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BIREFRINGENCE CHANGES IN VERTEBRATE STRIATED MUSCLE

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The changes in birefringence in the rigor to relax transition of single Triton-extracted rabbit psoas muscle fibers have been investigated. The total birefringence of rigor muscle fibers was dependent on sarcomere length and ranged from $(1.46 \pm 0.08) \times 10^{-3}$ to $(1.60 \pm 0.06) \times 10^{-3}$ at sarcomere lengths from 2.70 μm to 3.40 μm. An increase in total birefringence was measured dependent on sarcomere length when 55 single fibers were relaxed from the rigor state with Mg-ATP. Pyrophosphate relaxation produced a smaller increase in retardation when compared to Mg-ATP. The expected change in intrinsic birefringence during the rigor to relax transition was calculated assuming a hinge function of the subfragment 2 moiety of myosin. The changes in birefringence during isometric contraction and relaxation have been discussed in relation to possible structural changes.

INTRODUCTION

Actin and myosin filaments are highly organized within the sarcomeres of vertebrate striated muscle. The myosin aggregates of rabbit psoas muscle form a hexagonal lattice with the actin filaments interdigitating in the trigonal positions (1). Low angle X-ray diffraction patterns indicate that in living muscle and in relaxed glycerinated muscle there is a constant lattice volume at all sarcomere lengths, so that the center to center spacing between actin and myosin vary inversely as the square root of the muscle length (2–5). The actin to myosin spacing has a value between ca 290 Å and 230 Å over a range of sarcomere lengths between 2.30 μm and 3.60 μm. In comparison, the lattice spacing of actin and myosin in glycerinated muscle is dependent on the pH, ionic strength, and valency of cations in the bathing medium (6, 7). Glycerinated muscle in rigor exhibits an approximately linear variation of lattice spacing with sarcomere length in neutral salt solutions (7).

A simple mechanical interaction between actin and myosin filaments is not possible because of the variable lattice spacing between the filaments at different sarcomere lengths. A mechanism explaining the interaction between actin and myosin must permit the crossbridges to adapt to these changes in interfilament spacings (8).

The difficulties in explaining crossbridge angles, movements, and interfilament spaces at different sarcomere lengths were overcome when H. E. Huxley postulated that the subfragment 2 (S2) moiety of myosin might function as a "hinge" (8). The movement of S2 moieties away from the long axis of the myosin aggregates would permit the attachment of the subfragment 1 (S1) moieties of myosin at the same orientation to actin over a considerable range of filament spacings. This hypothesis was based primarily on X-ray

diffraction studies which indicated that the actin filaments themselves were rather invariant structures (9, 10). In contrast, Oosawa and his co-workers have suggested that changes in the flexibility of actin filaments, as measured by quasielastic light scattering (11) and linear dichroism (12), might be involved in the interaction of the S1 moieties of myosin with actin.

H. E. Huxley's suggestion that the S2 moieties of myosin angle away from the myosin long axis during the transition from relaxed to contracted or rigor state demands that the intrinsic birefringence of the myosin aggregates decrease by a predictable amount (Fig. 1). If the S2 moieties are capable of swinging out from the myosin long axis, then the amount of myosin oriented parallel to the long axis (slow crystalline axis) would decrease, resulting in a decrease in the intrinsic birefringence.

Conversely, the intrinsic birefringence would increase in the transition from rigor or contracted state to the relaxed state as the S2 moieties of myosin returned to positions more parallel to the long axis of the myosin aggregates (Fig. 1).

Linear birefringence measurements have been applied successfully to the study of intact muscle. Vertebrate striated muscle has been characterized as containing highly birefringent anisotropic (A) bands and only weakly birefringent isotropic (I) bands (12). Huxley and Hanson (13) demonstrated that myosin was the birefringent protein of the A band, while Colby (14) quantified the variations of intrinsic birefrigence along single myofibrils immersed in O-toluidine. The intrinsic birefringence in the region of actin and myosin overlap was found to be much less than that of thin filaments alone. This result demonstrated that interactions between thick and thin filaments involve some loss of intrinsic birefringence.

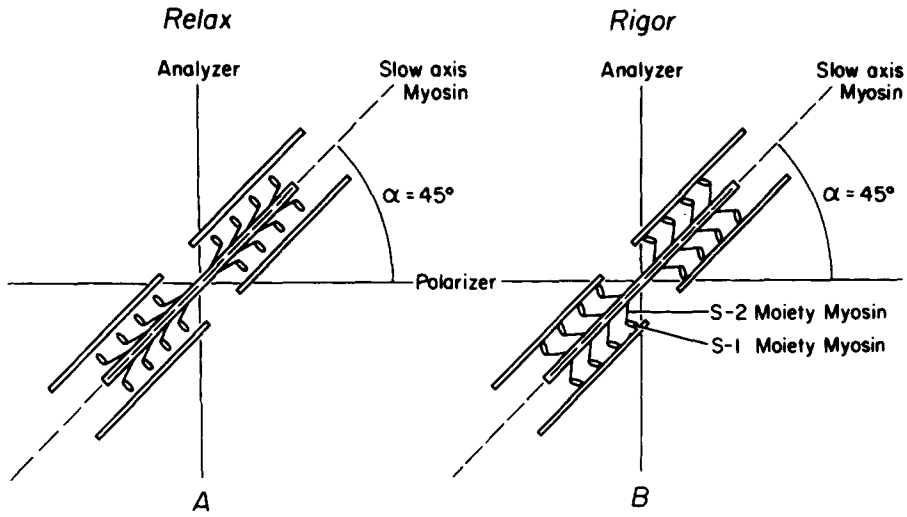

Fig. 1. The structural changes of myosin after Huxley (8) during the rigor to relax transition. If the S2 moiety of myosin functions as a hinge, then the positive intrinsic birefringence must increase during the transition. The slow axis of the myosin aggregate is parallel to the long axis and is shown here at 45° to the transmission plane of the polarizer.

Birefringence measurements have been utilized classically in studying the dynamics of muscle contraction (15). Early investigators measured a biphasic decrease in birefringence during isometric contraction (16, 17). In contrast, Eberstein and Rosenfalck (18) measured a monophasic decrease in total birefringence during isometric contractions of fresh fibers isolated from frog muscle. They measured a ca. 5.0% decrease in total birefringence that reached a minimum before the tension reached a maximum at a sarcomere length of ca. 2.50 μm (rest length in frog muscle).

Useful information may be gained when the polarization properties of muscle are related to data derived from X-ray diffraction and electron microscopy (5–7, 9, 14). Polarized-light techniques, including linear birefringence, linear dichroism, difluorescence, and circular dichroism, have the advantage that relatively small volumes may be measured during a short time interval. This property of polarized light techniques is particularly valuable in studying the dynamics of contraction within single muscle fibers, myofibrils, and nonmuscle motile systems (19). Birefringence measurements were made initially since they required no extrinsic labeling of the proteins.

It is the aim of this paper to discuss the changes in birefringence in single muscle fibers and to relate these changes to possible structural changes during the contraction cycle (20).

MATERIALS AND METHODS

The phase modulation microspectrophotometer (PM-MSP) was utilized in the birefringence mode where it functions as an automatic, photoelectric, null-seeking birefringence detection system (20–23). The PM-MSP system was used in conjunction with a Zeiss photomicroscope I equipped with selected strain-free polarization optics and a 150-watt xenon arc light source. The same measurements have been made with a birefringence detection system functioning as an automatic de Sénarmont compensator (Custom Instrumentation, Ravena, New York).

Small strips of rabbit psoas muscle were prepared at different sarcomere lengths by stretching the resting muscle in situ. Bundles of psoas muscle were tied to wooden sticks and placed in a Triton extraction solution for 6 hr at 0°C. (24). The Triton extraction solution was subsequently replaced with a glycerol storage solution modified after Rome (6). These muscles were used any time between 4 and 42 days after preparation.

The Triton extraction solution consisted of 20.0 mM phosphate buffer, 80 mM KCl, 5.0 mM EGTA, 2.5 mM dithiothreitol, and 1.0% Triton X-100, pH. 7.0. The glycerol storage solution contained 20.0 mM phosphate buffer, 80.0 mM KCl, 1.0 mM EGTA, 2.5 mM dithiothreitol, and 50.0% glycerol, pH 7.0.

Rigor was maintained in a solution modified after Rome (5) (5.0 mM PIPES buffer, pH 7.0, 5.0 mM EGTA, 6.0 mM $MgCl_2$, and 98.0 mM KCl). The muscle fibers relaxed without shortening in 5.0 mM PIPES buffer, pH 7.0, 5.0 mM EGTA, 5.0 mM $MgCl_2$, 3.0 mM Na_2 ATP, and 85.0 mM KCl. A pyrophosphate relaxing solution was prepared by replacing the ATP in the standard relaxing solution with 3.0–5.0 mM pyrophosphate.

Single fibers were teased out of a glycerinated bundle with fine watchmaker's

forceps and attached to an observation chamber (20). The muscle fibers were rotated on a motor-driven stage while the sinusoidal fluctuations of phase retardation were recorded (Fig. 2). After the phase retardation of rigor muscle fibers had been measured, the perfusion chamber volume was exchanged with relaxation solution and the measurements were repeated. A detailed description of the optical and experimental techniques is given elsewhere (20).

RESULTS

Birefringence of Muscle Fibers in Rigor

Assuming the fiber thickness to be equal to the fiber diameter, the total birefringence of rigor muscle fibers was dependent on sarcomere length and ranged from $(1.46 \pm 0.08) \times 10^{-3}$ to $(1.60 \pm 0.06) \times 10^{-3}$ at sarcomere lengths from 2.70 μm to 3.40 μm. The total birefringence decreased to $(4.0 \pm 0.5) \times 10^{-4}$ after the myosin was extracted (ca. 10.0% nonmyosin protein is also extracted using the pyrophosphate myosin extraction procedure [13]).

Increase in Birefringence during the Transition from Rigor to Relaxed State

An increase in total birefringence was measured dependent on sarcomere length when 55 single fibers were relaxed from the rigor state with Mg-ATP. The increase in total birefringence at rest length (ca. 2.70 μm) was $(1.67 \pm 0.05) \times 10^{-3}$, or ca. 14.0% greater than rigor fibers (20). The increase in total birefringence during Mg-ATP relaxation was smaller at longer sarcomere lengths where there was a decrease in actin and myosin overlap, as well as a decrease in lattice spacing (Fig. 3). The rigor to relax transition involved small decreases in fiber diameter at sarcomere lengths between 2.70 μm and 3.20 μm. After myosin extraction, no change in birefringence was detected upon the addition of the Mg-ATP relaxing solution.

Increase in Birefringence during the Transition from Rigor to Pyrophosphate Relaxed State

Pyrophosphate relaxation produced a smaller increase in retardation compared to Mg-ATP (Fig. 3). At rest lengths, pyrophosphate relaxation increased the total birefringence by ca. 3.0% and no further increase in birefringence was observed by increasing the magnesium and pyrophosphate concentration to 10.0 mM. No detectable change in fiber diameter occurred during pyrophosphate relaxation.

Muscle fibers relaxed for less than 5 min in pyrophosphate increased in birefringence after the addition of the Mg-ATP relaxing solution (Fig. 4). There was a greater increase in birefringence with rest length fibers than in fibers with longer sarcomere lengths (Fig. 4a, b).

DISCUSSION

Molecular Basis of the Variations in Muscle Fiber Birefringence

The total birefringence of muscle fibers depends primarily on a combination of form and intrinsic components. Theoretically, the form birefringence of muscle fibers should

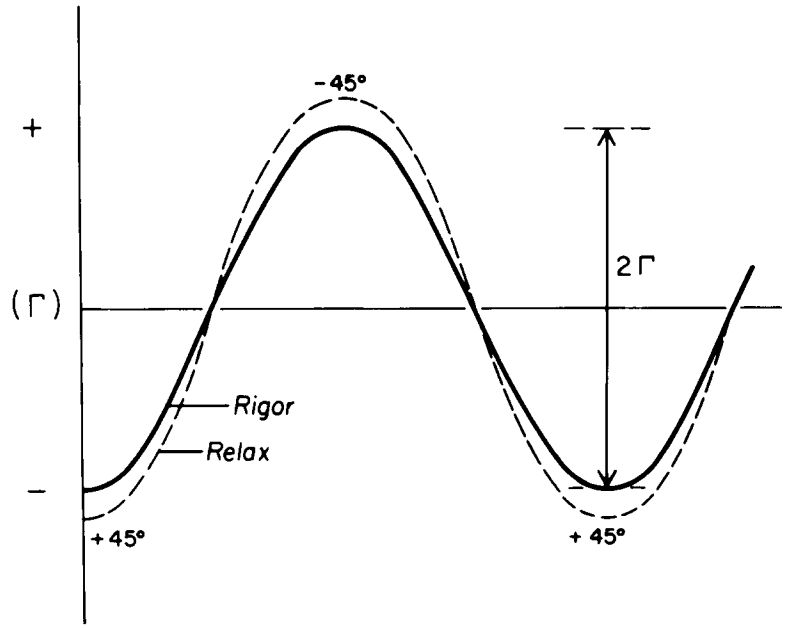

Fig. 2. Sinusoidal variation in phase retardation (Γ) of rigor (——) and relaxed (- - -) single fibers during rotation on the microscope stage. The maximum phase retardation occurs when the crystalline axis of the fibers are 45° to the polarizer transmission plane. The increase in phase retardation (Γ) during the rigor to relax transition is symmetrical around zero (20).

vary in a competing fashion at increasing sarcomere lengths. The constant volume characteristic of muscle will cause an increase in the volume fraction of filaments as the lattice spacing decreases at longer sarcomere lengths which will increase the form birefringence. In contrast, the number of filaments overlapped per unit length will decrease with increasing sarcomere length as the actin filaments are withdrawn from the myosin aggregates. A decrease in the number of overlapped filaments should diminish the form birefringence.

The intrinsic birefringence of muscle could vary with sarcomere length if portions of the contractile proteins changed conformation or orientation. Tropomyosin and myosin are the two major intrinsicially birefringent proteins in muscle. Tropomyosin is believed to change position within the actin helix by slight "rolling" movements (25). These movements should not affect the intrinsic birefringence to a large degree. On the other hand, large variations in the whole actin backbone, as suggested by Oosawa (10, 11), could affect the orientation of tropomyosin. However, X-ray diffraction measurements indicate that actin is a rather invariant structure (9).

If the S2 moieties of myosin function as hinges in bridging the gap between actin and myosin, then the intrinsic birefringence of the myosin aggregates would depend on the angle between the S2 moieties and the light meromyosin (LMM) backbones of myosin.

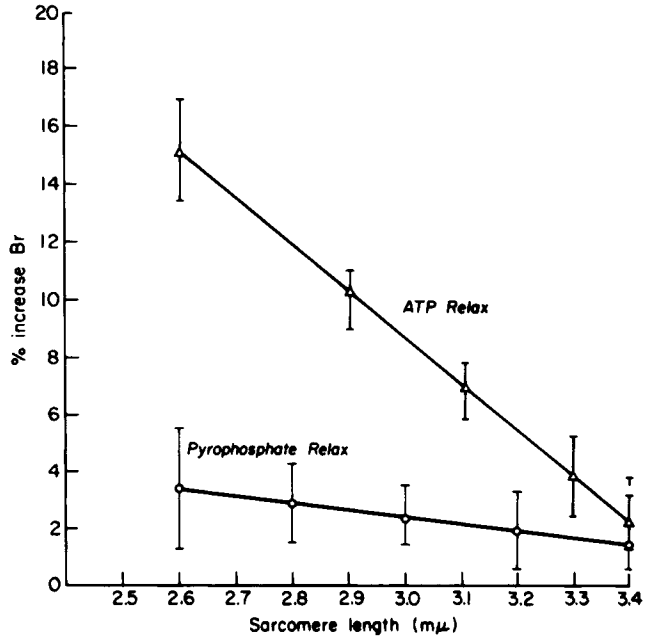

Fig. 3. Percent increase in total birefringence during the rigor to ATP relaxed state (△–△–△) and the pyrophosphate state (○–○–○) at various sarcomere lengths (20).

Decreasing angles between the S2 moieties and the LMM backbone would be required to bridge the gap between actin and myosin with increasing sarcomere lengths where the interfilament distance decreases. Therefore, the intrinsic birefringence of rigor fibers would increase at longer sarcomere lengths, since the S2 moieties would be more parallel to the myosin long axis (slow crystalline axis, Fig. 1).

The total birefringence of intact vertebrate striated muscle has been reported over a range from 1.70×10^{-3} (26) to 2.30×10^{-3} (27). The former value is quite close to the value reported here for relaxed glycerinated muscle at rest length (1.67×10^{-3}).

Fischer (27) demonstrated that relaxed smooth muscle as well as relaxed striated muscle increased in birefringence at longer sarcomere lengths. Striated muscle exhibited a ca. 4.0% increase in birefringence with a 25.0% increase in length. This increase in length corresponds to an increase in sarcomere length from 2.70 μm to 3.40 μm. In contrast, the present results indicate that the birefringence increases by ca. 10.0% over the same range of sarcomere lengths in rigor fibers. Therefore, a ca. 6.0% difference in birefringence exists between rigor and relaxed fibers at the same sarcomere length. Changes in form birefringence based on lattice spacing and relative number of filaments do not account for the total change in birefringence with sarcomere length (20). The fact that Colby (14) found a decrease in the intrinsic birefringence in the region of actin and myosin overlap

in rigor fibers demonstrates that structural alterations occur when the myosin is interacting with actin.

Rome (6, 7) demonstrated that the actin to myosin lattice spacing decreased as the ionic strength increased or when glycerinated muscle fibers at sarcomere lengths greater than 2.70 μm were relaxed (7). It has been demonstrated that muscle fiber diameters decreased by 2.0% at a sarcomere length of 2.90 μm when the ionic strength was increased from 0.04 to 0.14 or when the fibers were relaxed with Mg-ATP (20). Perturbing relaxed fibers by increasing the ionic strength of the bathing medium caused an increase in birefringence close to that predicted by changes in form birefringence. Conversely, there was almost a twofold birefringence increase during the rigor to relax transition, as compared to the control experiments decreasing the fiber diameter alone (20). A comparison of the changes in birefringence during these separate experimental treatments demonstrates that the changes in lattice spacing alone did not account for the total change in birefringence during relaxation (20).

The increase in total birefringence measured in the rigor to relax transition involves an increase in form birefringence due to the increased volume fraction of filaments, as well as an increase in intrinsic birefringence. It has been determined that the changes in form birefringence induced in muscle fibers by varying the ionic strength were consistent with the changes predicted by Wiener's equation (20). Therefore, the increased volume fraction of filaments in the rigor to relaxed transition accounted for more than half the total increase in birefringence at the sarcomere length studied (2.90 μm). Initial imbibition experiments have indicated that increases in the intrinsic birefringence accounted for the remaining birefringence (20).

The pyrophosphate relaxation experiments indicate that the dissociation of the actin and myosin with pyrophosphate does not induce the same structural changes as Mg-ATP (Fig. 3). The stepwise increase in birefringence from rigor to pyrophosphate relaxation to ATP relaxation (Fig. 4) indicates that the pyrophosphate relaxed state represents an intermediate structural state between rigor and Mg-ATP relaxation (28–30).

Predicted Intrinsic Birefringence Assuming a Hinge Function of S2

Determining the expected changes in birefringence, to a first approximation, during the rigor to relax transition involves several simplifying assumptions. (a) Angular movements of the S2 moieties of myosin are considered in two rather than three dimensions. Actually, the crossbridges are arranged helically around the myosin aggregates. (b) The polarizabilities of S2 and light meromyosin are treated separately. (c) The center to center spacing of actin to myosin at rest length (2.70 μm) is 280 Å. (d) The actin filaments are 60 Å in diameter. (e) The myosin aggregates are 120 Å in diameter. (f) The calculated surface to surface spacing between actin and myosin is ca. 190 Å. (g) The light meromyosin rods are 1,000 Å in length (ca. 0.67 total length of myosin). (h) The S2 moieties of myosin are 500 Å in length (ca. 0.33 total length of myosin). (i) The length of the S1 moiety of myosin is presently defined over a large range. Three possible lengths are 100, 120, and 140 Å. (j) The S1 moieties are angled 45° to the actin filaments in rigor. (k) The S2 moieties of myosin are parallel to the light meromyosin backbones in the relaxed state (Fig. 1). (l) The S1 moieties are optically isotropic; therefore, they could

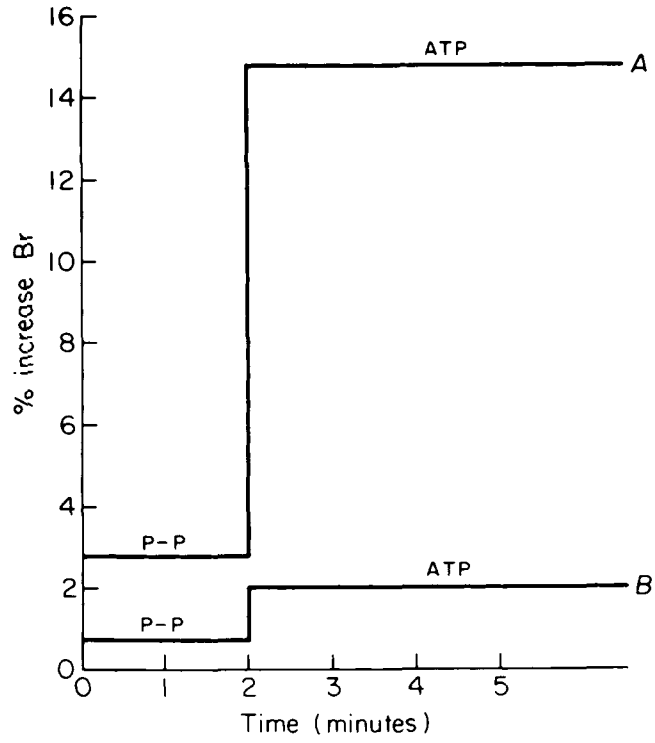

Fig. 4. Percent increase in birefringence during the rigor to pyrophosphate relaxed (P – P) and subsequent ATP relaxed (ATP) state. Fibers at rest length, 2.70 μm (A) and 3.40 μm (B) (20).

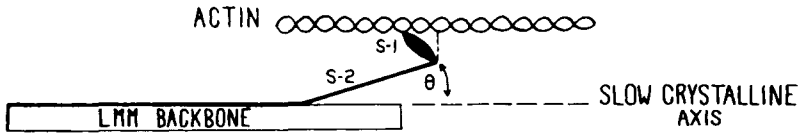

Fig. 5. Model of the interaction between a single myosin molecule (part of a myosin aggregate) and an actin filament, assuming a hinge function of the S2 moiety. The slow crystalline axis of the myosin aggregate is labeled. The angle θ depends on the interfilament spacing (see text).

affect the form birefringence and not the intrinsic birefringence.

The calculated "gap" between actin and the S1 moiety of myosin is determined by subtracting the effective length of the S1 when it is attached to actin at 45° (Fig. 5). The effective length of the S1 in rigor would be 71, 85, and 99 Å for S1 lengths equal to 100, 120, and 140Å, respectively. Therefore, the distance between actin and the tip of the S1 would be 119, 105, and 91 Å for three possible lengths of S1.

The angle θ between the myosin backbone and the S2 moiety that would permit the S1 to attach to actin over the above range of spacings is the angle satisfying the relation,

sin θ equal to the distance between the filaments divided by the length of the S2 (Fig. 5). The calculated angles are 14.0°, 12.3°, and 10.6° for S1 lengths equal to 100, 120, and 140 Å, respectively.

In rigor, when the whole muscle fibers (long axis of the myosin aggregates) are oriented ± 45° to the transmission plane of the polarizer, the S2 moieties of myosin would be oriented ± 31.0°, 32.7°, and 34.4° to the transmission plane of the polarizer for S1 lengths equal to 100, 120, and 140Å, respectively.

The light intensity passing crossed polars from a birefringent object depends on the angle between the slow axis of the object and the polarizer (31). Using the following relationship, the intensity ($I_\perp$) can be calculated on the basis of angular movements of the S2 moieties, while the LMM backbones remain oriented at ± 45°.

$$I_\perp = I' \sin^2 2\theta$$

$I_\perp$ = intensity passing crossed polars; I' = intensity of incident light; θ = specimen angle relative to the polarizer.

(1) Relaxed State

$$I_\perp = I' \sin^2 2(45°)$$

$$\therefore I_\perp = I'$$

(2) Rigor State

$$I_\perp = (0.67)\, I' \sin^2 2(45°) + (0.33)\, I' \sin^2 2(\theta'),$$

$$\theta' = 31, 32.7, \text{ or } 34.4°.$$

θ' represents the angular position of S2 moieties.

The light intensity passing crossed polars in the rigor state is equal to either $I_\perp$ = 0.92 I', 0.94 I', or 0.95 I' for the three assumed lengths of S1. This represents anywhere between a 5.0% and an 8.0% increase in light intensity during the rigor to relax transition.

The relationship between the predicted changes in light intensity passing crossed polars and the changes in phase retardation can be determined to a first approximation using the following equation (31):

$$I_\perp = I' \sin^2 \frac{\Delta}{2}.$$

I' = incident light intensity; Δ = phase retardation.

The intrinsic birefringence component in single muscle fibers varies from ca. 200 to 400 Å phase retardation (20). Within this range, the percent change in light intensity between the rigor and relaxed states is approximately equal to twice the percent change in phase retardation. The predicted percent increase in light intensity in the rigor to relax

transition (ca. 8.0% for an S1 length equal to the 100 Å) would represent an increase in phase retardation of ca. 4.0%. Therefore, if the S2 moieties of myosin function as hinges, then the intrinsic birefringence at rest length must increase by ca. 4.0% or less during the rigor to relax transition. The percent increase in intrinsic birefringence should decrease at longer sarcomere lengths, since the interfilament spacing decreases.

Relationship Between Birefringence Changes and Contractile State

The predicted change in birefringence from the rigor to relaxed state based on the hypothesis that actin filaments are rather invariant structures (9) and the presence of a hinge between S2 and light meromyosin is consistent with the measured changes in birefringence during contraction and relaxation. Eberstein and Rosenfalck (18) measured a ca. 5.0% decrease in total birefringence in isometrically contracting frog fibers at rest length (ca. 2.5 μm). The percent decrease in birefringence declined at longer sarcomere lengths, but slight decreases in birefringence were still recorded at nonoverlap sarcomere lengths (18). The slight decreases in birefringence at nonoverlap sarcomere lengths suggest that myosin aggregates may be controlled independently from actin (32).

The ca. 14.0% increase in total birefringence during the rigor to relaxed state transition involves an increase both in form and in intrinsic birefringence (20). A complete investigation relating the changes of the intrinsic birefringence with sarcomere length has not been completed, but initial measurements indicate that at least half the increase in total birefringence is due to form birefringence (20). Therefore, the increase in intrinsic birefringence would be equal to ca. 5.0% to 7.0% at rest length. The ca. 3.0% increase in total birefringence during pyrophosphate relaxation is compatible with minimal movements of the crossbridges (29, 30).

The complementary birefringence data from isometrically contracting (18) and relaxed single muscle fibers are consistent with the hypothesis that the S2 moieties of myosin function as hinges to permit the attachment of S1 moieties to actin. However, the measured changes in birefringence are slightly greater than the maximum predicted values. It is possible that other structural changes such as the "rolling" movements of tropomyosin (25), as well as conformational changes within the myosin backbone (32) affect the changes in intrinsic birefringence.

Future investigations will explore separately the changes in birefringence within the (I) and (A) bands. These experiments should identify the relative involvement of (I) band proteins to myosin aggregates in the total changes of birefringence. Furthermore, a combination of polarized-light techniques should identify dynamic events selectively during muscle contraction and relaxation. Difluorescence measurements on muscle fibers labeled with a probe specific for the rod portions of myosin should permit the hinge hypothesis to be tested directly. In addition, the application of these techniques to isometrically contracting muscles will complement similar investigations using X-ray diffraction (9, 32) and other light optical methods (11, 28, 33).

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