The relaxed crossbridge pattern in isolated rabbit psoas muscle thick filaments

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SUMMARY

Rabbit muscle is a major source of material for biochemical experiments and spin labelling studies of contraction, and so it is important to establish how closely this material resembles the frog and fish muscles usually used for structural studies. Previous studies have shown that relaxed rabbit muscle thick filaments lose the characteristic order of their crossbridges when they are cooled below about 15-19°C, whereas the order of fish and frog muscles is retained above 0°C. The lack of order has frustrated attempts to examine rabbit thick filament structure and has raised questions about how closely they might resemble other thick filaments. We have therefore developed a procedure for preserving the crossbridge order in isolated filaments. Electron microscopy of these thick filaments after either negative staining or metal shadowing has shown that the crossbridge pattern has a 43 nm axial repeat and is based on three near-helical strands. Computed transforms of either type of image show a series of layer lines confirming that the native relaxed pattern has been preserved, and computer reconstructions show the individual crossbridges lying on a slightly perturbed 3-stranded lattice. These data indicate an unexpectedly high degree of similarity between the rabbit and frog patterns and indicate that, in fully preserved material, there is little structural difference between the two thick filaments at the temperature at which each normally functions. Overall, therefore, our results validate the integration of biochemical data from rabbit muscle with structural data from frog and indicate that the differences between the two muscle types do not result from fundamental structural differences in the arrangement of myosin molecules in their thick filaments.

Key words: vertebrate thick filaments, crossbridges, structure, electron microscopy, image processing

INTRODUCTION

The structure and function of vertebrate skeletal muscle has been studied extensively. Numerous biochemical studies of the contractile proteins (reviewed by Squire, 1981; Holmes et al., 1990) combined with physiological and mechanical studies (Huxley and Simmons, 1971; Ford et al., 1977, 1986; Brenner, 1987, 1989; Schoenberg, 1988) have provided a wealth of information about the mechanism of contraction and strongly support the sliding filament model of muscle contraction (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). However, biochemical studies have concentrated on using material prepared from rabbit or chicken muscle, whereas structural studies have tended to use frog muscle and occasionally fish muscle. Although the symmetry and crossbridge arrangement of fish and frog muscle has been established (Kensler and Stewart, 1989; Stewart and Kensler, 1986), that of rabbit muscle has not. Electron microscopy has shown that rabbit filaments are bipolar structures about 1.5 μm long (Huxley, 1963) and has given some important information on the location of several accessory proteins (Starr and Offer, 1971, 1983; Craig and Offer, 1976; Starr et al., 1985; Bennett et al., 1986). X-ray diffraction studies have shown a clear pattern of layer lines similar to that obtained from frog muscle (Rome, 1972), although the strength and quality of these patterns was often variable and sometimes inferior to those obtained with frog muscle (Rome, 1972; Huxley and Brown, 1967). Electron microscopy has also produced variable results. Some studies have indicated almost complete disorder under relaxing conditions (Trinick and Elliott, 1979, 1982) whereas other studies (Ip and Heuser, 1983; Menetret et al., 1990) using rapid freezing have supported a helical arrangement of crossbridges. A possible explanation for this variability has come from Wray’s X-ray diffraction studies (Wray, 1987), which showed that the helical ordering of the crossbridges in rabbit muscle was temperature sensitive. At temperatures below ~15-19°C, the crossbridges lose their ordered arrangement, whereas at higher temperatures the filaments appear to have a highly
ordered arrangement and gave X-ray diffraction patterns similar in quality to those obtained for frog (Huxley and Brown, 1967) or fish muscle (Harford and Squire, 1986).

Because of the widespread use of rabbit muscle in biochemical and other experiments, it is important to establish how closely it resembles fish and frog muscle. Therefore we have developed an isolation procedure for rabbit psoas thick filaments that retains the ordered arrangement of the myosin crossbridges, thus facilitating their examination using the electron microscopy and image processing methods employed for fish and frog muscles (Kensler and Stewart, 1983, 1986, 1989; Stewart and Kensler, 1986). This examination has demonstrated an unexpectedly high degree of similarity in the structure of rabbit and frog thick filaments near the temperature at which the muscles function, and so justifies the integration of biochemical data from rabbit and structural data from frogs when probing the mechanism of contraction in vertebrate skeletal muscle. Moreover, our results may account for some paradoxical results obtained when using molecular probes to follow crossbridge changes in rabbit muscle.

MATERIALS AND METHODS

Thick filaments were isolated from rabbit psoas muscle by a modification of the procedures previously reported for the isolation of frog (Kensler and Stewart, 1983, 1986) and fish (Kensler and Stewart, 1989) thick filaments. Long strips (6.25 cm) of the psoas muscle were excised quickly and placed in small amounts of an EGTA-saline solution containing 0.1 M NaCl, 2 mM EGTA, 1 mM diithiothreitol, 5 mM MgCl2, and 10 mM imidazole buffer (pH 7) at 4°C. After 10 min, each muscle strip was gently teased into a mesh of finer diameter bundles (0.5 mm - 1 mm) using the fine tips of one set of forceps to separate the bundles while a second pair of forceps was used to hold one end of the muscle strip. The teased muscle bundles were then transferred into fresh relaxing solution of the same composition except for the addition of 2.5 mM ATP. After 4 changes (1 h each) into fresh solution, the muscle was left overnight at 4°C in relaxing solution of the same composition. The muscle was then incubated for one additional hour in fresh relaxing solution prior to small portions of the muscle being minced with a razor blade immediately before homogenization. Homogenization was performed on ice with two 10 s bursts (separated by 30 s) at setting 6.5 of a Sorvall Mixer (Omni Corporation, Inc., Waterbury, Connecticut). The homogenate was diluted with additional relaxing solution to approximately 15 ml and centrifuged at 3,000 rpm for 5 min to pellet the large debris. The separated thick and thin filaments remained suspended in the supernatant and were examined with the electron microscope after either negative staining or platinum shadowing.

Negative staining was performed with slight modifications of our previously published tannic acid-uranyl acetate procedure (Kensler et al., 1985; Kensler and Stewart, 1986, 1989). In this procedure, thick filaments absorbed onto thin carbon films (5-7 nm thickness) supported on perforated Formvar-coated grids, were rinsed sequentially with eight drops each of half-strength relaxing solution, aqueous 0.25% tannic acid (Mallinkrodt AR 1764, Mallinkrodt, Inc., St. Louis, MO). half-strength relaxing solution, and negatively stained with 1% uranyl acetate. The half-strength relaxing solution contained 50 mM KC1, 1 mM ethylene glycol-bis(b-aminoethyl ether) N,N',N''-tetraacetic acid (EGTA), 2.5 mM MgCl2, 1 mM diithiothreitol, 2.5 mM ATP, and 5 mM imidazole buffer at pH 7.0. An important modification of the procedure from our previous description was that all rinse and stain solutions were maintained at a constant temperature of 25°C with a Thermolyne Type 17600 Dribath temperature incubator. This modification helped to avoid the loss of helical ordering of the myosin heads that has been reported to occur at temperatures less than 15 - 19°C (Wray, 1987).

Platinum shadowing was performed as described previously (Kensler and Stewart, 1986, 1989) except that a rinse with tannic acid was employed to help reduce the flattening of the filaments during drying. In this procedure thick filaments, adsorbed to standard thickness carbon films, were rinsed sequentially with 8 drops each of half-strength relaxing solution, 0.25% tannic acid, half-strength relaxing solution, 1% uranyl acetate, and 20 drops of 10% glycerol, prior to drying under vacuum in a Denton DV502 vacuum evaporator (Denton Vacuum, Cherry Hill, New Jersey) at a pressure <10−6 Torr for 1 h. Unidirectional shadowing of platinum from a tungsten wire was performed at a shadowing angle of 20 - 30° and a specimen-to-electrode distance of 7.5 cm.

Electron microscopy of the negatively stained and platinum shadowed preparations was performed either with a Philips EM300 electron microscope (Philips Electronic Instruments, Mahwah, NJ) or a JEM-1200EX-II electron microscope (JEOL USA Electron Optics, Peabody, MA). Magnification was calibrated using catalase crystals (Wrigley, 1968).

Optical diffraction and computer image processing was performed as previously described (Kensler and Levine, 1982; Kensler and Stewart, 1983, 1986; Kensler et al., 1985; Stewart and Kensler, 1986). Spacings on the diffraction patterns were calculated relative to the spacing (1/14.3 nm−1) of the meridional reflection on the third layer line (Wray, 1982). Images with 256 gray-levels were either photographed off the screen of a Focus Imagecorder plus graphics display system or photographed from laser printer generated gray scale images.

RESULTS

Negative staining

The filament isolation procedure described above yielded rabbit thick filaments that frequently displayed well ordered arrays of crossbridges in negatively stained preparations. In high magnification images (Fig. 1A-C) the filaments displayed a periodic appearance that can clearly be seen by sighting along the filament axis. The crossbridge pattern appeared to have an axial repeat every third crossbridge (42.9 nm), and in many places (arrows, Fig. 1A-C) crossbridges could be seen projecting from one side of the backbone with this periodicity. Careful inspection of the images suggested that the crossbridge pattern lacks the bilateral symmetry that would be expected (Moody, 1967) if the crossbridges were arranged along an even number of helical or near-helical strands. This was particularly evident (Fig. 1) where crossbridges could be seen projecting from one side of the backbone (Fig. 1A-C, arrows), while equivalent crossbridges did not project from the other side.

Although most of the filaments in the best preparations showed order, it was not uncommon to find filaments that appeared to lose the crossbridge order along one arm or showed well ordered regions broken by short regions of disorder (asterisks, Fig. 1A and C). This was much less frequently seen in negatively stained preparations of either the isolated frog or fish thick filaments (Kensler and Stewart,
and appeared to be consistent with the sensitivity of the rabbit muscle filaments to such environmental conditions as temperature and ionic strength (Wray, 1987, Yu and Brenner, 1989; Peterson and Kensler, 1992).

Fig. 1. (A-C) A gallery of high magnification images of negatively stained rabbit psoas muscle thick filaments. The arrows indicate the 43 nm axial periodicity, which can be seen along the filament arms in most regions, except for occasional small regions in which the filaments appear to lose order (asterisks in (A) and (C)). Note that the crossbridge array appears to lack the bilaterally symmetrical appearance that would be expected if the crossbridges lay along an even number of helical or near-helical strands. (D-F) High-magnification images of rabbit psoas muscle thick filaments unidirectionally shadowed with platinum. Note the periodic right-handed near-helical appearance of the shadowed filaments. This appearance can be seen to result from subunits lying along a series of near-helical strands, which occur every 43 nm axially, except at the bare zone. The sets of diagonal lines in (D) illustrate a region in which the right-handed near-helical arrangement of the subunits can be clearly seen, while the arrows in (D-F) illustrate the 43 nm axial periodicity of the helical strands along the filament. (A) ×104,000; (B) ×112,000; (C) ×112,000; (D) ×112,000; (E) ×112,000; (F) ×112,000.
Platinum shadowing

Unidirectional platinum shadowing of the isolated rabbit thick filaments was performed to determine the hand of the long pitch helical or near helical strands along which the myosin crossbridges lie. In high magnification images (Fig. 1D-F) many of the filaments showed a distinct right-handed helical arrangement of subunits with a 43 nm axial repeat (arrows). This helical pattern extended uniformly across both arms of the filament, but was absent at the bare zone, consistent with the idea that the helical pattern of subunits corresponded to the crossbridge arrangement. Each helical strand of subunits (corresponding to one-half turn of the full helix) appeared to extend over 4-5 crossbridge levels, and in some cases 4-5 subunits could be seen along each of the strands, consistent with the 9 subunits per full turn expected for a three-stranded helical arrangement with a helical repeat every 43 nm and an average axial spacing of 14.3 nm between crossbridge levels. The general appearance of these shadowed filaments was similar to that previously shown for both shadowed frog thick filaments (Kensler and Stewart, 1986) and shadowed fish filaments (Kensler and Stewart, 1989).

Computer image processing

Negatively stained filaments

Optical diffraction and computed Fourier transforms were used to assess the periodicity of the images of the negatively stained rabbit filaments. Both the optical diffraction patterns (data not shown) and computed Fourier transforms (Fig. 2C) showed a strong series of layer lines indexing close to the expected orders of a 43 nm helical or near helical repeat, with meridional reflections on the third and sixth layer lines, as expected from X-ray diffraction studies of living vertebrate skeletal muscle (Huxley and Brown, 1967; Rome, 1972; Harford and Squire, 1986; Huxley and Faruqi, 1983; Huxley et al., 1980, 1982; Padron and Huxley, 1986; Wray, 1987; Squire et al., 1991). The patterns typically extended to the 5th or 6th layer lines (Fig. 2C) and appeared similar to the optical diffraction patterns previously shown for the isolated frog thick filaments (Kensler and Stewart, 1983, 1986) in that the strength of the meridional reflection on the third layer line at 1/14.3 nm\(^{-1}\) was variable in intensity from filament to filament. Meridional reflections not expected from ideal helical symmetry were observed frequently both in the optical diffraction patterns and in the computed transforms, particularly on the 2nd layer line (1/21.5 nm\(^{-1}\)). These reflections probably correspond to the ‘forbidden meridional reflections’ previously described in the X-ray diffraction patterns from vertebrate muscle, and are thought to indicate the presence of a regular perturbation from ideal helical symmetry in the filaments (Huxley and Brown, 1967; Yagi et al., 1981; Squire et al., 1982). The similarity of the patterns to that expected from X-ray diffraction studies of the living muscle confirms that the regular arrangement of crossbridges had been preserved in isolated rabbit thick filaments, and suggests that the images reflect the in situ structure well.

The phase data for the primary maxima on the first layer line in the computed transforms from five negatively stained rabbit filaments were examined. Fig. 2K shows the amplitude and phase data along the first layer line for one of these filaments. Helical diffraction theory (Klug et al., 1958) and also the more general formulation (Stewart and Kensler, 1986) in terms of cylindrical symmetry predicts that these maxima should be in phase if the crossbridges lie along an even number of strands but 180° out of phase for an odd number. As shown in Fig. 2K the phase difference for the maxima was close to 180° out of phase, consistent with an odd-stranded structure. A similar phase difference of close to 180° was found for all five filaments, supporting this conclusion.

Further evidence for this conclusion was provided by filtered images (Fig. 2D-F) of the filaments obtained by computing the inverse Fourier transform of the layer line data for the first six layer lines of the transforms for each of the filaments. The maxima along the layer lines were not selectively masked and no assumptions about the symmetry, except that the structure had a 43 nm axial repeat, were included in the filtering. The filtered images showed the repeat in the crossbridge pattern every third crossbridge level (43 nm) as expected from the original images, the transforms, and the filtering operation itself. In filtered images the crossbridge pattern also lacked bilateral symmetry, consistent with the phase data, which indicated that the crossbridges lie along an odd number of strands. The appearance of the filtered images was similar to those

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Fig. 2. High magnification images of negatively stained filaments (A and B), and computer processing data for both the negatively stained filaments (C-F, K) and the unidirectionally platinum shadowed filaments (G-J). (A) and (B) show two very high magnification images of the same filament differentially labelled to show both the distinct 43 nm periodicity in the crossbridge array (A) and the helical paths along which the crossbridges appear to lie (B). The arrowheads in (A) indicate where crossbridges appear to project from the backbone of the filament at regular axial repeats of 43 nm. In (B) the solid lines and dotted lines illustrate the helical paths as seen on both the front (solid lines) and back (dotted lines) of the filament image. Note that the helical tracks appear to delineate a three-stranded helical pattern. (C) illustrates computed transforms of a negatively stained filament showing a series of layer lines (indicated by the arrowheads) indexing close to the expected orders of a 43 nm helical (or near-helical) periodicity, with a meridional reflection on the 3rd layer line. (D-F) show images from three different filaments filtered using the data along the first six layer lines. In (G) the transform of a shadowed filament shows a series of layer lines (arrowheads) indexing close to the expected orders of a 43 nm helical (or near helical repeat). The asterisks indicate maxima on the 1st layer line, which would not be expected to occur for a one-sided ideal helical arrangement, consistent with the presence of a regular perturbation from helical symmetry in the structure. (H-J) show filtrations using the 1st four layer lines for three different shadowed filaments. In each image four to five subunits can be seen along each of a set of helical (or near-helical) tracks. This is illustrated for a single track in (J) by the black dots at the center of each subunit, which indicate that each track extends over 5 crossbridge levels per half-turn of the helix. A similar pattern can be recognized for the other images. This number of subunits per half-turn of each strand is consistent with the nine crossbridges per full turn expected for a three-stranded arrangement of the myosin heads. (K) Phase and amplitude data along the 1st layer line as a function of the radial spacing from the meridian for a transform similar to (C) is shown. x346,000.
obtained earlier from both frog (Kensler and Stewart, 1983) and fish (Kensler and Stewart, 1989) thick filaments and was most consistent with a three-stranded arrangement of the crossbridges.

Additional direct support for this interpretation came from examination of the pattern of the helical or near helical paths along which the crossbridges appear to lie on very high magnification images (Fig. 2A and B) of better preserved filaments. In Fig. 2B the helical paths on the near and far sides of the filament have been marked, and it can
be seen that these appear to delineate a three-stranded pattern, which gives rise to the 43 nm periodicity (arrowheads, Fig. 2A).

Platinum-shadowed filaments

Fourier transforms of images of platinum shadowed rabbit thick filaments (Fig. 2G) showed the expected series of layer lines corresponding to orders of a 43 nm axial repeat, analogous to the results for negative staining. As we reported previously for both isolated frog (Kensler and Stewart, 1986) and fish (Kensler and Stewart, 1989) thick filaments, the patterns frequently showed departures from that expected for ideal helical symmetry. On the first, and frequently on the second layer lines, off-meridional maxima were often present on both sides of the meridian at similar radial spacings (asterisks, Fig. 2G), rather than on one side as expected from ideal helical symmetry. The intensity of these reflections in the rabbit appeared to be intermediate between the relatively strong similar reflections seen in the transforms of the frog (Kensler and Stewart, 1986) and the relatively weak reflections seen in the isolated fish thick filaments (Kensler and Stewart, 1989), although we have not quantified these results. Since we have seen these reflections in transforms of both lightly-shadowed and heavily-shadowed filaments, the idea that these reflections may result from residual negative staining seems unlikely. In the case of the frog and fish filaments we have shown that these reflections may result from a regular perturbation in the crossbridge array from ideal helical symmetry (Kensler and Stewart, 1986, 1989; Stewart and Kensler, 1986). Filtered images (Fig. 2H-J) of the unidirectionally shadowed rabbit filaments showed a right-handed helical or near-helical arrangement of subunits along the arms of the filaments, with an axial repeat every third crossbridge level. The filtered images typically showed either four or five subunits per half turn of each helical or near helical track, consistent with the nine subunits per turn expected for a three-stranded arrangement of the myosin crossbridges.

DISCUSSION

Rabbit skeletal muscle thick filaments are three-stranded

We have developed a protocol that preserves the relaxed crossbridge order of rabbit skeletal muscle thick filaments and have obtained electron microscopy evidence that establishes unequivocally that they have a three-stranded approximately helical symmetry. In negatively stained preparations the periodic appearance of the rabbit filaments was very similar to that seen previously in both frog (Kensler and Stewart, 1983) and fish thick filaments (Kensler and Stewart, 1989). There was a clear axial repeat in the crossbridge pattern every third crossbridge level (43 nm), and optical diffraction patterns and computed Fourier transforms of the negatively stained rabbit filaments generally resembled those obtained from low angle X-ray diffraction studies of relaxed living vertebrate muscle (Huxley and Brown, 1967; Haselgrove, 1975, 1980; Haselgrove and Rodger, 1980; Harford and Squire, 1986). As we have previously noted in our studies of the frog and fish thick filaments (Kensler and Stewart, 1983, 1989), this similarity suggests that the order we see in the crossbridge arrangement is a reflection of the in situ crossbridge arrangement, rather than an artifact of the isolation procedure. Although some details such as the precise tilt angles of the myosin heads may vary from the in situ structure, major features such as the strandedness of the crossbridge arrangement are unlikely to differ. Electron micrographs of negatively stained rabbit thick filaments also lacked the bilateral symmetry in the crossbridge pattern that would be expected for an even number of helical strands, thus suggesting that the crossbridges were arranged along an odd-number of helical strands. Analysis of the phase differences for the primary maxima on the first layer line of the computed transforms of the negatively stained rabbit filaments confirmed that there were an odd-number of strands. This was consistent with the three-stranded arrangement of the crossbridges that we have demonstrated for both the frog (Stewart and Kensler, 1986) and fish (Kensler and Stewart, 1989). Furthermore, the radial position of the primary maxima along the first layer line was only consistent with a three-stranded structure. Finally, examination of the computer filtered images of the negatively stained and platinum shadowed rabbit filaments also supported a three-stranded arrangement of the crossbridges in these filaments; the similarity in appearance of the computer filtered images of the negatively stained rabbit filaments to the filtered images previously obtained from the frog and fish filaments (Kensler and Stewart, 1983, 1989; Stewart and Kensler, 1986) further supports this conclusion.

Perturbations from ideal helical symmetry

X-ray diffraction data from frog muscle indicate that the myosin heads are perturbed from an ideally helical arrangement (Huxley and Brown, 1967; Yagi et al., 1981; Squire et al., 1982). The forbidden meridional reflections in optical and computed transforms of negatively stained filaments, together with additional off-meridional reflections not expected from ideal helical symmetry in transforms of shadowed filaments, indicated rabbit filaments also had some crossbridge perturbation. Frog thick filaments, which have substantial crossbridge perturbation, show similar patterns (Stewart and Kensler, 1986). Weaker reflections of this type are present in transforms of fish thick filaments (Kensler and Stewart, 1989), which are believed to have less perturbation from ideal helical symmetry (Harford and Squire, 1986). The present results do not enable the degree or nature of the perturbation or the precise shape and orientation of the heads to be determined accurately, which will require a full three-dimensional reconstruction from tilt series. However, the present results do demonstrate that this is feasible for rabbit filaments, and support the possibility of delineating both the similarities and differences in crossbridge arrangement of the thick filaments from different vertebrate classes.

Comparison with other vertebrate thick filaments

Overall our results suggest that relaxed rabbit skeletal muscle thick filaments are more similar to frog thick filaments than to fish. The rabbit filaments appear to differ
from the fish filaments in lacking the pronounced striping of the filament at the 14.3 nm axial spacings of each crossbridge level. The rabbit filaments are similar in this respect to the frog thick filaments, which also lack this strong striping (Kensler and Stewart, 1983, 1989). This may result from the myosin heads in both the rabbit and frog thick filaments lying more closely along the helical paths and overlapping with each other at adjacent crossbridge levels, thus reducing the density modulations at the crossbridge levels along the helical paths. This would be consistent with the weaker and more variable third layer line meridional maxima at 1/14.5 nm⁻¹ observed in both frog and rabbit filaments as compared to fish.

The high degree of similarity between the crossbridge arrangement in relaxed frog and rabbit skeletal muscle thick filaments indicates that biochemical and structural information from both sources can be integrated when investigating the mechanism of muscle contraction and thick filament assembly. However, the differences between these thick filaments and those from fish suggest that more caution may be needed in integrating information from these sources.

**Lability of crossbridge order**

An important difference between the rabbit filaments and both the frog and fish filaments is in the apparent lability of the crossbridge order. Although we were able to routinely obtain rabbit filaments with well-ordered arrays of crossbridges, the ordered relaxed crossbridge arrangement in these filaments appeared to be far more easily perturbed than that of either the frog or fish thick filaments. This observation would be consistent with X-ray diffraction studies that indicated that the relaxed crossbridge order of rabbit thick filaments was temperature sensitive, with filaments appearing to lose their crossbridge order at temperatures below ~15-19°C. This was also noted in the preliminary electron microscope study of Schröder et al. (1990) of these filaments. We have confirmed this lability, and shown that fish filaments do not show this temperature sensitivity (Peterson and Kensler, 1992). A more detailed account of these results will be published elsewhere.

This lability of the crossbridge order in rabbit thick filaments may explain the tendency of some of the rabbit filaments to show the loss of crossbridge order we observed along one arm of the filament, or in small patches along an otherwise well-ordered filament. This increased lability of the crossbridge order in the rabbit filaments may also explain the failure of many probe studies (Thomas and Cooke, 1980; Thomas et al., 1980; Barnett et al., 1986) to show the ordered relaxed arrangement of crossbridges expected from X-ray diffraction of the living muscle (Wray, 1987). As shown here, however, if temperature and other variables of the isolation procedure are carefully regulated, preparations of relaxed rabbit filaments with ordered arrays of crossbridges can be obtained.

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