X-Ray studies of order-disorder transitions in the myosin heads of skinned rabbit psoas muscles

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ABSTRACT Using x-rays from a laboratory source and an area detector, myosin layer lines and the diffuse scattering between them in the moderate angle region have been recorded. At full overlap, incubation of rigor muscles with S-1 greatly reduces the diffuse scattering. Also, three of the four actin-based layer lines lying close to the meridian (Huxley, H. E., and W. Brown, 1967. *J. Mol. Biol.* 30:384–434; Haselgrove, J. C. 1975. *J. Mol. Biol.* 92:113–143) increase, suggesting fuller labeling of the actin filaments. These results are consistent with the idea (Poulsen, F. R., and J. Lowy, 1983. *Nature* [*Lond.*]. 303:146–152) that some of the diffuse scattering in rigor muscles is due to a random mixture of actin monomers with and without attached myosin heads (substitution disorder). In relaxed muscles, regardless of overlap, lowering the temperature from 24 to 4°C practically abolishes the myosin layer lines (a result first obtained by Wray, J.S. 1987. *J. Muscle Res. Cell Motil.* 8:62 (*a*). Abstr.), whilst the diffuse scattering between these layer lines increases appreciably. Similar changes occur in the passage from rest to peak tetanic tension in live frog muscle (Lowy, J., and F. R. Poulsen. 1990. *Biophys. J.* 57:977–985). Cooling the psoas demonstrates that the intensity relation between the layer lines and the diffuse scattering is of an inverse nature, and that the transition occurs over a narrow temperature range (12–14°C) with a sigmoidal function. From these results it would appear that the helical arrangement of the myosin heads is very temperature sensitive, and that the disordering effect does not depend on the presence of actin. Measurements along the meridian reveal that the intensity of the diffuse scattering increases relatively little and does so in a nearly linear manner: evidently the axial order of the myosin heads is much less temperature sensitive. The combined data support the view (Poulsen, F. R., and J. Lowy. 1983. *Nature* [*Lond.*]. 303:146–152) that in relaxed muscles a significant part of the diffuse scattering originates from disordered myosin heads. The observation that the extent of the diffuse scattering is greater in the equatorial than in the meridional direction suggests that the disordered myosin heads have an orientation which is on average more parallel to the filament axis.

INTRODUCTION

X-Ray patterns from relaxed vertebrate skeletal muscles show sharp reflections which fade out beyond 3 nm (Huxley and Brown, 1967), and diffuse scattering which is approximately diamond shaped and extends to higher angles (Lowy and Poulsen, 1990). In this paper we deal mainly with the diffuse scattering, particularly its relation to those sharp reflections which arise from the helical arrangement of myosin heads around the surface of the thick filaments: the myosin layer lines first observed by Elliott (1964) and studied in detail by Huxley and Brown (1967).

Poulsen and Lowy (1983) reported results from experiments with relaxed frog muscles which they interpreted to indicate that a significant amount of the diffuse scattering is produced by positional disorder of the myosin heads. This could arise from type-1 or substitution disorder. Type-1 disorder involves departure of the repeating motifs from the positions they would occupy in a crystalline lattice due to, for example, thermal disordering or azimuthal rotation. In muscle, substitution disorder would result from "holes" left in the helical arrangement of myosin heads attached to the actin filament in the A-bands. Such disorder has a twofold origin: actin monomers outnumber myosin heads, and attachment of heads to actin can take place from three different myosin filaments. Thus there will be a random mixture of actin monomers with and without attached myosin heads and this will generate substitution disorder along the actin filament.

Both type-1 and substitution disorder ought to give a well-defined intensity minimum in the center of the pattern under conditions where the total volume is constrained so that there will be appreciable interference between individual scattering centers. But in the system under investigation such a minimum would be seen only at angles much smaller than those accessible to the equipment used by Poulsen and Lowy (1983), and that also applies to the present study. Here we did indeed find that the diffuse scattering diminishes progressively at lower angles but our results do not reveal what happens at spacings larger than ~40 nm (see Fig. 1).

So far we have assumed that the myosin head is rigid. However, it is known that it contains two main structural domains separated by a cleft, and that the region of the neck close to the head-rod junction is flexible (Vibert, 1988). The possibility exists, therefore, that in addition

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to positional disorder of the myosin molecules, there are fluctuations in their scattering density resulting from conformational variations. Because we could not take account of this in the present work, the problem as to whether the diffuse scattering arises solely from positional disorder of the myosin molecules must remain unsolved.

Assuming the predominance of type-1 disorder, Poulsen and Lowy (1983) explained the simultaneous existence of myosin layer lines and diffuse scattering in terms two populations of myosin heads which differ in the r.m.s. displacement of their mass centers from their equilibrium positions. Heads with smaller displacement would have a helical arrangement and thus contribute mainly to the layer lines, whereas the ones with a larger displacement would principally generate the diffuse scattering. Accordingly, it was postulated that the helically arranged heads would be located near the filament backbone, whilst the disordered ones would be further away. Evidence consistent with this has come from electron microscope observations of negatively stained myosin filaments which showed two configurations of heads: ordered ones located near the backbone, and disordered ones lying far away from it (Knight and Trinick, 1984). More recently Padron and Craig (1989) studied rapidly frozen thin longitudinal sections prepared from skinned frog sartorius muscles and observed that depletion of ATP produces a rigor-like array in the overlap zone whereas in the nonoverlap region (H-band) the myosin heads move away from filament backbone and lose their helical order.

Poulsen and Lowy's view of the origin of the diffuse scattering was supported by two additional lines of evidence. (*a*) In relaxed unstriated muscles like the anterior byssus retractor of Mytilus (ABRM) and the taenia coli of the guinea pig (TCGP), no signs can be detected of ordered heads, i.e., one can see strong diffuse scattering but no myosin layer lines (Lowy et al., 1970; Lowy and Vibert, 1972). (*b*) The same state of affairs exists in thread preparations which contain only oriented filaments composed of myosin molecules (Poulsen et al., 1987).

As regards the situation under dynamic conditions, three features are of particular interest in patterns from isometrically contracting frog muscles: the myosin layer lines lose all sampling and practically all intensity; the intensity of the equatorial (1,1) reflection increases whilst that of the (1,0) decreases; and the spacing of the meridional peak on the third myosin layer line increases by ~1%. (Huxley and Brown, 1967; Haselgrove, 1975). The interpretation of the first two changes was, respectively, that the helical order of the heads is lost; and that the heads move into the close vicinity of actin (Huxley and Brown, 1967; Haselgrove, 1975). Recordings of the diffuse scattering between the off-meridional myosin layer lines in the moderate angle region showed that its intensity increases considerably (Lowy and Poulsen, 1987) and this was interpreted as being due to an increase in the population of disordered heads.

Time-resolved experiments (Huxley, 1979; Huxley et al., 1982; Lowy and Poulsen, 1990; Bordas et al., 1991) revealed that all the above x-ray effects have an appreciable lead over tension rise, indicating that they might arise from structural changes which must occur before tension can be generated. With the object of obtaining further information along these lines we embarked on a study of a static situation where, as in isometrically contracting frog muscle, the sampling and practically all intensity of the myosin layer lines is lost. This happens when relaxed rabbit psoas muscle either skinned (Wray, 1987) or live (Wakabayashi et al., 1988) is cooled from 24 to ~4°C. We have extended this work using skinned psoas preparations. Here we describe the results, together with data from other experiments where we investigated, at various filament overlaps, the effects of depletion of ATP, and depletion of ATP together with added myosin subfragment-1.

MATERIALS AND METHODS

Psoas muscles from giant Belgian rabbits were skinned and stored as described by Poole et al. (1988). Bundles (1–3 mm thick) dissected from animals freshly killed at room temperature were tied to plastic sticks and immersed in skinning solution at 0°C. The latter contained 0.5% Triton in a relaxing solution (ionic strength 100 mM, pH 6.85) of the following composition: 70 mM K Propionate, 8 mM Mg Acetate, 5 mM EGTA, 10 mM ATP, 20 mM MOPS, 1 mM NaN_3. Muscles were kept in the skinning solution under slight stirring for 4–6 h at 4°C. They were then washed several times in relaxing solution, transferred to 50% glycerol in relaxing solution, and stored at −30°C. Muscles were used within 2 wk of being skinned. After that time the x-ray pattern deteriorated considerably, showing a higher overall background and weaker layer lines.

Thin strips (1 or 2 fibers thick and 10 fibers wide) were mounted in a specially designed perspex chamber with adjustable mylar windows. The chamber was inserted into a temperature-controlled mount which had a force transducer attached.

Patterns were obtained using a GEC-Elliott GX 18 rotating anode generator operated at 35 kV and 50 mA. Two 20-cm quartz mirrors produced a focus 400 μm in diameter. The patterns were recorded with a two-dimensional Nicolet detector with a specimen-detector distance of either 50 or 80 cm. Exposure times ranged between 0.5 and 2 h.

After every exposure the muscle mount was moved by ~1 mm to avoid beam damage. To obtain a blank, the muscle was removed from the chamber at the end of the experiment and the total background scattering was recorded for the same time as the test exposure.

The parts of the pattern to be analyzed were located either along the meridian or in a region parallel to the meridian (details in legend to Fig. 1). The background of the slices was fitted using spline functions, and the integrated intensity of the background as well as of the peaks

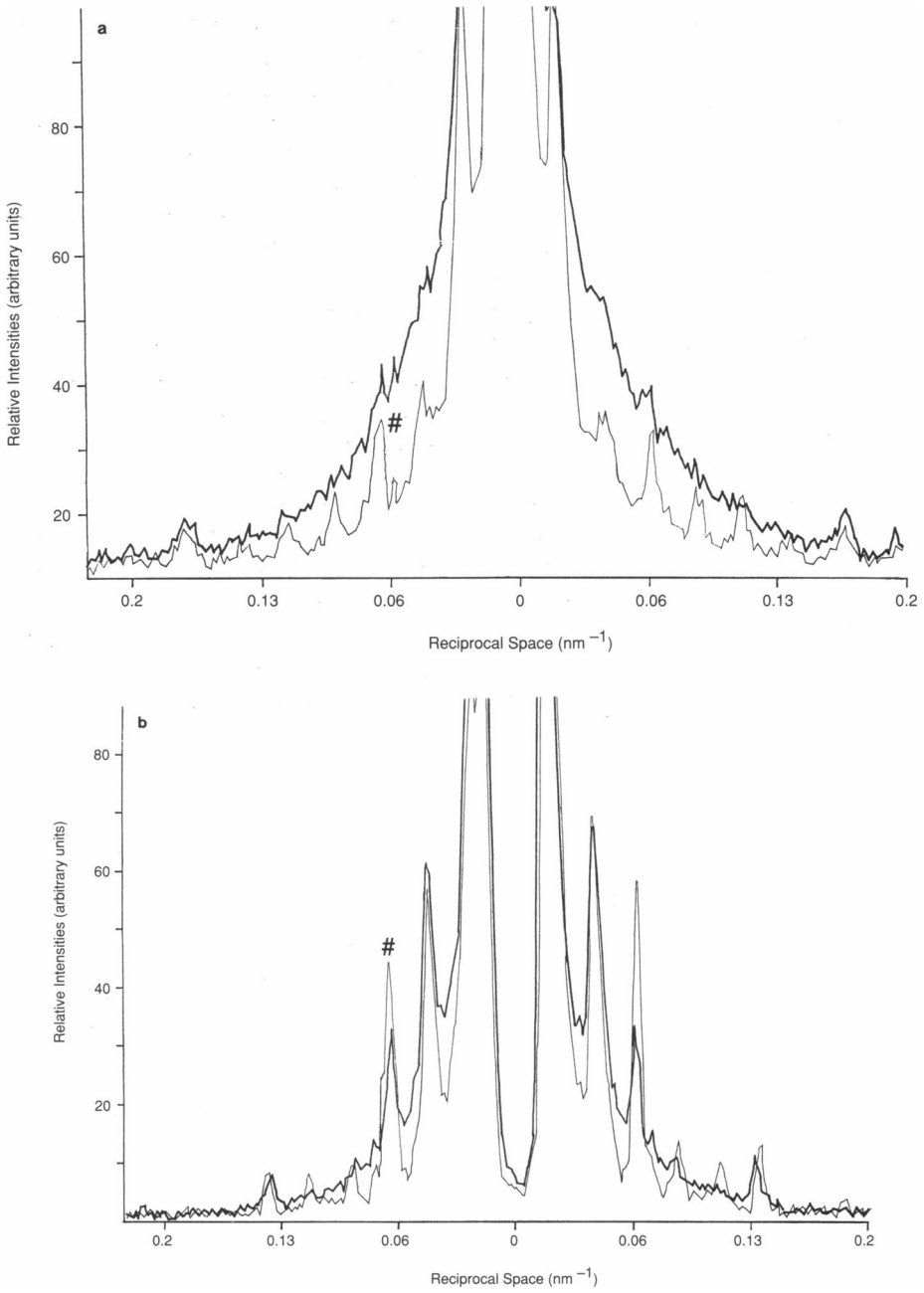


Figure 1 (continued)

above the background were determined. The analysis used a set of programmes (Holmes, K.C., personal communication) which involved centering, reduction of the data to one quadrant, background fitting, correction for disorientation (Holmes and Barrington-Leigh, 1974), and deconvolution of the layer line intensities by a least square method like that of Makowski (1987).

All patterns were normalized against the total integrated intensity. Patterns from the same muscle in various states showed a variation in the total integrated intensity of $<1\%$. Similar results were obtained using as a reference standard the diffuse scattering in the region around 0.17 nm^{-1} where it shows practically no changes (method of Poulsen and Lowy, 1983).

The patterns were also corrected for the detector response. Errors for this correction and in the determination of the background amounted to $<2\%$. The overall error in measuring any of the diffraction features in the patterns was between 1 and 2%.

It will be noted that with our method of measurement one cannot obtain absolute values for the amount of diffuse scattering due to any particular structure. Our observations only relate to the changes that take place during order-disorder transitions in different states of the

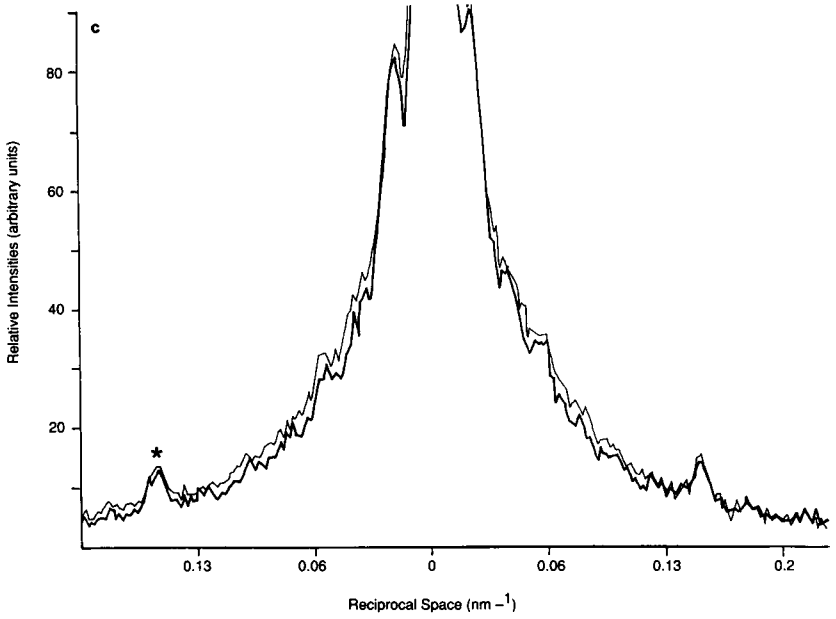


FIGURE 1 *a* and *c* = examples of typical tracings from patterns of fulloverlap muscles showing the intensity distribution in an axial slice parallel to the meridian which includes all the off-meridional myosin layer lines but excludes the meridian and equator. The data were obtained by integrating over the whole width of the myosin layer lines from an inner value of ~ 0.018 nm^{-1}, to an outer value of ~ 0.14 nm^{-1}. *b* = tracing showing the intensity distribution along the meridian. The 14.3 nm myosin reflection is marked (#) in *a* and *b*. The 5.9 nm actin reflection is marked (*) in *c*. (*a*) Relaxed muscle: thick tracing at 2°C, thin tracing at 24°C. At the lower temperature note the almost complete disappearance of the myosin layer lines, and the large increase in the background. (*b*) Same relaxed muscle as in *a*, showing meridional intensity distribution: thick tracing at 2°C, thin tracing at 24°C. Note that at the lower temperature the background increases, but many of the myosin reflections remain relatively strong. The spacings of the 7.15 and 14.3 nm reflections increase by $\sim 1\%$. (*c*) Records from an experiment at 4°C: thin tracing from a muscle in normal relaxing solution; thick tracing from the same muscle in a relaxing solution of low ionic strength (20 mM). Note in the latter state the partial return of the myosin layer lines. Note that during the various order-disorder transitions the total number of counts (peaks and diffuse scattering together) changes by $< 2\%$.

muscle. Thus the present results give relative values, and they cannot therefore be directly compared with the original data of Poulsen and Lowy (1983) who estimated the number of scattering units from the intercept of the Guinier straight-line plot with the intensity axis.

RESULTS

Relaxed muscles

Shape of the diffuse scattering

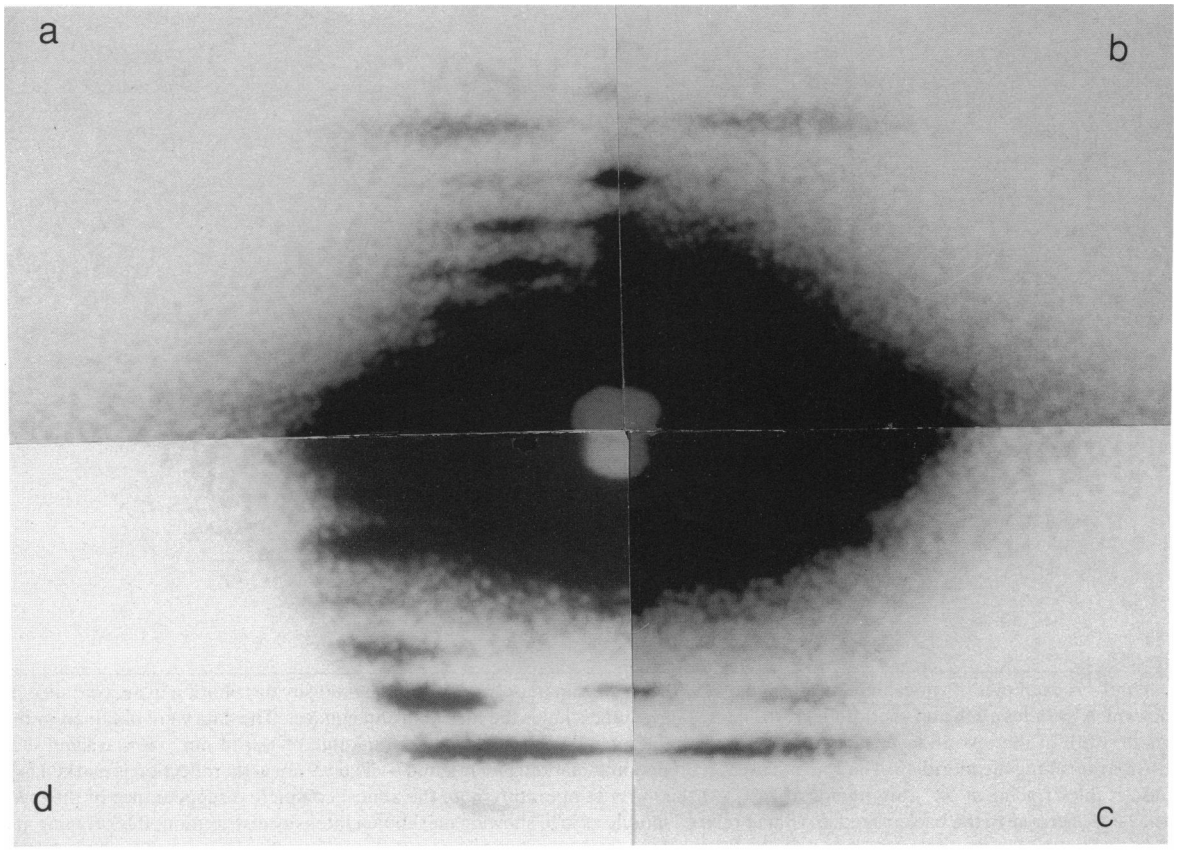
Area detector results from the skinned psoas confirm the asymmetric shape of the diffuse scattering seen previously in relaxed live frog muscles (Lowy and Poulsen, 1990). As in frog muscles, the patterns from the rabbit muscle show that the extent of the diffuse scattering is greater in the equatorial than in the meridional direction, thus giving an approximately diamond shape (Plates I and II, patterns *a*). This also applies to the unstriated ABRM and TCGP muscles where the pattern in the relaxed state shows no myosin layer lines but plenty of strong diffuse scattering (Lowy and Vibert, 1972; Popp, Stewart and Lowy, unpublished results).

The fact that the extent of the diffuse scattering is more pronounced in the equatorial direction means that there is a preferred orientation in the arrangement of the disordered structures of the muscle cell. In terms of Poulsen and Lowy's (1983) view, such an equatorial elongation would indicate that in relaxed muscles the disordered myosin heads have an orientation which is on average more parallel to the filament axis.

At the present stage of the work we considered it adequate to characterize the shape of the diffuse scattering by one measurement taken in the meridional direction (meridional extent) and another in the equatorial direction (equatorial extent), using patterns such as the ones shown in Plates I and II.

Effects of temperature changes

In their x-ray studies Wray (1987) and Wakabayashi et al. (1988) found that cooling relaxed rabbit psoas muscles produces axial deregister of the myosin filaments and azimuthal disordering of the myosin heads. The loss of three-dimensional order is completely reversible, and also occurs in nonoverlap muscles (Wray, 1987). Previ-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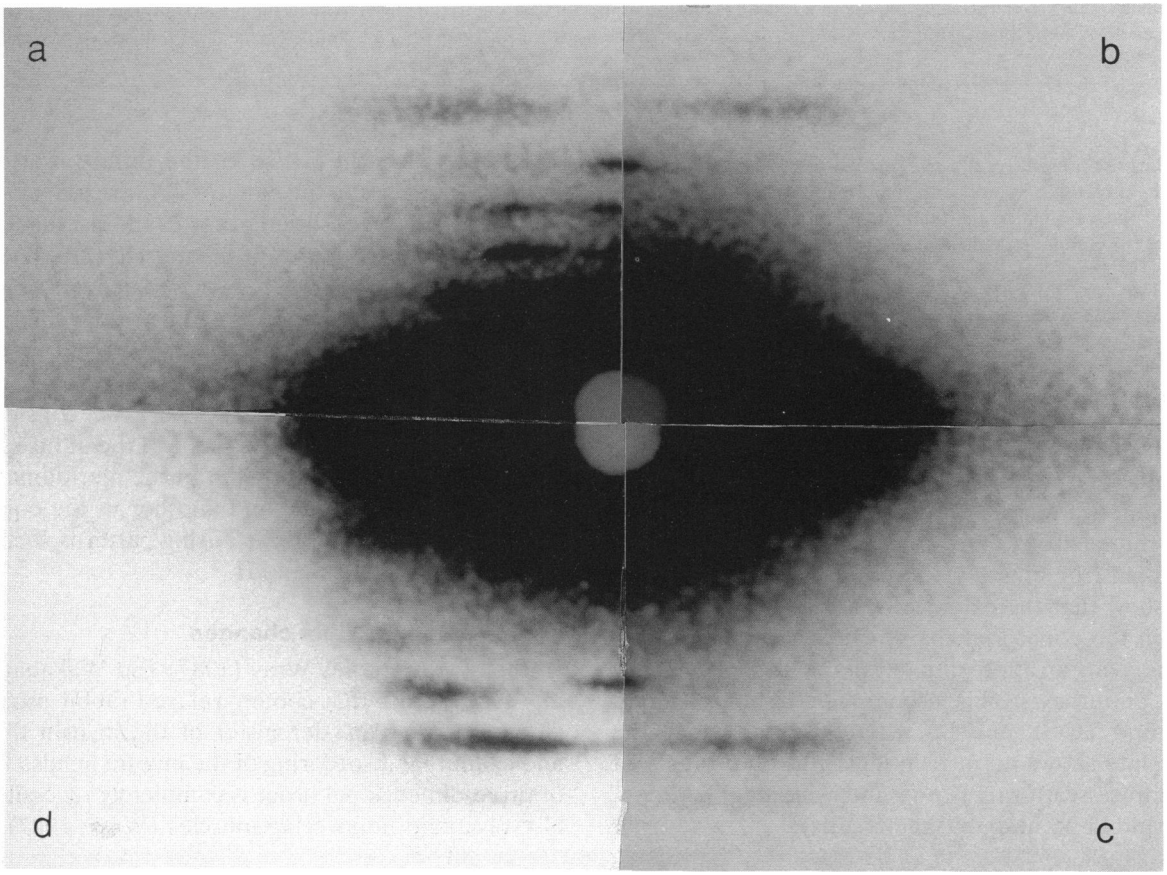
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ously, Haselgrove (1975), using nonoverlap frog muscles, had shown that depletion of ATP resulted in the loss of myosin layer lines. Wray (1987) interpreted these results to indicate that the disordering is an intrinsic property of the relaxed myosin filament independent of actin, and that the relation of the head to the filament backbone depends on the state of the nucleotide bound at the active site of myosin. He envisaged that increasing temperature displaces the equilibrium between the unhydrolyzed (M.ATP) and hydrolyzed (M.ADP.Pi) myosin-nucleotide complex in favor of the latter, and that hydrolysis of the nucleotide leads to the ordering of the head on the filament backbone.

Our experiments with skinned rabbit muscles provide additional information. Thus we find that on cooling and regardless of overlap, as the sampling and intensity of the myosin layer lines is lost, the intensity of the off-meridional diffuse scattering between the layer lines recorded in the moderate angle region increases appreciably (Fig. 1 *a*), maximally by ~12% (Figs. 2, 4, and 6). The relation between the layer lines and the diffuse scattering is of an inverse nature (Fig. 2). It will be noted that the transition occurs over quite a narrow temperature range (12–14°C), and that its function is sigmoidal. The results suggest that the arrangement of myosin heads in helical tracks along the filament is very temperature sensitive, and that the disordering effect does not depend on the presence of actin.

Measurements along the meridian show that with progressive cooling, the intensity of the diffuse scattering increases relatively little and does so nearly linearly (Fig. 3 *a*), as does its meridional extent (Fig. 3 *b*). This temperature effect indicates that the axial arrangement of the myosin heads along the filament is much less temperature sensitive than the azimuthal arrangement, i.e., that along the helical tracks. This is also evident from the fact that cooling affects the intensity of the meridional reflections much less than it does that of the layer lines (Figs. 1, *a* and *b*). There is clearly a difference in the nature of the azimuthal and axial order of the myosin heads.

Another result is that the spacing of the 7.15 and 14.3 nm meridional myosin reflections increases by ~1%. This was first noted in cooled, relaxed psoas muscles (Rinne and Wray, 1990).

Cooling increases the meridional extent of the diffuse scattering by ~40% (Figs. 3 *a* and 5), whereas its equatorial extent undergoes only a very small change namely a decrease by ~2% (Plate I, *a* to *b*).

We interprete the combined results to indicate that a certain amount of the diffuse scattering is due to disordered myosin heads, and that on cooling the resulting order-disorder transition is accompanied by a change in the disordered heads from an average orientation more parallel to the filament axis to one more perpendicular to that axis.

Here it is relevant to mention the behavior of the (1,1) and (1,0) equatorial reflections in full-overlap vertebrate skeletal muscles during contraction and on cooling. We find that in both cases the intensity of the (1,1) equatorial reflection increases by ~50% (results not shown here). The (1,0) reflection decreases by 25–50% during contraction (Haselgrove and Huxley, 1973), but only by ~10% on cooling (Popp, 1989). The point to note is that whether these changes are due to more or less random radial outward movement and/or aximuthal disordering

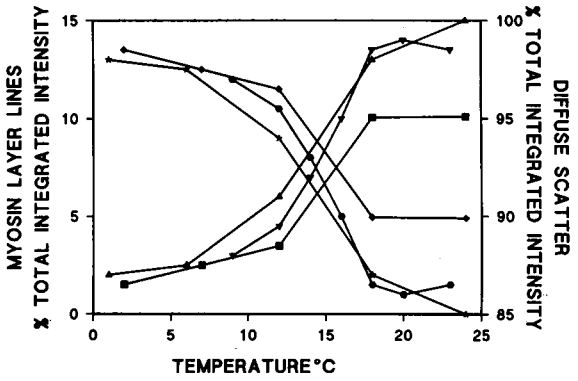

FIGURE 2 Effects of changes in temperature from results of three different experiments with relaxed muscles at full-overlap (sarcomere length 2.5 μ). Relative intensities were measured from axial slices as described in Fig. 1. The diffuse scattering at 2 or 4°C was taken as 100%. As the temperature is lowered the diffuse scattering increases (maximally by ~12%) whilst the myosin layer lines decrease at the same time. Note the inverse relation betwen the layer lines and the diffuse scattering.

PLATES I AND II Composite photographs made of four patterns from experiments with muscles at fulloverlap; sarcomere length 2.5 μ (Plate I) and nonoverlap; sarcomere length 4.5 μ (Plate II). Abbreviations: I(DS) = intensity of the diffuse scattering measured as percentage of the total number of counts. Highly simplified characterization of the shape of the diffuse scattering in terms of extents measured in the meridional (ME) and equatorial (EE) directions, from patterns like the ones shown in Plates I and II. *a* and *b* in the relaxed state at 24 and 4°C, respectively; *c* ATP depleted; *d* ATP depleted and incubated with S-1. With the data in Figs. 4–7, and measurements of ME and EE, these patterns can be taken to illustrate the behavior of the diffuse scattering: in Plate I *a–b*: 12% increase I(DS), 40% increase ME, 2% decrease EE; *b–c*: 4% decrease I(DS), 40% decrease ME, 5% decrease EE; *c–d*: 15% decrease I(DS), no change ME, no change EE. In Plate II *a–b*: 12% increase I(DS), 40% increase ME, no change EE; *b–c*: 2% decrease I(DS), 15% decrease ME, no change EE; *c–d*: no change I(DS), no change ME, no change EE.

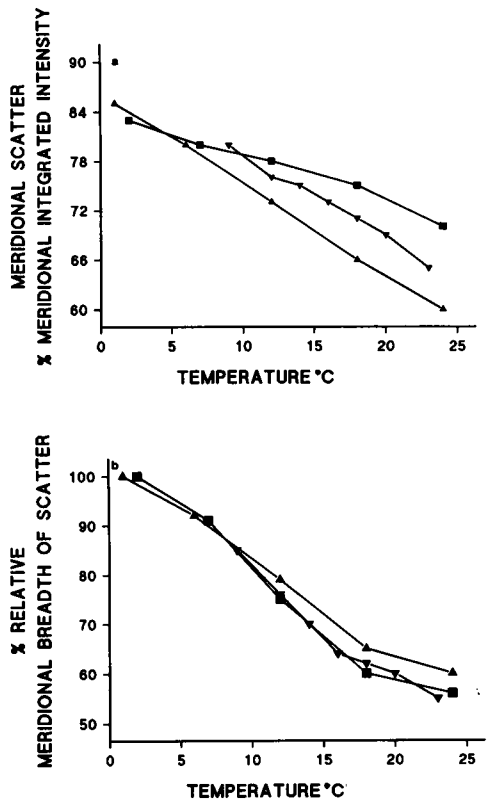

FIGURE 3 Effects of changes in temperature from results of three different experiments with relaxed muscles at full-overlap (sarcomere length 2.5 μ). In *b* the extent of the diffuse scattering measured in the meridional direction at 2 or 4°C was taken as 100%. In this, as in all other experiments, estimates were also made of the extent in the equatorial direction, but the data are not shown as this parameter changes appreciably only during the transition from the relaxed state to rigor at full-overlap (see Plate I, *a* or *b* to *c*). (*a*) Intensity of diffuse scattering measured along the meridian. As the temperature is lowered, the diffuse scattering increases nearly linearly (maximally by ~24%); and as shown in *b* its extent in the meridional direction increases in a similar manner (maximally by ~40%).

of the myosin heads, the result would still be a simultaneous decrease of the myosin layer lines.

It is interesting that in experiments where the temperature was reduced progressively from 25 to 4°C, study of the equatorial reflections revealed that their intensity changes show the same behavior as the azimuthal disordering of the myosin heads (Rapp et al., 1991).

ATP-depleted muscles

Full-overlap

Early x-ray studies with static muscles showed that comparing resting and rigor states, the myosin layer lines disappear and are replaced by a new set which looks somewhat weaker, is more diffuse, and has a repeat which corresponds to the pitch of the actin helix: the interpretation was that in rigor most heads attach to actin at specific sites, thus generating "decorating actin layer lines" (Huxley and Brown, 1967).

On the assumption that the myosin heads are subject to displacement disorder (Poulsen and Lowy, 1983), one would therefore not expect to see much diffuse scattering in rigor. The observation that rigor patterns still showed a considerable remnant of diffuse scattering was explained in terms of substitution disorder along the actin filaments (Poulsen and Lowy, 1983). Accordingly, the diffuse scattering due to displacement disorder of the myosin heads in the relaxed state should decrease in the transition to rigor, provided that most heads attach to actin at specific sites. It also follows that incubating a rigor muscle with myosin subfragment-1 (S-1) should abolish substitution disorder along the actin filament and thus produce a further decrease in the diffuse scattering. The first prediction was tested here, the second in the experiments described in the next section.

Fig. 4 shows that in rigor the diffuse scattering either increases or decreases (in both cases by ~4% maximally), and one sees (Fig. 5) either a small increase

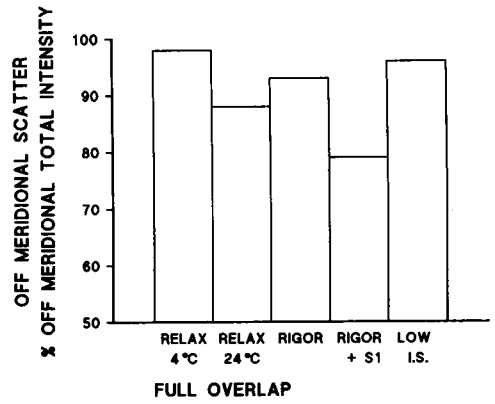

FIGURE 4 Results averaged from five different experiments with muscles at full-overlap (sarcomere length 2.5 μ). Relative intensities were measured from axial slices as described in Fig. 1. The diffuse scattering at 2 or 4°C was taken as 100%. In relaxed muscles the transition from the high to the low temperature state is accompanied by an increase in the diffuse scattering (maximally by ~12%). In the transition from the relaxed to the rigor state, the diffuse scattering is increased or decreased by ~4% starting at 24°C or 4°C, respectively. For further details see text. Incubation of a rigor muscle with myosin subfragment S-1 causes the intensity of the diffuse scattering to be reduced to ~15% of its rigor value. At the same time the intensity of the decorated actin layer lines increases considerably (Plate I, *c* to *d*). In a relaxed muscle at low ionic strength, the intensity of the diffuse scattering decreases by ~2% (Fig. 4) as the myosin layer lines return to a small degree (Fig. 1 *c*).

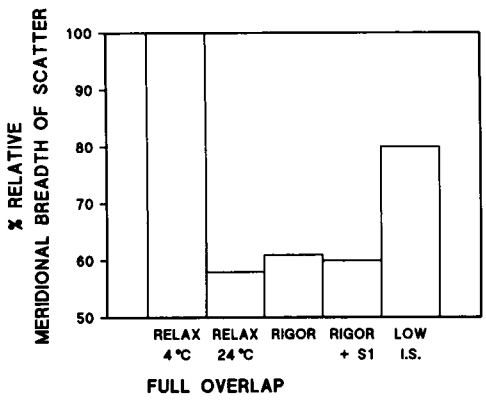

FIGURE 5 Results averaged from five different experiments with muscles at full-overlap (sarcomere length 2.5 μ). The extent of the diffuse scattering measured in the meridional direction at 2 or 4°C was taken as 100%. During the transition from 24 to 4°C, the extent of the diffuse scattering in the meridional direction increases by ~40%, whereas its extent in the equatorial direction undergoes only a very small change, namely a decrease by ~2% (Plate I, *a* to *b*). During the transition from the relaxed to the rigor state at 4°C, the meridional extent decreases by ~40%, and increases by ~4% starting at 24°C. In both cases the equatorial extent decreases by ~5% (Plate I, *a* or *b* to *c*). Incubation of a rigor muscle with S-1 does not affect either the meridional or equatorial extents (also Plate I, *c* to *d*). In relaxed muscles at low ionic strength, the meridional extent decreases by ~20%, but the equatorial extent remains much the same (data not shown here).

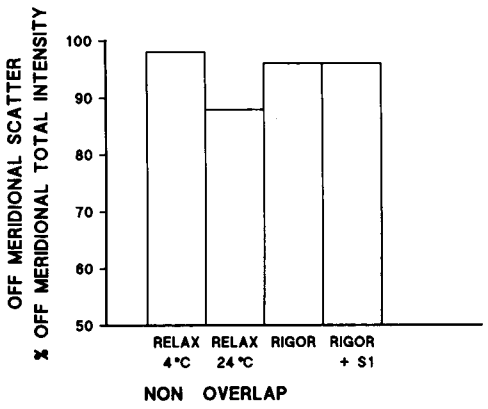

FIGURE 6 Results averaged from four different experiments with muscles at nonoverlap (sarcomere length 4.5 μ). Relative intensitities were measured from axial slices as described in Fig. 1. The diffuse scattering at 2 or 4°C was taken as 100%. In the transition from the high to the low temperature state the diffuse scattering increases (maximally by ~12%). Starting at 4°C, depletion of ATP reduces the diffuse scattering by ~2%. Starting at 24°C produces an increase of ~10%. Plate II (*c*) shows that decorated actin layer lines are not seen. Incubation of the ATP depleted muscle with S-1 causes no detectable change in the diffuse scattering.

(~4%) or a relatively large decrease ~40%) in its meridional extent, depending, respectively, on whether in the relaxed state the myosin layer lines are present (at 24°C) or absent (at 4°C). At both temperatures the equatorial extent decreases in rigor by ~5% (Plate I, *a* or *b* to *c*).

At the high temperature, the events are similar to those seen on cooling. At the low temperature, the large decrease in the meridional extent during the passage from the relaxed state to rigor (Fig. 5) is of some interest because under these conditions it would appear that the myosin heads adopt an average orientation which is more parallel to the filament axis. A more precise interpretation should become possible when the shape of S-1 is taken into account.

Nonoverlap

Following depletion of ATP at 24°C, decorated actin layer lines are not seen, but the relaxed state myosin layer lines disappear (Plate II *b* to *c*). This is what happens in frog muscles at low temperatures (Haselgrove, 1975; Poulsen and Lowy, 1983) and indicates that the myosin heads become largely disordered. Accordingly, the diffuse scattering should increase: Fig. 6 does indeed show an increase by ~10%.

In the experiments which start at 4°C, the myosin heads are already disordered (Plate II *a*). Therefore, following depletion of ATP, one would not expect to see much change in the diffuse scattering, and in fact we find only a very small (~2%) decrease (Fig. 6).

Following depletion of ATP, there are considerable differences in the changes of the meridional extent of the diffuse scattering seen in fulloverlap as compared with nonoverlap muscles (Figs. 5 and 7). Starting at 4°C and fulloverlap or nonoverlap, the meridional extent decreases by ~40% and 15%, respectively. Starting at 24°C and fulloverlap or nonoverlap, the meridional extent increases by ~4% and 25%, respectively. Also, whereas in fulloverlap at both temperatures the equatorial extent decreases by ~5% (Plate I, *a* or *b* to *c*), in nonoverlap such a change is not seen (Plate II, *a* or *b* to *c*). Considering the very considerable variability of the shape of the diffuse scattering in the different states of the muscle, these results provide further support for the view that a certain amount of the diffuse scattering must come from disordered myosin heads.

Myosin subfragment-1 (S-1) attached to actin

Fulloverlap

In agreement with the results from similar experiments on insect flight muscles (Goody et al., 1985), we found

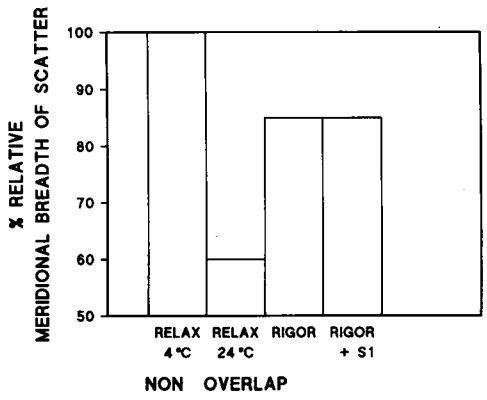

FIGURE 7 Results averaged from four different experiments with muscles at nonoverlap (sarcomere length 4.5 μ). The extent of the diffuse scattering measured in the meridional direction at 2 or 4°C was taken as 100%. During the transition from the high to the low temperature state, the meridional extent is reduced by ~40% without there being an appreciable change in the equatorial extent (also Plate II, a to b). Starting at 4°C, depletion of ATP reduces the meridional extent by ~15%; starting at 24°C produces an increase of ~25%. In both cases there is not much change in the equatorial extent (Plate II, *a* or *b* to *c*). Incubation of the ATP depleted muscle with S-1 does not affect either the meridional or equatorial extents (also Plate II, *c* to *d*).

that incubation of the rabbit muscle for several hours in a rigor solution containing 10–20 mg/ml S-1 leads to a 4–5 times increase in the intensity of the decorated actin layer lines. Furthermore, in the rabbit muscle incubation with S-1 causes an increase in the intensity ratio of the equatorial reflections (1,1)/(1,0), from the rigor value of 2.4 to a maximum of 5.3, thus indicating that in these experiments myosin heads had attached to all free actin monomers.

Following incubation of an ATP-depleted muscle with S-1, the diffuse scattering is reduced by ~15% of its value in rigor (Fig. 4). Presumably S-1 has filled the "holes" left in the helical decoration of the actin filaments, that is S-1 has attached to the actin monomers previously not labeled with endogenous heads in the A-band. Therefore one would not expect to see diffuse scattering due to substitution disorder. The finding of a 15% decrease in the diffuse scattering after addition of S-1 to an ATP-depleted muscle demonstrates the presence of substitution disorder along the actin filaments in the rigor state and provides some measure of its amount.

S-1 will of course also have labeled actin monomers in the I-band so that the actin helix will be fully decorated.

Incubation with S-1 does not affect either the meridional or equatorial extents of the diffuse scattering (Fig. 5; Plate I, *c* to *d*). Therefore it would appear that the orientation of the myosin heads is not altered.

In rigor patterns of frog muscles, Huxley and Brown (1967) and Haselgrove (1975) observed four layer lines lying relatively close to the meridian at spacings of ~36, 24, 18, and 14.4 nm. We have also recorded these inner layer lines from the rabbit psoas in rigor, and find that after incubation with S-1, except for the one at 24 nm, the other three become more prominent (results not shown here). This will be discussed later.

Nonoverlap

Incubating an ATP-depleted muscle with S-1 has no effect at all either on the intensity of the diffuse scattering (Fig. 6) or on its meridional and equatorial extents (Fig. 7; and Plate II, *c* to *d*). This is as expected because S-1 having attached to all the actin monomers at specific sites, it will not be possible to produce diffuse scattering changes in the fully decorated actin helix.

Effects of low ionic strength in fulloverlap muscles at 4°C

In relaxed muscle at normal ionic strength most myosin heads do not interact with actin (Huxley and Brown, 1967; Schoenberg, 1988). In relaxed muscle at low ionic strength stiffness increases and it is believed that myosin heads are weakly bound to actin (Brenner et al., 1982; Schoenberg, 1988; Brenner, 1990). Muscles at low ionic strength have been studied mainly with the object of exploring the idea that the change in the structure of the head between this state and rigor may be similar to that which occurs during tension generation (Matsuda and Podolsky, 1984; Huxley and Kress, 1985; Eisenberg and Hill, 1985; Yu and Brenner, 1989).

Recording the pattern at low ionic strength, we observed a decrease of ~2% in the diffuse scattering (Fig. 4), a result in line with our finding of only a small increase in the intensities of the myosin layer lines (Fig. 1 *c*). Measurements of the meridional extent of the diffuse scattering revealed a decrease by ~20% (Fig. 5), but hardly any variation in its equatorial extent (results not shown here). This can be interpreted to indicate that some change has taken place in the orientation of the heads.

Upper limit for the r.m.s. displacement of ordered myosin heads

Here we wish to consider very briefly some results from measurements on the six myosin layer lines. Fig. 8 reveals that no significant changes occur in their axial width at higher angles, which is in line with the view that the helically arranged myosin heads are subject to type-1 disorder. The presence of this type of disorder would be expected from the fact that the heads are tethered to the filament backbone, thus preserving long-range order

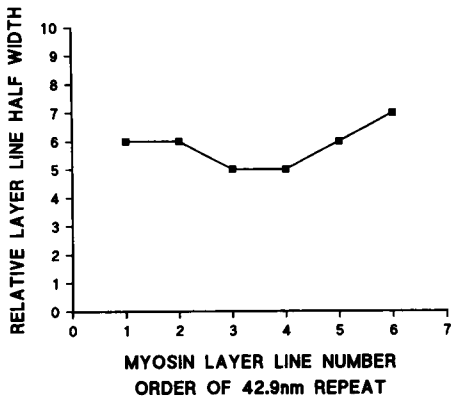

FIGURE 8 Measurements of the axial width on six myosin layer lines. Note that this does not change significantly at higher angles, indicating type-1 disorder. Data from experiments with 5 different relaxed full-overlap muscles at 24°C.

and therefore excluding type-2 (liquid-like) disorder (Poulsen et al., 1987).

Assuming that type-1 is the dominant disorder, and that the Debye-Waller factor is isotropic (i.e., ignoring that type-1 disorder is strictly speaking represented by a tensor), one can obtain an estimate of the upper limit for the r.m.s. displacement of the ordered myosin heads as follows.

Measurement of the integrated intensity on each of the six myosin layer lines shows how the intensities diminish (Fig. 9). The intensity decrease will be determined in part by the Debye-Waller factor (D), in part by other factors listed below. To determine D we use the

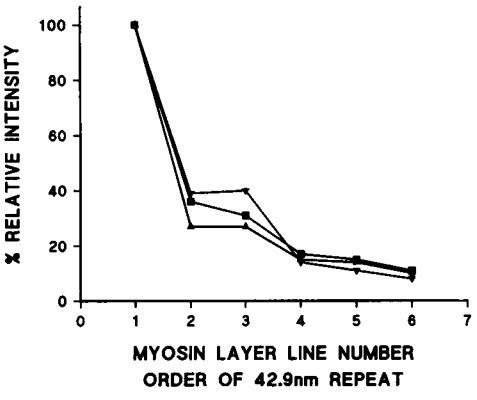

FIGURE 9 Relative intensities of six myosin layer lines. Symbols indicate data from experiments with three different full-overlap muscles at 24°C. The intensity of the first at 429 nm was taken as 100%. Note that the intensity falls quite steeply, leaving only 30–40% on the second layer line. For further details see text.

formula:

$$D = \exp(-4\pi^2\Delta^2 s^2), \quad (1)$$

where Δ is the r.m.s. displacement in any direction, and s is the distance in reciprocal space from the origin in any direction.

Factors which will also affect the intensity of the layer lines are: (*a*) the backbone structure of the myosin filament; (*b*) the size of the myosin heads, the detailed contribution being dependent on their shape and orientation; (*c*) the interference between the two heads of each molecule; (*d*) the disposition of the molecules within the 42.9-nm unit cell, the existence of the "forbidden meridionals" (Huxley and Brown, 1967) showing that the molecules are displaced in a systematic way from an exact 14.3 nm repeat.

Disregarding all these factors, and taking the intensity of the first myosin layer line as 100%, application of formula (1) shows that the data for the second, third, and fourth layer lines fit reasonably well with a r.m.s. displacement between 4 and 7 nm.

Lower limit for the r.m.s. displacement of disordered myosin heads

Again assuming that the disordered myosin heads are subject to type-1 disorder, the intensity distribution $I(s)$ of the diffuse scattering will be determined in part of the Debye-Waller factor (D) where:

$$D = \exp(-4\pi^2\Delta_1^2 s^2),$$

and in part by the intensity distribution $I(S1)$ due to the shape of the head where:

$$I(\bar{s}) = (1 - D)I(\bar{s}_1)$$

and Δ_1 is the r.m.s. displacement of the heads in any direction.

In the following we use the method introduced by Poulsen and Lowy (1983) to calculate a lower limit for Δ_1. The method is based on the fact that the intensity distribution of the diffuse scattering due to type-1 disorder decreases toward the center of the pattern. The larger Δ_1, the closer one must go to the center to detect this decresae. Thus Δ_1 can be estimated from the minimal value of the distance from the center (s min) where there is still no drop in intensity.

We find that the intensity distribution of the diffuse scattering has an approximately Gaussian shape with a maximum at ~ 0.04 nm^{-1}. Taking this value as s min, gives a Δ_1 of at least 9 nm. Using essentially the same method, data from live frog skeletal muscle patterns give

a similar value for Δ_1 (Bordas, J., personal communication).

DISCUSSION

Diffuse scattering in ATP-depleted muscles

As regards the diffuse scattering due to substitution disorder along the actin filaments, the following additional considerations are relevant. In his electron microscope studies of rigor insect flight muscles, Reedy (1968) found a specific distribution of attached myosin heads along each actin filament such that pronounced periodicities at 38.5 and 115 nm are generated. This led to an explanation of the rigor pattern in terms of "target areas" located on each actin filament where attachment of myosin heads would be most likely to occur (Holmes et al., 1980). Squire and Harford (1988) used this concept to account for the presence of the four inner layer lines in rigor patterns of frog muscle by suggesting that the target areas are defined solely by the azimuthal position of the actin-binding sites. On this view, our finding that after incubation of rigor muscles with S-1, the intensities of the inner rigor layer lines increase, indicates that the actin filaments have become more fully labeled. Most likely this is because the exogenous heads will attach at specific actin sites in the absence of any strain due to myosin subfragment-2 (see below). Thus our results can be taken to provide further evidence for the existence of substitution disorder along the actin filaments in the full-overlap rigor state. However, in nonoverlap muscles depleted of ATP, most of the diffuse scattering is probably produced by disordered myosin heads.

Further evaluation of our experiments with ATP-depleted muscles, particularly where S-1 was added, naturally raises the question as to what structures could be responsible for the substantial amount of diffuse scattering still seen under these conditions, regardless of overlap. Soluble sarcoplasmic proteins are not likely to be involved because these components have been largely removed during the skinning process (see also Padron and Huxley, 1984). At this stage we have no other data which could help toward a solution of the problem. Attempts are being made to obtain clues from experiments with myosin-extracted muscles.

Diffuse scattering from disordered attached myosin heads

We wish to put forward arguments which suggest that attached myosin heads might also produce a certain amount of diffuse scattering. Combined studies of equatorial reflections and actin layer lines indicate that in rigor frog muscles the majority of heads are attached at specific actin sites (Huxley and Brown, 1967; Huxley and Kress, 1985). This would not hold if, as suggested by Egelmann et al. (1982), the actin filaments possessed a great deal of azimuthal flexibility. Against that idea, Squire and Harford (1988) cite evidence from experiments with relaxed fish muscles which show sampling on actin layer lines thus demonstrating that along most of their length within the A-band, actin filaments can be in good helical register with their neighbors. These authors argue that if the actin azimuth was unfavorable for attachment, the actin filament may nevertheless undergo local azimuthal changes so as to accommodate head labeling. In their discussion, Squire and Harford (1988) intimate that a local distortion could be produced in the attached head to relieve the strain in myosin subfragment-2 (S-2) that would otherwise result. This raises the possibility that heads may attach over several azimuthal angles, thus producing diffuse scattering.

From these several considerations we conclude: (*a*) that in fulloverlap rigor vertebrate skeletal muscles a certain amount of diffuse scattering is produced by substitution disorder along the actin filaments; (*b*) that some diffuse scattering comes from heads attached over several azimuthal angles due to strain exerted via S-2; and (*c*) that both phenomena might also occur in the contracted state.

Relation to other work

Our findings from the experiments with relaxed skinned rabbit muscles differ in several respects from those obtained in experiments at 2°C with relaxed live frog muscles by Poulsen and Lowy (1983). But for reasons explained in Methods, it is not really possible to compare the two data sets. In any event, the behavior of disordered heads may well be somewhat different in the two cases owing to differences in experimental conditions and type of muscle. For example, stretching the live relaxed frog muscle reduces the intensity of the myosin layer lines (Haselgrove, 1975; Poulsen and Lowy, 1983), whereas this is not seen in the skinned psoas (Patterns *a* in Plates I and II).

The data presented here generally confirm Poulsen and Lowy's (1983) view that a significant amount of the diffuse scattering in relaxed muscles originates from disordered myosin heads. But considering the highly asymmetric shape of the diffuse scattering evident in all states of all the muscles which we as well as Lowy and Poulsen (1990) have investigated, the deductions by

Poulsen and Lowy (1983) based on Guinier plots are not likely to be valid.

It is worth noting that, as observed in cooled psoas muscles (Figs. 4 and 6), live frog muscles contracting isometrically at 4°C (Lowy and Poulsen, 1987, 1990), also show an increase of intensity in the diffuse scattering between the off-meridional myosin layer lines in the moderate angle region. But there is an important difference as regards the diffuse scattering along the meridian: in the first case one sees an increase (Fig. 3 *a*), whereas in the second there is hardly any change (Bordas, J., personal communications). The significance of this difference will be dealt with elsewhere.

With reference to the situation in other live contracting muscles, the reduction in the diffuse scattering which occurs in the psoas at a low temperature during the passage from the relaxed to the rigor state (Fig. 4) resembles that seen in live ABRM muscles where myosin layer lines are absent in the relaxed state at 20°C (Lowy and Vibert, 1972), and the diffuse scattering decreases during contraction (Lowy and Poulsen, 1982; Popp, Stewart, and Lowy, unpublished results). What happens in the ABRM could be explained if, starting at rest with all the myosin heads in a disordered state, many heads attach in an ordered configuration during the cross-bridge cycle. The reason might be that around each ABRM thick filament, up to 17 actins are available for interaction, and the actin filaments are labeled with myosin heads from one side only (Lowy and Hanson, 1972; Sobieszek, 1973). It would seem that the structural arrangements underlying the acto-myosin interaction are quite flexible thus making it relatively easy for the heads to attach in an orderly manner.

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