AN X-RAY DIFFRACTION STUDY OF CONTRACTING MOLLUSCAN SMOOTH MUSCLE

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ABSTRACT The living anterior byssus retractor muscle of *Mytilus* (ABRM), a smooth, "catch" muscle, has been studied by X-ray diffraction while relaxed and while tonically contracted. X-ray reflections were observed from the actin and paramyosin filaments and from the α-helical substructure of the paramyosin filaments. No differences in spacings or relative intensities were observed when the relaxed and contracting muscle patterns were compared. This result is consistent with a sliding filament mechanism involving an interaction between actin and paramyosin filaments.

INTRODUCTION

In the last few years it has become possible to study the structure of living muscle during contraction (Elliott et al., 1965, 1967; Huxley et al., 1965; Huxley and Brown, 1967). In this paper we report results obtained from a low-angle X-ray diffraction study of molluscan smooth or catch muscle.

Molluscan catch muscle differs from vertebrate striated muscle both in contractile properties and in structure (see Millman, 1967). In comparison to the single type of contraction found in vertebrate striated muscle, catch muscle shows two distinct types both of which are slower than the contraction of vertebrate muscle. Catch muscle contains the protein tropomyosin A (or paramyosin); vertebrate striated muscle does not. Catch muscle is a smooth muscle with thick filaments 30 μ or more long and 500–1000 A in diameter, whereas vertebrate striated muscle has shorter (1.6 μ) and thinner (120 A) thick filaments. On the other hand, the basic mechanical properties (except for those mentioned above) are similar in both types of muscle. Both types of muscle have two kinds of filaments containing actin, myosin, and tropomyosin B.

The catch muscle we have used is the ABRM, and we have compared the X-ray diffraction pattern obtained from the muscle when relaxed with that obtained when the muscle was in a prolonged (or tonic) contraction. This muscle is specialized for

holding tension over very long periods of time. It can give two types of contraction which differ dramatically in their relaxation rates. In both, the speed of shortening or tension development is the same, but in one case (tonic contraction) relaxation is about 1000 times slower than in the other (phasic contraction, see Lowy and Millman, 1963). Tonic relaxation can be changed to phasic relaxation by repetitive electrical stimulation or by the direct application of 5-hydroxytryptamine to the muscle. The muscle appears to be doubly innervated (Twarog, 1967): one set of nerves releasing acetylcholine to produce tonic contraction, a second set releasing 5-hydroxytryptamine to give rapid relaxation. Phasic contractions are produced by simultaneous stimulation of both sets of nerves.

The ABRM, like most other molluscan muscles, contains large quantities of the protein tropomyosin A (sometimes called paramyosin), as well as actin, myosin, and tropomyosin B which are normally found in other types of muscle. The tropomyosin A is found in large filaments which were called "paramyosin" filaments by Hall et al. (1945) referring to their characteristic structure as seen by electron microscopy or X-ray diffraction (Bear and Selby, 1956). These thick filaments are surrounded by thin filaments which appear identical with the actin-containing filaments found in all other types of muscle (Selby and Bear, 1956; Lowy and Hanson, 1962).

Detailed X-ray diffraction patterns can be obtained from the ABRM which show meridional and near-meridional reflections from both actin and paramyosin filaments. These patterns give information about the packing of actin, tropomyosin A, and myosin molecules into filaments. In addition, a diffuse reflection is seen on the equator at about 130 A. This was at first believed to arise from internal layered structures in the paramyosin filament (Elliott and Lowy, 1961). More recently, Lowy and Vibert (1967) have found that under certain conditions (living muscle near the freezing point after an isotonic stretch) there is a sharp sampling along the 59 A actin layer line at about the position corresponding to the equatorial reflection. From this observation they concluded that the equatorial reflection arises from a partial ordering of actin filaments in certain regions of the muscle. This is in agreement with electron microscope observations of cross-sections of the ABRM which show regions where only thin filaments are found see Figs. 3, 4, and 5 of Twarog, 1967; Hanson and Lowy, unpublished results; H. E. Huxley, unpublished results). In these regions, the actin filaments appear to be arranged at a regular average spacing as indicated by the equatorial reflection but without any long range order.

Two different mechanisms have been proposed as the basis of tonic contraction in molluscan muscles. These mechanisms differ essentially in the role ascribed to tropomyosin A. In one (Lowy et al., 1964), the tropomyosin A is assumed to have a largely passive role as a structural protein in the center of the thick filaments. According to this hypothesis, both tonic and phasic contractions are caused by actin-myosin "linkages" which break at rates controlled externally by the concentration of 5-hydroxytryptamine. In tonic contractions the linkages break slowly; in phasic contractions they break rapidly.

In the second mechanism, two distinct systems are proposed for contraction, one involving actomyosin, the other tropomyosin A (Ruëgg, 1958; Johnson et al., 1959). According to this hypothesis, phasic contraction and the first part of tonic contraction are produced by the actomyosin system. During tonic contraction the tropomyosin A system takes over through association or "crystallization" of the tropomyosin A, to maintain the contraction for long periods. In support of this hypothesis, tropomyosin A has been shown to have solubility properties that depend on pH and ionic strength and which are reflected in the behavior of glycerol-extracted fibers from the ABRM (Ruëgg, 1961, 1964). These mechanisms have been extensively discussed and compared by Lowy et al. (1964) and Ruëgg (1964).

In the experiments reported here, we have attempted to find changes in molecular structure which are correlated with contraction of the muscle. No such specific changes have been found. We believe, however, that our experiments tend to support the single system mechanism for tonic contraction in catch muscles.

MATERIALS AND METHODS

Most of the X-ray patterns used in this study were obtained using a microfocus X-ray generator (Hilger and Watts, London, England, model Y33) with low-angle X-ray cameras of the basic Franks (1955) design, as modified by Elliott and Worthington (1963). The size of the X-ray beam could be varied either by changing the type of filament on the X-ray generator or by rearranging the focal distances in the camera. With these adjustments, the exposure required for a particular pattern and the detail visible within that pattern could be optimized for the conditions under which the muscles were being studied. For most of the experiments reported here the high power line focus was used (35 kv at 10 amp) and an arrangement of the camera that gave a foreshortened spot size of about 50 μ in width and a length that could be varied from 0.5 to 2.0 mm. Normally exposures in the range from 3 to 24 hr were required and these were recorded on Ilford Ilfex X-ray film (Ilford Ltd., Ilford, Essex, England). Films were measured using a measuring microscope or an optical comparator.

The muscles (living ABRM) were used near their equilibrium or reference length (see Lowy and Millman, 1963). All experiments were done at room temperature (approximately 20°C).

The meridional diffraction pattern was studied in the relaxed and tonically contracted muscle. The muscle was hung vertically and bathed by a constant flow of natural (Plymouth) seawater. Contraction was recorded by means of an isometric lever and smoked drum. Tonic contraction was produced and maintained by single electric stimuli at a frequency of 2/min (Lowy and Millman, 1963). Contractions lasting as long as 24 hr were produced by this means.

Patterns from the relaxed muscle were obtained either before or after the contraction and under either isometric or isotonic (with a 2–10 g load) conditions. It was not in general possible to record detailed patterns from the relaxed muscle both before and after the contraction because of the length of time required for a single exposure. When this was done, no difference could be detected between patterns obtained before and patterns obtained after the contraction, nor was there a difference between the patterns from relaxed muscle obtained under isometric and isotonic conditions.

Some muscles were examined under isotonic conditions in a large Perspex box which enclosed the muscle, the guard slits, and the backstop. This was filled with a water-saturated

atmosphere of either pure oxygen or pure hydrogen. The muscle was kept completely wet by means of a slow drip of seawater which ran over the muscle at a rate of about 1 drop/sec. This gave a much lower background scattering of the X-ray beam than the normal procedure.

To obtain equatorial diffraction patterns, the muscle was suspended in a manner similar to that for the meridional experiments. The cathode filament was mounted so that its long axis was vertical. Because of the intensity of the equatorial reflections, a much shorter exposure (1–2 hr) was required. In these experiments, patterns from the relaxed muscle could be obtained both before and after the contractions, and the spacings for the relaxed muscles were calculated by averaging the measurements from patterns obtained before and after the contractions. As in the case of the meridional patterns, contraction was produced by slow, repetitive, electrical stimulation. About one-half of these experiments were done at Brock University using Instant Ocean artificial seawater (Aquarium Systems, Wickliffe, Ohio). These later results did not differ significantly from earlier ones.

RESULTS

The small and moderate angle X-ray diffraction pattern of the living ABRM is shown in Fig. 1 under (*a*) relaxed and (*b*) tonically contracted conditions. Two series of reflections are seen on or near the meridian. One is a series of sharp reflections, orders of a basic periodicity of about 720 A which have been attributed to the thick, paramyosin filaments (Bear and Selby, 1956). The other is a more diffuse series, extending to higher angles than does the paramyosin series, which has been attributed to the thin, actin filaments (Selby and Bear, 1956). In addition, there is a near-meridional reflection at about 5.1 A and some diffuse equatorial and near-equatorial reflections at about 10 and 20 A which have been attributed to the coiled-coil α-helical structure of the paramyosin in the thick filaments (Cohen and Holmes, 1963; see also Elliott et al., 1968). A cursory comparison of Figs. 1 *a* and 1 *b* indicates that the relaxed and contracting patterns from the muscle are very similar.

In a preliminary report of this work (Millman and Elliott, 1965), we showed that the spacings of the low-angle meridional X-ray reflections from both paramyosin and actin filaments were unchanged during tonic contraction. Since then, we have added no further experimental data from contracting muscles to our results on meridional reflections. We have, however, extended our analysis of these results to include a qualitative analysis of the relative intensities of the various orders of the reflections. We have also included several further experiments on living, relaxed muscles in the calculation of the basic actin and paramyosin periodicities. The basic paramyosin periodicity (calculated from the 5th and 10th orders) and the actin spacing measured are shown for the relaxed and contracted muscle in Table I. We have also measured the meridional reflection at about 5.1 A associated with the α-helical structure of the paramyosin filaments for both relaxed and tonically contracting muscle (Table I). These spacings do not differ significantly between the relaxed and contracted conditions of the muscle.

The intensities of the 5th–45th order paramyosin reflections from several muscles

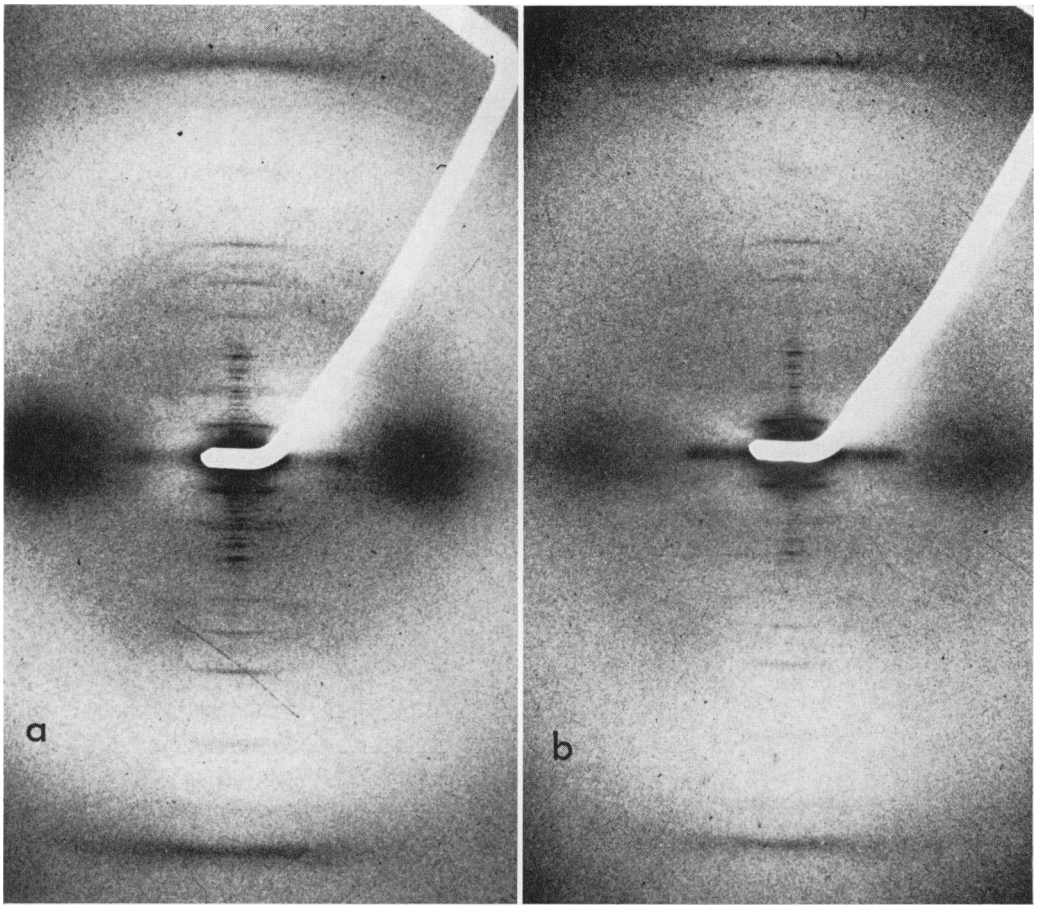

FIGURE 1 X-ray diffraction patterns from living ABRM's. (*a*) Relaxed muscle; specimen: film distance, 4.63 cm; exposure, 6 hr. (*b*) Different muscle, tonically contracted by electrical stimulation at 2/min; specimen: film distance, 4.55 cm; exposure, 8.6 hr. Original X-ray patterns enlarged × 3.7.

TABLE I

MERIDIONAL AND NEAR-MERIDIONAL PERIODICITIES IN THE ABRM

(Compared for relaxed and tonically contracted muscle)

	Relaxed muscle			Contracted muscle		
	A			*A*		
Paramyosin periodicity	723.1	±1.1*	(27)‡	720.4	±1.4	(12)
Actin spacing	58.95	±0.10	(27)	58.51	±0.14	(12)
α-Helix spacing	5.080	±0.005	(24)	5.069	±0.005	(11)

* Standard error of the mean.

‡ Number of muscles.

TABLE II

RELATIVE INTENSITIES OF MERIDIONAL AND NEAR-MERIDIONAL PARAMYOSIN REFLECTIONS*

Spacing (approximate)	Spacing order (of 720 A)	Row line	Average intensity	
			Relaxed muscle	Contracted muscle
A				
144	5	0	8.9 (vvs)	9.0 (vvs)
103	7	1	2.7 (w)	2.2 (vw)
72	10	0	7.1 (s)	7.4 (s)
60	12	1	0.6 (vvw)	– (–)
55	13	1	4.1 (mw)	3.4 (w)
48	15	0	4.3 (mw)	3.8 (mw)
40	18	1	2.2 (vw)	2.0 (vw)
36	20	0	2.7 (w)	2.4 (vw)
31	23	1	2.0 (vw)	1.4 (vvw)
29	25	0	4.1 (mw)	3.6 (mw)
24	30	0	2.1 (vw)	2.4 (vw)
22.5	32	1	2.1 (vw)	1.4 (vvw)
21.8	33	1	2.6 (w)	1.6 (vw)
20.6	35	0	1.1 (vvw)	1.0 (vvw)
19.5	37	1	2.7 (w)	2.2 (vw)
18.9	38	1	4.1 (mw)	3.4 (w)
18.0	40	0	1.6 (vw)	1.6 (vw)
16.0	45	0	0.5 (vvw)	0.8 (vvw)

* Averaged from 14 relaxed and 5 tonically contracted ABRM's. The intensity scale used is as follows: very very strong, 9; very strong, 8; strong, 7; medium strong, 6; medium, 5; medium weak, 4; weak, 3; very weak, 2; very very weak, 1. (It should be noted that this scale is in no way a linear intensity scale.)

TABLE III

RELATIVE INTENSITIES OF MERIDIONAL AND NEAR-MERIDIONAL ACTIN REFLECTIONS*

Spacing (approximate)	Row line	Average intensity	
		Relaxed muscle	Contracted muscle
A			
59	1	6.3 (ms)	6.5 (s)
28	0 or 1	2.8 (w)	2.5 (w)
19.1	1	1.4 (vvw)	2.1 (vw)
13.9	0	1.2 (vvw)	0.9 (vvw)
11.4	1	3.1 (w)	2.5 (w)
9.3	0 or 1	4.2 (mw)	4.1 (mw)
7.0	1	2.4 (vw)	2.6 (w)
5.8	1	1.2 (vvw)	1.2 (vvw)

* Averaged from 18 relaxed and 8 tonically contracting ABRM's. The intensity scale used is that of Table II.

were examined and rated according to a visual scale. The averages are compared for the relaxed and contracting muscles in Table II. No significant differences appear in the intensity patterns under the two conditions. It is possible that a more detailed examination of the patterns (e.g., using a microdensitometer) might show differences between the two conditions. Such a comparison, however, would hardly be significant unless greater precautions were taken to ensure that the mounting of the muscle and other experimental details (e.g., area of muscle in the beam, fiber angle) were precisely controlled. Such controls would be very difficult to arrange.

A similar comparison of intensities was made for the actin filaments (Table III), and as in the case of the paramyosin filaments, no significant difference was found in the patterns from the relaxed and contracting muscles. The distance of the 59 A reflection from the meridional axis was compared for six muscles under the two conditions. This reflection was broad in the equatorial direction, partly because of the width of the X-ray beam. It extended from 70 to 170 A with a mean position of 97.2 A (SD of the mean = 1.0 A). The position did not change on contraction; the ratio of the contracting to the relaxed spacings averaged 1.00 (SD of the mean = 0.015).

The equatorial reflection at about 130 A was also compared during relaxation and during tonic contraction of the ABRM. As was found by Elliott and Lowy (1961) there was considerable variation in this spacing from muscle to muscle and the reflection tended to become diffuse, or to disappear, in a muscle in poor physiological condition.

In these experiments the spacing determined in a tonically contracting muscle was compared with the average spacing obtained from resting muscle patterns taken immediately before or immediately after the contracting muscle pattern. The resting muscle spacing ranged from 117 to 145 A with an average of 134 A. The contracting muscle pattern showed no significant change when compared with the resting pattern. Spacing changes for 13 individual muscles ranged from +5 to −8 A with an average of +0.1 A (SE of the mean = 1.0 A).

DISCUSSION

The experiments described in this paper and others reported elsewhere (Elliott et al., 1967) have shown the versatility of the Franks type of low-angle camera when combined with the various focal sizes available with the Hilger microfocus X-ray tube.

When the ABRM contracts tonically, there is no change in the spacings or the intensities of the meridional and near-meridional reflections from either actin or paramyosin filaments. This is similar to the finding in vertebrate striated muscle (Elliott et al., 1965; Huxley et al., 1965) and indicates that in neither case do the filaments change their over-all structure during the contraction. This does not, however, rule out irregular or cyclical changes that only affect small portions of a filament at any instant. In both types of muscle, this finding indicates a sliding fila-

ment contraction mechanism. In vertebrate muscles there are certain reflections (lying on myosin layer lines) that are associated with projections from the thick filaments and which disappear on contraction. Although projections have been seen in electron micrographs of the ABRM (Hanson and Lowy, 1959) we have been unable to detect any layer-line reflections associated with them. This is probably because the projections in this muscle are much less well ordered than those in vertebrate striated muscle. For this reason, a direct demonstration of the involvement of such projections in tonic contraction of the ABRM is not possible at present.

We found no change in the high-angle α-helical pattern on tonic contraction. This contradicts suggestions that contraction is associated with a reduction of α-helical structure (e.g., Pautard, 1959; Mandelkern et al., 1959). Astbury (1947) reported an increase in the angular dispersion of the 5.1 A meridional reflection on contraction of the living ABRM, but we did not observe this. It is probable that the shortening permitted in Astbury's experiments (up to 58%) led to disorientation of the muscle filaments.

The equatorial reflection associated with the actin filaments did not change on tonic contraction, nor did we observe any other changes in the pattern which would suggest a regular aggregation of either thick or thin filaments. One would expect that if a crystallization of paramyosin filaments were associated with tonic contraction, as has been suggested by Rüegg (1958) and Johnson et al. (1959), that this would be detected as some change in the X-ray diffraction pattern.

We did not observe sampling on the 59 A actin layer line, as reported by Lowy and Vibert (1967), in either the relaxed or the tonically contracted ABRM. We do not believe that such sampling was missed in our patterns because of the width of our X-ray beam, but rather that the sampling was not present in our muscles. Our reasons are twofold: (*a*) our 59 A actin reflections did not show variation of intensity in the equatorial direction, and (*b*) the mean spacing of our reflections in the equatorial direction was 96 A, not 136 A as found in our measurements of the reflection on the equator. Our experiments were done at room temperature whereas Lowy and Vibert observed the sampling at temperatures near 0°C and after a period of isotonic stretching. We believe that the sampling of the 59 A reflection is not normally found at room temperature, and that the presence of such sampling is not an indication of the ability of the muscle to contract. It should be noted that Lowy and Vibert found that their muscles lost the sampling within 48 hr, whereas the muscles remained able to contract for 3–4 days.

The equatorial spacing does show considerable variation from one muscle to another. This variation cannot be correlated with muscle length (Elliott and Lowy, 1961; Vibert, 1968; Cottrell and Millman, unpublished results). Recent studies on vertebrate striated muscle (Rome, 1967, 1968) and guinea pig taenia coli muscle (Elliott and Lowy, 1968) have suggested that it might depend on small changes in cellular pH or ionic strength from muscle to muscle. Vibert (1968) and Cottrell and Millman (unpublished results) have found that the spacing of this reflection can

indeed by changed by varying the osmolarity of the bathing solution. It seems likely that variation in ionic strength and temperature underlie the natural variation of this spacing from one muscle to another.

All our results are consistent with a contraction mechanism in which tension (or shortening) is generated by actin-myosin linkages. During tonic contraction, these linkages turn over exceedingly slowly, giving rise to a prolonged contraction (see Lowy and Millman, 1963). In this mechanism, the chief function of tropomyosin A would be to give mechanical strength to the long thick filaments (Lowy et al., 1964). It is possible, however, that the actin-myosin interaction is controlled by tropomyosin A, perhaps in conjunction with calcium ions, as has been suggested by Twarog (1967) and Szent-Györgyi et al. (1971). This conclusion is in agreement with the hypothesis that tonic contraction in molluscan smooth muscles is by a mechanism essentially similar to that found in vertebrate striated muscle (see Lowy et al., 1964). We have not yet been able to obtain X-ray diffraction patterns during the rapidly relaxing (phasic) contractions of the ABRM. Such patterns could reveal further information about the basic contractile mechanism of these muscles.

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