COMPARATIVE STUDIES OF LIGHT MEROMYOSIN PARACRYSTALS DERIVED FROM RED, WHITE, AND CARDIAC MUSCLE MYOSINS

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ABSTRACT

Tryptic and chymotryptic light meromyosin paracrystals from red and cardiac muscles of rabbit show a negative and positive staining pattern with uranyl acetate and phosphotungstate that sharply differs from that of white muscle light meromyosin paracrystals. The main periodicity of about 430 Å is the same regardless of the source of light meromyosin. The results are discussed in terms of the molecular structure and the functional properties of various myosins.

INTRODUCTION

Red and white muscles have long been known to differ in their velocity of contraction and relaxation (red, slow; white, fast).

Myosins isolated from red and white skeletal muscles differ with respect to ATPase activity (1, 2), their stability at alkaline and acid pHs (3, 4), and susceptibility to tryptic digestion (1). There are many similarities in these properties between cardiac muscle myosin and myosin from red skeletal muscle (5, 6). Bárány has suggested that the apparent correlation between the myosin ATPase activity and the speed of contraction in red and white muscles applies in general if one compares muscles from a variety of species (7).

The above results suggest that the differences among myosins from different types of muscles are localized in the HMM portion of the molecule. They are also reflected in reported differences in the methylated lysine and histidine content of red and white muscle myosins (8, 9).

Although the physicochemical parameters of white, red, and cardiac muscle myosins appear to be essentially identical, it seemed worthwhile to carry out a comparative investigation on the light meromyosin portions of the different myosins. The property of LMM to form aggregates (paracrystals) at low ionic strength (10) and the well-defined negative staining pattern (11) of conventional, presumably mainly white muscle, LMM paracrystals offered a suitable approach to the problem. Electron micrographs of negatively stained aggregates, the so-called paracrystals, of LMM prepared by tryptic digestion of red skeletal muscle and of cardiac myosin showed a striation pattern different from that seen with white muscle LMM paracrystals. The main repeat period, however, was the same. Although the precise molecular basis of these differences is not clear, the results suggest that red and cardiac muscle myosins, while similar to each other, differ in their...
structure from myosin of white muscle. These results assume new interest in the light of recent reports suggesting that changes in the properties of myosin can be brought about by changing the innervation of the muscle (12).

EXPERIMENTAL PROCEDURE

Myosin was extracted from white (m. vastus lateralis and adductor magnus), red (m. soleus, semitendinosus, and intertransversarius), and cardiac muscles of rabbit as described earlier (6, 13), freed of actin by dissociation with ATP in the presence of MgCl₂ (14), and purified by precipitation at low ionic strength, as originally described by Szent-Györgyi (15). All solutions used in the preparative procedure contained 1 mM EDTA. Myosin obtained in this way has higher ATPase activities with either Ca²⁺ or K⁺-EDTA as the activator (6).

Digestion with Trypsin and Chymotrypsin

White muscle myosin: to a solution of myosin, approximately 10 mg per ml in 0.42 M KCl, 0.008 M N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), 0.16 M phosphate, pH 7.5, trypsin dissolved in 10⁻⁴ M HCl was added, usually in a 1:200 (w/w) trypsin/myosin ratio, 20°C. The digestion was stopped at 12 min by adding soya bean trypsin inhibitor in an amount twice that of trypsin. In some experiments digestion with chymotrypsin was used and the reaction was stopped with diisopropylfluorophosphate (16).

Red and cardiac muscle myosins: digestion was carried out in the same medium except that the trypsin to myosin ratio was 1:50 and the digestion time was varied up to 30 min.

Purification of LMM

The digests were dialyzed overnight against large volumes of a solution containing 0.05 M KCl, 5 mM phosphate, pH 6.5, and 1 mM DTT at 2°C. Aggregates of LMM and undigested myosin were removed by centrifugation for 30 min. The sediment was dissolved in 0.5 M KCl and 50 mM phosphate, pH 7.0. Further purification was carried out by precipitation with 3 volumes of ethanol essentially according to Szent-Györgyi, Cohen and Philpott (17). The precipitate was removed by centrifugation at 105,000 g for 30 min. The pellet was then resuspended in a small amount of a solution containing 0.5 M KCl, 50 mM phosphate, pH 7.0, and dialyzed overnight at 2°C against a large volume of a solution containing 0.5 M KCl, 5 mM phosphate, pH 6.5, and 0.1 mM DTT. The precipitate formed was removed by centrifugation for 10 min at top speed in a clinical centrifuge and the supernatant was used (LMM F1). LMM F1 aggregates were formed by dialyzing the solution overnight at 2°C against a solution containing 0.05 M KCl, 5 mM phosphate, pH 6.3, and 0.1 mM DTT.

Electron Microscopy

For negative staining, the sample was placed on a carbon film mounted on a 400 mesh grid without a supporting collodion film. Negative staining was done with 1% uranyl acetate or 1% potassium phosphotungstate, pH 7.2, essentially according to Huxley and Zubay (18), with or without fixation. 4% glutaraldehyde or 4% formaldehyde, pH 7.0, was used as fixative. Some preparations were examined after positive staining with 1% uranyl acetate or 1% phosphotungstate. A Philips EM 200 electron microscope was used with double condensor illumination (300 µ Pt aperture), 30 µ or 40 µ Pt or molybdenum objective aperture, accelerating voltage of 60 kv, and an emission current of 36 µA.

RESULTS

White Muscle LMM Aggregates

In order to be able to make a comparison among LMM aggregates from different types of muscles, we first reexamined the various forms of aggregation exhibited by white muscle LMM. Some of the patterns have been described before, and while this work was in progress renewed attention was drawn by Podlubnaya et al. (19) to the polymorphism of light meromyosin aggregates. Fig. 1 shows some of the typical forms encountered. The spindle-shaped aggregates (a) previously described occur in various sizes. They show the characteristic staining-band patterns with alternating 100 A wide, lightly stained bands and 330 A wide, intensively stained ones, and a main periodicity of about 430 A. Subperiodicities consisting of 15–20 A wide dark lines appear. In some instances, not shown in Fig. 1, aggregates of the same size and shape were found but they lacked a striation pattern.

Fig. 1 b shows another pattern of aggregation consisting of thin sheetlike aggregates. They contain longitudinally running thin filaments, sometimes with a faint indication of a 430 A periodicity.

We also encountered the open lattice type arrangement (Fig. 1 c) described previously by Huxley (11) and Lowey et al. (20). Similar lattices were observed earlier by Philpott and Szent-Györgyi (10) with the use of the shadow casting
Figure 1 Various types of white muscle light meromyosin aggregates. Negative staining with 1% uranyl acetate. (a) Paracrystals are seen with regular striation patterns. There is a 430 A main periodicity with alternating 100 A wide, lightly stained bands and 330 A wide, intensely stained bands. X 62,000. (b) Thin sheetlike aggregates. X 43,000. (c) Open (square) lattice formed by white muscle LMM aggregation. The distance between two lattice points is about 400 A. The filaments forming the lattice are 80–100 A wide. Sometimes transitions from a square lattice to a hexagonal one can be seen (arrow). In the right upper and left lower corner, paracrystals not showing any cross-band pattern can be seen. X 43,000.
technique. Both square and hexagonal type lattices can be seen, and often the two patterns merge into each other. Another type of aggregate, having the same over-all dimensions as the first two, is characterized by a system of dark lines repeated every 145Å with fainter dark lines appearing within each period (see Fig. 5 a). This type of pattern has been described by Lowey et al. (20) for the so-called helical subfragment of heavy meromyosin, subfragment 2, and has recently been found by King and Young (21) in what they describe as crystals of cyanogen bromide-cleaved LMM. Although it is difficult to lay down the exact conditions for the formation of the band pattern with a 145Å period, it appeared that longer trypsic digestion at a higher enzyme concentration in 0.167M phosphate buffer favors it. In 30mM Tris buffer the 145Å band pattern tended to be present on shorter digestion (10 min instead of 15) and lower (1 to 200 rather than 1 to 50) trypsin to myosin ratios.

Red Muscle

It has been shown previously (1) that, as with cardiac myosin, the digestion of red muscle myosin requires higher trypsin to protein ratios and longer periods of digestion. It appears that, while the splitting of the links responsible for the separation of HMM and LMM goes on at a reduced rate in red muscle LMM, the products formed are more readily degraded and thus the yield is lower.

In contrast with the polymorphism observed in preparations of white muscle LMM, aggregates of red muscle LMM usually showed only one type of aggregate although in some preparations the sheetlike structureless aggregates appeared. The typical appearance of red muscle LMM paracrystals is shown in Fig. 2.

The important features of these paracrystals can be summarized as follows. The main periodicity is 430Å, as with white LMM paracrystals. There are, however, wider, 250-280Å, lightly stained bands and narrower, 180-150Å, strongly stained bands. Thus the situation prevailing in white LMM paracrystals (wider dark bands, narrower light bands) is reversed. The light band is further divided by two doublets of 15-20Å wide, dark lines separated from each other by 20-30Å. The dark band itself is bounded by a pair of 20Å wide, more intensely stained lines. The existence of these lines is particularly clear when the electron micrograph plates are traced with a microdensitometer.

No true polarity has been revealed in the paracrystals, but some irregularities are seen at their tips. An interesting example occurs in Fig. 2 a, where the tip is bent back 180°. It seems that it is the dark bands in which the bending occurs, leading to a widening and compression at the two ends of the dark bands, respectively. This observation suggests that in the dark bands the forces holding the molecules together are weaker. This may be due to less close packing of the protein molecules which is reflected in the greater penetration of the dye.

Further features of end portions of WM and RM LMM paracrystals are shown in Fig. 3. The width of the paracrystal seems to be decreasing in a stepwise fashion in both cases, and the main periodicity is well preserved to the very end. In the case of WM LMM paracrystals, the decrease occurs distal to each light band. In the case of the RM LMM paracrystal, the dark band gets less distinct towards the end, but the distal one of the two lines bounding the dark band remains quite distinct. The decrease in width of the paracrystal seems to occur distal to these lines. It should also be noted that, at the very end, molecules seem to be protruding after the last sharp transverse line over a length corresponding to one 430Å period. This would suggest that in the case of WM LMM aggregates molecules coming through the dark band terminate at the farther end of the light band, while in the case of RM LMM aggregates, some LMM molecules coming through any given dark band terminate before entering the next light band.

Negative and Positive Staining

The interpretation of negatively stained band patterns can be made easier by correlating them with positively stained preparations and establishing the connection by gradual washing out. This procedure has been applied by Caspar et al. (22) to tropomyosin B crystals and by Kendrick-Jones, Cohen and Szent-Györgyi (unpublished) to paramyosin (tropomyosin A) aggregates. A comparison of negative staining, negative staining followed by washing, and positive staining of RM LMM is shown in Fig. 4. It should be noted that in negatively stained LMM the width of the paracrystals seemed to be slightly larger in the light bands than in the dark bands. This property and the characteristic doublets seen in the original light bands
Figure 2  Purified light meromyosin aggregates from red muscle. (a) Purified red muscle light meromyosin forming spindle-shaped paracrystals with a band pattern different from that found in white muscle LMM paracrystals. The paracrystal on the left is bent back about 180°. The bending seems to affect dark bands. For details see the text. Negative staining with 1% uranyl acetate. × 49,000. (b) Red muscle light meromyosin paracrystal band pattern at higher magnification. The regularly repeating light and dark band pattern is clearly visible. The light band is about 250–280 Å wide, the dark band about 180–150 Å wide; repeat 430 Å. The light band is divided into three equal bands (30–50 Å) by two doublets which are composed of two dark lines (each 15–20 Å wide). These two dark lines are separated from each other by about 20–30 Å. There is a strong dark line (less than 20 Å) where the light and dark bands meet. Negative staining with 1% uranyl acetate. × 270,000.
can be used to establish correspondence between the various staining results. These experiments clearly show that the so-called negative staining pattern of RM LMM paracrystals includes both negative and positive staining components. As the stain was washed out, the narrow band became less electron-opaque and each of the two doublets in the wider band fused into a single broad dark line. On the other hand, the strong dark lines separating the narrow and wide bands remained distinct even after prolonged washing. The washed, negatively stained pattern becomes very similar to that obtained on positive staining. Philpott and Szent-Györgyi (10) have shown that K+ ions accumulated in the positively stained LMM paracrystals at lines perpendicular to the long axis spaced 420-430 Å apart, and that these lines could be removed by washing with distilled water or by omitting the K+ ion from the staining solution. In negatively stained preparations K+ ions do not show any direct effect on the band pattern, except that KCl solutions seem to be less effective than distilled water in washing out the stain.

**Mixture of Red and White LMM**

An interesting relation between the two kinds of LMM aggregates emerges from observations made on a system in which aggregates were allowed to form in solutions containing both RM and WM LMM (Fig. 5). In some cases one type of aggregate is on top of an aggregate of the other type either in or out of register. In Fig. 5 a a white LMM aggregate, well characterized by its 145 Å repeat pattern, lines up spontaneously with a RM LMM aggregate. The doublets in RM LMM are in register with the lines separated by 145 Å; in the WM LMM, three 145 Å periods correspond to one period in the RM LMM. Fig. 5 b illustrates an overlap where the two different kinds of pattern are less clearly discernible.

The different time course of the appearance of the two distinct types of LMM aggregates can be shown by examination of digests of mixtures of red and white muscle myosins. As shown in Fig. 6, during the first stages of digestion, only paracrystals characteristic of white muscle LMM are seen. As the digestion proceeds the sheetlike aggregates appear, as well as filamentous aggre-
Figure 4  Negative or positively stained red muscle light meromyosin paracrystals. (a) Negative staining with 1% uranyl acetate; (b) Negative staining followed by washing with 0.05 M KCl after drying; (c) Negative staining followed by washing with 0.05 M KCl before drying; (d) Negative staining followed by washing with distilled water after drying; (e) Negative staining followed by washing with distilled water before drying; (f) Positive staining with saturated uranyl acetate after fixation with 4% glutaraldehyde. × 125,000.
FIGURE 5 Mixed aggregates of white muscle light meromyosin and red muscle light meromyosin. WM LMM and RM LMM were mixed in a 1:1 weight ratio in 0.5 M KCl. The mixture was dialyzed overnight against 200–500 volumes of 0.05 M KCl, 5 mM phosphate, pH 6.5, 0.2 mM DTT. (a) The right half of this paracrystal shows the 145 A repeat of white muscle light meromyosin. The left half shows a typical red muscle LMM pattern. For discussion see the text. Markers show the positions of the doublets and 145 A periods in RM LMM and WM LMM, respectively. (b) Red and white type LMM paracrystals are nearly superimposed. Two distinct band patterns can, however, be discerned. Markers point to pairs of doublets characteristic of RM LMM. Negative staining with 1% uranyl acetate. X 125,000.
**Figure 6** Light meromyosin paracrystal from tryptic digest of red and white myosin mixture. R/W ratio 4:1 w/w; trypsin-myosin ratio, 1:100 by weight (a) 5 min digestion (× 68,000); (b) 10 min digestion (× 68,000); (c) 15 min digestion (× 40,200). Marker points to WM LMM band pattern. Arrow points to RM LMM band pattern; (d) 20 min digestion (× 68,000).
gates, revealing the band pattern characteristic of red muscle LMM. If white muscle myosin alone is digested as long as 20–30 min paracrystals showing the 145 Å band pattern are seen, but never the inverted staining pattern with a wider light band containing the two doublets found in digests containing red or cardiac muscle LMM.

**Phosphotungstate Staining**

The differences between paracrystals from white and red muscle LMM are also clearly discernible on examining prefixed preparations negatively stained with phosphotungstate (Fig. 7). Both types of paracrystals show the 430 Å period, but again the same inversion of the subperiodicity observed on uranyl acetate-stained preparations occurs. In white muscle LMM there is an 80–100 Å wide lightly stained band and a 330–350 Å strongly stained band. In the preparations from red muscle there is an intensely stained narrow band and a poorly stained wide band. The ratios of the widths of the narrow and wide bands are the same in the two types of LMM when negative staining is done with phosphotungstate. This is in contrast with uranyl acetate-stained preparations where the ratio of the two bands within the period is roughly 4:1 in white LMM but about 2:1 in red LMM. The light band of phosphotungstate-stained white muscle LMM contains a single dark line. The two doublets seen in the uranyl acetate-stained red muscle LMM paracrystals appear only faintly in the light bands of phosphotungstate-stained RM LMM, but the contrast between the edges and the center of the narrow dark band is much stronger with phosphotungstate than with uranyl acetate.

![Figure 7](https://jcb.rupress.org/content/jcb/49/4/892/F7.large.jpg)

**FIGURE 7** Red and white LMM paracrystals negatively stained with 1% PTA (pH 7.4). Paracrystals were fixed with 4% formaldehyde (80 mm PO₄ buffer, pH 7.0) and, after washing, were negatively stained with 1% PTA, pH 7.4. (a) White LMM paracrystal; (b) Red LMM paracrystal. X 116,000.
FIGURE 8  White muscle light meromyosin paracrystals from α-chymotrypsin digest. (a) White muscle light meromyosin produced by digestion with α-chymotrypsin (ratio 1:200). The spindle-shaped paracrystal shows 430 Å main periodicity. The bands are limited by strong dark lines. The contrast between light and dark bands is less than in tryptic paracrystals. × 60,000. (b) Chymotryptic paracrystals at higher magnification. The main periodicity is 430 Å, and the light band is about 150 Å: slightly wider than the tryptic type. Dark lines on each side of the light band are quite distinct. Tryptic LMM paracrystals do not show this feature. Negative staining with 1% uranyl acetate. × 118,000.
FIGURE 9 Two types of red muscle chymotryptic light meromyosin aggregates. Digestion was carried out at a 1:100 enzyme to myosin ratio, 15 min. (a) Many small spindle-shaped aggregates form large, loose parallel bundles. Some of them show a 145 Å repeat. × 102,000. (b) Sheet-like aggregates: Negative staining with 1% uranyl acetate. × 80,000.
Chymotryptic Digestion

Differences between the two types of LMM also appeared after chymotryptic digestion. Fig. 8 shows chymotryptic LMM paracrystals obtained from WM LMM. Although the overall shapes of trypic and chymotryptic meromyosins are not substantially different, the staining pattern of this aggregate shows some differences compared with the trypic one. The band pattern is sharper than in the case of trypic LMM and strong lines at the edges of the bands are prominent. It also appears that the ratio of the widths of the light and dark bands is slightly shifted in favor of the former compared with the trypic aggregates. In contrast to the typical appearance of the chymotryptic LMM from white muscle myosin, the chymotryptic RM LMM shows thin sheetlike aggregates with very little cross striation pattern (Fig. 9), but there is an indication of a 430 or 860 Å periodicity.

Cardiac LMM

A comparison of cardiac myosin (CM) LMM paracrystals with RM and WM LMM paracrystals

Figure 10  Comparison of the band pattern of cardiac, red and white muscle light meromyosin paracrystals (trypic digest). (a) Cardiac muscle; (b) Red muscle; (c) White muscle. Negative staining with 1% uranyl acetate. × 133,000.
DISCUSSION

The different staining patterns of light meromyosin paracrystals from white and red muscles described in this paper offer evidence that the differences between the two types of myosin, heretofore attributable only to differences in the structure of the HMM portion, also extend into the rodlike shaft of the molecule. It is clear from this and previously published reports that white muscle LMM can assume various patterns of aggregation (11, 19, 20, 21). Red muscle LMM aggregates show considerably less polymorphism; the band pattern found in red muscle LMM paracrystals is never found, even under a variety of conditions of digestion, in white muscle preparations. Thus the inversion of the staining pattern obtained with both uranyl acetate and phosphotungstate, when one compares red LMM with white LMM, cannot be attributable to the length of the digestion or the concentration of enzyme required for obtaining LMM from red muscle myosin. It is well known that higher amounts of trypsin and longer times of digestion are required for obtaining red muscle LMM. It is in accord with this fact that in tryptic digests of mixtures of red and white myosins there first appear aggregates characteristic of white muscle LMM followed by the appearance of the staining pattern characteristic of red muscle LMM.

Attempts have been made in this work to correlate the negative staining pattern of red LMM with that of positively stained preparations. If one allows the stain to be washed out from negatively stained preparations some features found in positively stained preparations emerge, suggesting that the so-called negatively stained pattern must have contributions from positive staining of certain portions of the constituent molecule. The differences between phosphotungstate and uranyl acetate in their negative staining patterns may also reflect differing contributions from positive staining with the two reagents. Huxley (11) had previously discussed the staining pattern of white muscle LMM in terms of a staggered array of LMM molecules in which the length of the molecule would be a multiple of the repeat period + one lightly stained zone, the latter being identified with a zone of overlap. We have tried to work out various possible models that might explain the band pattern in red and cardiac LMM aggregates, but in view of the existence of positively stained contributions this approach does not seem to be promising at present. If the relation between molecular overlap and length and the periodicity of paracrystals proposed by Huxley were to apply to red and cardiac LMM aggregates, the length of the LMM molecules from red and cardiac muscle myosins would have to differ from that of LMM derived from white muscle myosin. For the main period observed in the paracrystals is the same while the staining pattern indicative of the overlap is different. Physical measurements of LMM and heavy meromyosin from cardiac muscle suggest that the proteolytic fragments have the same over-all dimension as those obtained from skeletal, presumably white, muscle. Thus it is unlikely that the same simple relation between periodicity and particle length would prevail. On the other hand, one may wonder whether the 430 A period observed in all of these paracrystals may not represent some property common to all three types of myosin. Ultimately, the reason for the morphological differences among LMM aggregates must be found in terms of differences in the amino acid sequence of the different types of myosin.

The differences described in this work among LMM aggregates from different types of muscle and the similarities between red and cardiac muscles suggest that this kind of approach may be useful in situations involving changes in the protein complement of a given muscle. Thus it has been suggested that cross innervation leads to a transition from a white or fast type of myosin to a red or slow muscle as the physiological properties show a corresponding change (12). It might also be possible that with changes of cardiac muscle corresponding to changes in its physiopathological state, e.g. hypertrophy or failure, there may be a change in the structure of myosin. Finally, it might be of interest to carry out a more detailed investigation into the structure of the thick filaments of cardiac and red muscles since the structure of the thick filaments depends largely on interactions among the LMM portions of the myosin molecule (24). It has been recently suggested that under certain conditions in the presence of calcium a
type of aggregation of LMM takes place that may throw light on the processes underlying the formation of thick filaments (25). Clearly, in the light of these results, it would be of interest to pursue similar studies with light meromyosin from red and cardiac muscles.

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