrum, it will be necessary to produce uniform verifiable phosphorylation in individual isolated filaments.
Relation Between Crossbridge Structure and Actomyosin ATPase Activity

If the changes in the structure of the crossbridges have a significant effect on the kinetics of cycling, there should be some correlation between the structure of the crossbridges and both actomyosin ATPase activity and maximum velocity of unloaded shortening. The ATPase activity at maximum Ca\textsuperscript{2+} activation and the maximum velocity are believed to be determined by the rate constants for the attachment and detachment of the crossbridges from actin.

Two components of the diffraction pattern of the thick filament, the intensity of the first maximum along the 43-nm layer line and its distance from the meridian, which indicate, respectively, relative flexibility and position, were compared with the ATPase activity and maximum velocity of shortening. Four conditions were examined: preparations with >95% \(\alpha\)- or \(\beta\)-MHC and with and without PKA-mediated phosphorylation.

There is some disagreement in the literature concerning the effect of \(\beta\)-adrenergic stimulation on the kinetics of crossbridge cycling and the maximum velocity of unloaded shortening, but we feel that the preponderance of evidence favors an effect of PKA-induced phosphorylation on crossbridge cycling. Work by Hasenfuss et al., Hoh et al., Berman et al., Strang and Moss, and McClellan et al. all show an increase in the turnover rate of crossbridges with \(\beta\)-adrenergic stimulation or activation of PKA. An increase in actomyosin ATPase activity has been observed by use of quantitative histochemistry and, more recently, by use of the NADH-coupled technique. Indirect evidence for an increase in ATPase activity has been generated by myothermal measurements. Further support for an effect of PKA on crossbridge kinetics comes from studies of contractile economy and efficiency. There is a decrease in the efficiency and/or economy of contraction, implying an increase in ATPase activity that is greater than the increase in tension.

Hofmann and Lange and de Tombe and ter Keurs have failed to find any change in maximum velocity of shortening, but there are reasonable explanations for the different results in each of these studies. de Tombe and ter Keurs found shortening velocities of their untreated preparations to be >7 lengths/s, a value that is 50% higher than is usually found after \(\beta\)-adrenergic or PKA stimulation. It is quite likely that their preparations retained the effects of the strong \(\beta\)-adrenergic stimulation when the animal was killed for study. In contrast to the intact preparations of Hasenfuss et al., others, Hofmann and Lange used Triton-treated isolated cardiac myocytes, in which endothelial cells were clearly disrupted. McClellan and colleagues have shown that response of the crossbridge cycling to cAMP treatment can be inhibited by disrupting endothelial cells and that it can be altered by changes in the level of activity of \(\alpha\)-adrenergic activity and, probably, PKC activity.

There is an excellent correlation between the relative intensity of the 43-nm reflection and both ATPase activity and maximum velocity of unloaded shortening (Figure 10). Values used for comparing ATPase activity and velocity to filament structure have been taken from our published work. Whereas the apparent linearity of the relation may be fortuitous because of the small number of points, the important conclusion is the high degree of significance of the relation. Since the cause of most or all of the difference in intensity of the reflections is a difference in the flexibility of the crossbridges, it appears that the more flexible the crossbridge is, the lower the ATPase activity and the slower the cycling rate of crossbridges. This holds true whether the ATPase activity and the maximum velocity of shortening differ because of the sequence of amino acids in the 2 isoforms of MHC or a posttranslational modification by phosphorylation. The difference in crossbridge structure could lead to a lower rate of detachment of the crossbridge from the thin filament and a slower rate of crossbridge cycling. The range of movement of a cycling crossbridge within a thick filament may be larger as well. This change in kinetics could explain or at least contribute to the slower velocity of shortening, the lower rate of ATP hydrolysis, and the greater efficiency of energy transduction found with contraction of \(\beta\)-MHC–containing crossbridges.

Although a change in actomyosin ATPase activity is produced by PKA-mediated phosphorylation when the \(\alpha\)-isoform of MHC is present, no change in actomyosin ATPase activity occurs with only \(\beta\)-MHC present in myocytes. However, in cardiac myocytes with a mixture of \(\alpha\)- and \(\beta\)-MHC, PKA-induced phosphorylation decreases (and may completely inhibit) the ATP hydrolysis by the crossbridges containing \(\beta\)-MHC. This difference in response can be explained by the changes in the position of the crossbridges with \(\alpha\)- and \(\beta\)-MHC produced by phosphorylation of C protein. After PKA-induced phosphorylation of C protein, the less extended \(\beta\)-MHC–containing crossbridges could have a sufficiently lower probability of binding to actin than the more extended crossbridges containing \(\alpha\)-MHC to markedly reduce or even eliminate the attachment and transition of the \(\beta\)-MHC–containing crossbridges through a complete crossbridge cycle. In the absence of the more extended \(\alpha\)-MHC–containing crossbridges, this “competitive domination” of crossbridge cycling by the faster crossbridges would not occur.

Physiological Role of C Protein and Its Phosphorylation

Two general types of arguments against a role for phosphorylation of C protein in the physiological regulation of cardiac contraction have been put forward. The failure of phosphorylation of C protein to alter the ATPase activity in reconstituted systems has been cited. As discussed above, reconstituted
systems are poor models for detecting changes in the contraction that result from changes in the steric relation of proteins in the thick and thin filaments. The structure of the reconstituted filaments differs from that of native thick filaments. The strain on the crossbridge normally developed during the generation of force and important in setting the rate of crossbridge detachment cannot develop in a reconstituted protein system. The combination of failure to detect effects in reconstituted systems and the presence of effects in models with intact filament lattice provides support for the hypothesis that regulation of crossbridge kinetics through C protein phosphorylation occurs by changes in the steric relation of the crossbridge to the thin filament.

The presence of C protein in only part of the thick filament has also been given as a reason why its phosphorylation would not have a physiological regulatory effect. There are at least 3 different ways in which phosphorylation of C protein in a limited region of the thick filament could alter the contraction. First, the change in the structure of the myosin molecules in the region where C protein is present could be transmitted through the rest of the thick filament by protein-protein interactions among the rod portions of the myosin molecules in the backbone of the thick filament. Second, the structural change could also be transmitted through the interactions with titin. Titin binds to the thick filament with the same periodicity as C protein. Third, mechanical coupling among crossbridges in a thick filament interacting with a thin filament could obviate the need for structural alteration of all crossbridges to change the characteristics of the contraction. A change in the cycling rate of as few as 5% to 10% of the total population of crossbridges can alter the kinetics of the entire population of crossbridges.

A mutation in cardiac C protein has been discovered in some families with hypertrophic cardiomyopathy, indicating that C protein is necessary for the normal development and/or normal contractile function of heart muscle. The mutation leads to a truncated C protein that does not contain the myosin binding domain. From studies performed so far, it has not been possible to distinguish between defects in the development of the thick filaments and abnormalities in function of normally formed thick filaments resulting from the mutation, but it is clear that C protein binding to myosin is essential for normal function. No studies on the effect of phosphorylation of the mutant have been performed.

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Andrea Weisberg and Saul Winegrad

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