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We have investigated the orientation and rotational mobility of spin-labeled myosin heads in muscle fibers as a function of the sarcomere length in the absence of ATP. An iodoacetamidospin label was used to label selectively two-thirds of the sulphydryl-1 groups in glycerinated rabbit psoas muscle. Conventional electron paramagnetic resonance experiments were used to determine the orientation distribution of the probes relative to the fiber axis, and saturation transfer experiments were used to detect sub-millisecond rotational motion. When fibers are at sarcomere length 2.3 µm (full overlap), spin-labeled heads have a high degree of orientational order. The probes are in a single, narrow orientation distribution (full width 15°), and they exhibit no detectable sub-millisecond rotational motion. When fibers are stretched (sarcomere length increased), either before or after labeling, disorder and microsecond mobility increase greatly, in proportion to the fraction of myosin heads that are no longer in the overlap zone between the thick and thin filaments. Saturation transfer difference spectra show that a fraction of myosin heads equal to the fraction outside the overlap zone have much more rotational mobility than those in fibers at full overlap, and almost as much as in synthetic myosin filaments. The most likely interpretation is that some of the probes, corresponding approximately to the fraction of heads in the overlap zone, remain oriented and immobile, while the rest are highly disordered (angular spread > 90°) and mobile (microsecond rotational motion). Thus, it appears that myosin heads are rigidly immobilized by actin, but they rotate through large angles on the microsecond time scale when detached from actin, even in the absence of ATP.

1. Introduction

Muscle contraction involves the dynamic interaction of the two principal structural proteins of muscle: myosin and actin. For nearly 30 years discussions of the molecular mechanism of contraction have focussed on a proposed cyclical attachment, force-generating movement, and detachment of crossbridges between thick (myosin) and thin (actin) filaments (Huxley & Hanson, 1955; Huxley, 1957). The most widely discussed molecular model for this process involves the
rotational motion of the "head" or "S-1" region of myosin which forms part of a "crossbridge" to actin (Huxley, 1969; Huxley & Simmons, 1971; reviewed by Tregear & Marston, 1970; Morales et al., 1982). A definitive test of this model requires the direct measurement of myosin head rotation during muscle contraction. In making progress toward this goal, it is also essential to measure these orientations and motions in systems less complex than contracting fibers. Therefore, many measurements have been made on systems approximating intermediates in the contraction cycle: for example, purified myosin or relaxed fibers (ATP, no Ca²⁺), in which heads have little or no interaction with actin; and actomyosin or fibers in rigor (no ATP), in which heads are attached to actin.

Steady-state X-ray diffraction and electron microscopy have provided considerable information on the static (time-averaged) structure of myosin monomers, thick filaments, actomyosin and muscle fibers. One of the most consistent and reliable conclusions from these studies is that myosin crossbridges are highly ordered in rigor, due to the attachment of myosin heads to actin with distinctly different and relatively uniform orientations (Reedy et al., 1965; Reedy, 1965,1967; Huxley & Brown, 1967; Haselgrove, 1975; Holmes et al., 1980; Toyoshima & Wakabayashi, 1960; Taylor & Amos, 1981). Three-dimensional image reconstructions from electron micrographs have led to models in which S-1 attaches to thin filaments at angles of 75° (Toyoshima & Wakabayashi, 1980) or 55° ± 5° (Taylor & Amos, 1981), depending on how the respective authors chose to place the long axis of S-1 within their reconstructed images.

The orientation of crossbridges detached from actin (e.g. in relaxed muscle or in fibers stretched to eliminate overlap between thick and thin filaments) is considerably less certain. Electron micrographs appear to show much less orientational order in relaxation than in rigor (Heuser, 1983) and X-ray diffraction data from muscle in relaxation and/or at long sarcomere lengths are clearly consistent with considerable disorder of some kind (Huxley & Brown, 1967; Haselgrove, 1975; Poulsen & Lowy, 1983). However, the observation that relaxed muscle does exhibit clear X-ray layer-lines out to the sixth order (Huxley & Brown, 1967; Rome, 1972) implies some degree of helical order, and most models have depicted relaxed crossbridges as having fairly well-defined orientations that are different from those in rigor (Haselgrove, 1980; Reedy et al., 1965).

However, these structural measurements are subject to limitations and ambiguities in interpretation. Neither electron microscopy nor conventional X-ray diffraction can be used to follow directly the structural dynamics of macromolecules. Electron microscopy can only be used to study static samples fixed by methods which can introduce artifacts (Reedy et al., 1983), and it is often difficult to clearly distinguish individual proteins, even in image reconstructions. Although most X-ray diffraction studies up to this point have provided only time-averaged information, recent time-resolved X-ray methods (Huxley et al., 1981,1982; Lowy & Poulsen, 1982) show great potential for studying structural

† Abbreviations used: s-1, subfragment 1; e.p.r. electron paramagnetic resonance; IASL, N-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl) iodoacetamide; MSL, N-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)isoleucine.
dynamics in muscle. However, in both steady-state and time-resolved X-ray
diffraction, it is often difficult to assign changes in the diffraction pattern to
specific structures, and it is even more difficult to interpret these changes
unambiguously in terms of molecular orientations and rotations (Hasegove &
Rodger, 1980).

Site-specific labeling with orientation-sensitive spectroscopic probes is an
approach that is well-suited to decreasing this ambiguity in the study of myosin
head orientation and rotational motion. Fluorescence anisotropy (Mendelson et al.,
1973; Mendelson & Cheung 1976, 1978) and conventional e.p.r. (Seidel & Gergely,
1973; Thomas et al., 1975) have been used to study the nanosecond rotational
motions of labeled myosin heads in solution, but these techniques are not sensitive
to the slower motions that occur in the protein assemblies of the muscle fiber.
Saturation transfer e.p.r. spectroscopy is optimally sensitive to rotational motion
in the microsecond time range (Thomas, 1978; reviewed by Hyde & Thomas,
1980), and it has been used to study the rotational dynamics of spin-labeled
myosin heads in purified myosin and in myofibrils (Thomas et al., 1975, 1979, 1980;
reviewed by Thomas, 1982). In those studies, spin labels were shown to be rigidly
fixed to the myosin head, so that the e.p.r. spectra could be used to monitor the
motion of the head as a whole, rather than motion of the probe relative to the
protein. Myosin heads were found to be immobile (on the microsecond time scale)
when attached to actin in the absence of ATP, either in purified actomyosin
(Thomas et al., 1975), or in myofibrils in rigor (Thomas et al., 1980). However,
considerable microsecond mobility was observed in synthetic myosin filaments in
the presence and absence of ATP (Thomas et al., 1980), suggesting a simple model
in which myosin heads have microsecond rotational mobility if, and only if, they
are detached from actin, regardless of the presence of ATP.

The validity of this model depends on the assumption that myosin head
rotational mobility is the same in synthetic thick filaments as in native thick
filaments in muscle fibers. Conventional e.p.r. has been used to probe the
orientation of labeled heads in glycerinated rabbit psoas fibers (Thomas & Cooke,
1980). In that study, the overlap between thick and thin filaments was partially
eliminated by increasing the sarcomere length, resulting in conventional e.p.r.
spectra of fibers containing detached myosin heads in the absence of ATP. Fibers
were also studied at full overlap under relaxing conditions (ATP, no calcium). It
was found that heads are highly oriented when attached to actin in rigor, but are
highly disoriented when detached from actin, either due to ATP (relaxation) or
due to increased sarcomere length. However, it was not determined whether the
disorder observed at long sarcomere length is static or dynamic on the
microsecond time scale; it remained possible that microsecond rotational motion
like that observed in relaxed myofibrils (Thomas et al., 1980) requires not only
detachment of heads from actin but also the presence of ATP. The present study
is designed to determine whether detachment of myosin heads from actin is
sufficient to permit microsecond rotational mobility. Accordingly, we have used
both conventional and saturation transfer e.p.r. to determine the dependence of
the orientation and the rotational mobility of spin-labeled myosin heads on the
sarcomere length of glycerinated fibers, in the absence of ATP.
2. Materials and Methods

(a) Preparation of fibers and proteins

Psoas muscle from New Zealand white rabbits was dissected into small strips (2 to 5 mm in diameter) and glycerinated as described (Cooke & Bialek, 1979). Usually after spin-labeling (procedure discussed in section (b)), the following procedure was used to vary the sarcomere length. Fiber bundles (about 1-0 mm in diameter) were clamped to opposite plates of a small lab jack (Little Jack, GCA/Precision Scientific, Chicago, IL) and drawn taut by increasing the separation between the plates. This apparatus was placed over a trough of relaxing solution (5 mm-ATP, 0-12 m-KCl, 1 mm-EGTA, 1 mm-Na3, 25 mm-MOPS, pH 7-0, 0°C), so that the fiber was immersed. After a 15-min incubation, the length was gradually increased until the desired sarcomere length was achieved. The mean sarcomere length was determined by examination of first-order diffraction line-spacing of the beam from a 0.5 mW He-Ne laser (Spectra-Physics model 155, Mt. View, CA). A fiber bundle was used for subsequent experiments only if this determination was uniform along the full length of the fiber bundle within ±0.01 μm. Sarcomere length was fixed by transfer of the stretched fiber bundle (while still clamped) into rigor solution (0-12 m-KCl, 5 mm-MgCl2, 1 mm-EGTA, 1 mm-Na3, 25 mm-MOPS, pH 7-0, 0°C) to remove ATP. If unlabeled fibers were stretched, they were labeled at this point (see section (b)). Taking the length of the hydrated thick filament to be 1.6 μm (Page & Huxley, 1963; Craig & Offer, 1976; Craig, 1977), the length of the bare zone to be 0.15 μm (Huxley, 1963; Page & Huxley, 1963), and the length of the I-filament to be 2.24 μm (Craig, 1977), we calculated the fraction of myosin heads in the overlap zone as follows: For sarcomere length (S.L.) of 1.6 to 2.4 μm, the fraction of overlap is 1. For S.L. values of 3.8 μm or greater, overlap is 0. For intermediate values of S.L. the overlap fraction is (3.8 μm − S.L.)/1.4 μm.

Myofibrils were prepared from two sources: 1 rabbit back and leg muscle, and 2 rabbit psoas muscle. The method for preparing myofibrils from rabbit back and leg muscle has been described (Thomas et al., 1980). Myofibrils were made from psoas fibers by a similar method, following homogenization with a ground-glass tissue homogenizer. Myosin was extracted from myofibrils as described (Thomas et al., 1980).

F-actin was prepared as described by Thomas et al. (1979). Actinomyosin was prepared by mixing myosin and F-actin in rigor solution such that the ratio of actin monomers to myosin was 5:1. Then 3 m-KCl was added to raise the KCl concentration to 0.6 M. The solution was mixed thoroughly and then dialyzed exhaustively against rigor solution at 0°C.

(b) Spin-labeling

Strips of glycerinated psoas fibers were dissected into bundles of 25 to 30 fibers, and these bundles were washed in rigor solution at 0°C for 30 min. The fibers were then transferred into a prelabeled solution (rigor solution plus 2 m-PPi) and incubated 30 min at 0°C. An iodoacetamide spin-label, designated IASL (SYVA, Palo Alto, CA), was added to a final concentration of 0.5 mM. After 1 h with gentle shaking, excess label was removed by washing the fibers for 30 min in prelabeled solution followed by 30 min in rigor solution. Labeling was followed by a 2-h incubation in 25 mM-K3Fe(CN)6, 10 mM-MOPS (pH 7) at 0°C. The ferricyanide treatment is used to selectively destroy the signal from probes not rigidly attached to myosin heads (Thomas et al., 1980; Graceff & Seidel, 1980). Ferricyanide was removed by washing several times in rigor solution. Spin-labeled fibers were either used immediately or stored in rigor solution containing 50% (w/v) glycerol for up to 1 week.

Rabbit myofibrils were labeled with IASL by the same method used for labeling fibers, except that myofibrils were pelleted by low-speed centrifugation, then resuspended to change solutions.
Protein concentrations were determined as described (Thomas, 1978; Thomas et al., 1980). ATPase assays were measured by determining the rate of release of inorganic phosphate (P_i). Myosin K'/EDTA ATPase activity was assayed at 25°C in a solution containing 1 mM-EDTA, 0.12 M-KCl, 10 mM-MOPS (pH 7.0). The reaction was started by the addition of 5 mM-ATP and portions were quenched by pipetting into the acidic P_i-determining solution used by Lanzetta et al. (1979). Protein concentrations were chosen so that 1, 3, and 5-min time points produced measurable absorbances at 660 nm that were linear with time.

(d) Tension measurements

Isometric tension development by psoas fibers was measured using a home-built tensiometer employing an Akers 801 transducer element (Akjeselskapet Mikro-Elektronikk, Horten, Norway). The design of the tensiometer is similar to that of Crowder & Cooke (1984). Single glycerinated fibers at full overlap (5 to 7 µm in diameter, 3 to 5 mm in length) were mounted horizontally in the trough of a temperature-controlled brass block (25°C). Each fiber was suspended between a fine glass hook attached to the micrometer and a stainless steel pin fixed to the transducer element, it was then glued in place using 10% Duco cement (Du Pont Co., Wilmington, Delaware) in acetone. The trough was then filled with relaxing solution and a zero tension reading was taken. The relaxing solution was rapidly exchanged with contraction solution (relaxing solution +2.5 mM excess CaCl_2) and the resultant tension was recorded. IASL-labeled fibers treated with ferricyanide for 2 h produced 85% of the tension of unlabeled control fibers.

(e) e.p.r. spectra

Conventional (V_1) and saturation transfer (V_2) e.p.r. experiments were performed as described (Thomas et al., 1976,1979,1980; Thomas & Cooke, 1980) using a Varian E-109 Spectrometer (Varian Associates, Palo Alto, CA). Spectra were digitized and analyzed by means of a North Star computer interfaced to the spectrometer. For psoas fiber samples, conventional e.p.r. can be used to determine the orientation distribution of probes relative to the axis of the applied magnetic field. The theory which explains the orientation sensitivity of conventional e.p.r. has been discussed (Thomas & Cooke, 1980). The key principle is that a spin-label’s contribution to a conventional e.p.r. spectrum depends almost exclusively on cos^2 θ, where θ is the angle between the probe’s principal axis and the applied magnetic field. Therefore, if psoas fibers are placed parallel to the applied field and the probes are bound at a single fixed angle with respect to the fiber axis, a characteristic e.p.r. spectrum is obtained which permits the determination of θ to within an accuracy of ± 1°. If there is some orientational heterogeneity of the probes, the unique orientational resolution of the e.p.r. spectrum permits an unambiguous determination of the orientation distribution ρ(θ). For example, if the distribution is a spherically weighted Gaussian, obeying the equation:

$$\rho(\theta) = \sin^2 \theta \exp \left[ -4 \ln 2 \left( \frac{\theta - \theta_0}{\Delta \theta} \right)^2 \right]$$

Fig. 1 shows how the spectra depend on θ_0 (the most probable angle) and Δθ (the full width at half maximum of the Gaussian factor). Each row demonstrates the sensitivity of the conventional e.p.r. spectrum to changes in θ_0. In each of the columns of Fig. 1, Δθ is varied from no angular spread (Δθ = 0°) to a random distribution (Δθ > 90°), to show the effects of orientational disorder on the lineshape. Through the use of simulated spectra like those in Fig. 1, both θ_0 and Δθ can be fitted with a high degree of precision (D. D. Thomas, K. Lindahl, C. Wendt & V. A. Barnett, unpublished work). In this study, muscle fibers
oriented parallel to the magnetic field axis are the only samples which show a preferred orientation in conventional e.p.r. experiments. The conventional e.p.r. spectra of myofibrils, myosin, and actomyosin match closely the spectrum of a random orientation distribution of probes (see the bottom row, Fig. 1).

Saturation transfer e.p.r. can be used to detect sub-millisecond rotational motions of spin-labeled proteins (reviewed by Hyde & Thomas, 1980). The effective rotational correlation times are determined by comparing experimental spectra with reference spectra obtained from isotropically tumbling spin-labeled hemoglobin in solutions of known viscosity and temperature. Assuming hemoglobin approximates a sphere of radius 29 Å, the rotational correlation time $\tau_c$ can be determined ($\tau_c = \eta V/kT$, where $\eta$ = viscosity, $V$ = volume of the sphere, $k$ = Boltzman's constant and $T$ = absolute temperature; see Fig. 2). In this study we have used the peak $L'/L$ to determine rotational correlation times (Thomas et al., 1976), using reference spectra obtained recently on our current spectrometer (T. C. Squier & D. D. Thomas, unpublished work).

To obtain a randomly oriented sample, fibers were minced with a scalpel and conventional e.p.r. was used to confirm the absence of a preferred orientation of probes before saturation transfer spectra were obtained. This was done to remove all orientational information from the saturation transfer spectra and ensure that only motional parameters were measured.

The relative number of spins per sample was determined by double integration of the digitized conventional ($V_1$) e.p.r. spectra, recorded at low (non-saturating) microwave power. Saturation transfer e.p.r. ($V_2$) spectra shown are all normalized to the same number of spins, by dividing each spectrum by a number proportional to the double integral of the
Fig. 2. Saturation transfer e.p.r. spectra of spin-labeled hemoglobin in glycerol/water solutions. The ratio of glycerol to water and the temperature were varied to change the rotational correlation time ($\tau$) of the hemoglobin tumbling in solution. Each spectrum above represents the same number of spins. The baseline for the spectra above and all the experimental spectra which follow is 110 G.

$V_1$ spectrum. The molar concentration of spins in a sample is estimated by comparison of the number obtained from double integration of the $V_1$ spectrum with the number obtained for a 0.5 mM IASL standard whose $V_1$ spectrum has been digitized and double-integrated in the same manner.

3. Results

(a) Extent and specificity of labeling

It has been shown (Thomas et al., 1980) that IASL reacts quite specifically with the fast-reacting sulphydryl (designated $SH_1$) on the myosin head, even when the reaction is carried out in intact myofibrils or fibers, and the reaction is allowed to continue until most of the $SH_1$ groups are labeled. IASL was chosen over other spin-labeling reagents because of the relative ease in obtaining site-specific labeling at $SH_1$ (Thomas & Cooke, 1980; Thomas et al., 1980). Since a slightly different labeling procedure is used in the present study, we have analyzed the labeling results for the present preparation.

The extent of labeling ([spin-labels bound]/[myosin heads]) was determined by calculating the molar concentration of spin-labels and the molar concentration of myosin heads, in a sample obtained by homogenizing labeled fibers and dissolving them in 4 M-urea. The concentration of spin-labels was determined from the e.p.r. spectrum as described in Materials and Methods, section (d), above. The
concentration of myosin heads was determined by measuring the protein concentration and multiplying by the fraction of myofibrillar protein which is assumed to be myosin. If we assume that this fraction of myosin is 0.54 (Potter, 1974), we obtain a value of 0.52±0.06 for the extent of labeling. If we assume that the fraction of myosin is 0.43 (Yates & Greaser, 1983), we obtain a value of 0.66±0.07 for the extent of labeling. The mean of these two values is 0.59. These values are from the final preparations, after ferricyanide treatment. The ferricyanide treatments destroyed no more than 10% of the total signal.

The modification of SH1 is known to cause characteristic changes in myosin ATPase activity (Sekine & Kielley, 1964). In particular, the fractional inhibition of the K+/EDTA ATPase has been demonstrated to be a good indicator of the fraction of SH1 groups modified. Assays on myosin extracted from fibers used in this study exhibited a 66%±6% inhibition of K+/EDTA ATPase activity, indicating that the fraction of SH1 groups labeled is 0.66±0.06. The ferricyanide treatment had no effect on the K+/EDTA ATPase activity of the labeled fibers. Thus, our estimates for the fraction of SH1 groups labeled (0.66±0.06) and the extent of total labeling ([labels]/[heads]) = 0.59±0.13 are in good agreement, indicating that most the labels are attached to SH1 groups. The extent of labeling (measured from the e.p.r. signal) appears to be slightly less than the extent of SH1 blocking, possibly due to the destruction by ferricyanide of some (but not more than 10%) of the probes on SH1.

(b) Orientation (conventional e.p.r.)

At full overlap (sarcomere length = 2.3 μm) the conventional e.p.r. spectrum of fibers oriented parallel to the references magnetic field show a high degree of orientation, with only a very small spectral component corresponding to randomly oriented probes (Fig. 3, 100% overlap), confirming the result of Thomas & Cooke (1980). At least 90% of the label is within a distribution centered at 68° (θ0) with respect to the fiber axis with a full width at half maximum of 15° (Δθ, see Fig. 1). Thomas & Cooke (1980) demonstrated that this uniform orientation was due to the bond between the myosin head and actin, since spin-labeled S-1 (or heavy meromyosin) diffused into unlabeled fibers produced the same spectrum as that of spin labels attached directly to myosin in glycerinated fibers.

When fibers are stretched, the shape of the conventional e.p.r. spectrum changes dramatically (Fig. 3, 33-6% and 9-3% overlap); there is a decrease in the spectral component corresponding to highly oriented probes (θ0 = 68°) and a concomitant increase in a component shaped like the spectrum of a randomly oriented distribution of probes (see Fig. 1, bottom row). This is clearly seen in the spectrum of fibers stretched to 33-6%±3-6% overlap (sarcomere length = 3.33±0.05 μm), the line shape appears to contain two components: one with sharp lines resulting from probes (and presumably heads) which have retained the rigor orientation observed at full overlap (Fig. 3, top), and the other with broad peaks in the wings of the spectrum resulting from heads that have adopted a broad orientation distribution like that observed for randomly oriented probes (e.g. probes attached to SH1 of spin-labeled synthetic myosin filaments;
Fig. 3. Conventional e.p.r. spectra of spin-labeled psoas fibers at varying percentages of overlap. Top to bottom 100%, 33.6%, 9.3% overlap, and synthetic myosin filaments. As the percentage of heads in the non-overlap region increases, so does the contribution to the spectrum from isotropic probes. The myosin filaments represent an essentially isotropic distribution of crossbridge orientations. The spectra have been normalized to represent the same number of spins.

Fig. 3, bottom). In the spectrum of fibers stretched to 9.3% ± 2.1% overlap (sarcomere length = 3.67 ± 0.03 μm) the orientation distribution, as indicated by the line shape, has shifted even more toward that observed for randomly oriented heads. Essentially the same results were obtained using fibers labeled either before or after stretching, and the effects of stretching on the e.p.r. spectrum were reversible upon fiber shortening. Thus, the labeling procedure is not specific for any subpopulation of heads (e.g., those in the overlap zone or those with a particular orientation).

Bundles of labeled fibers were treated with 0.5 M-KCl, a concentration which is sufficient to disrupt thick filament integrity. At full overlap, little or no effect on probe orientation was evident. In stretched fibers, however, the line shape of the conventional e.p.r. spectrum changed substantially. The spectral features indicating a random distribution of probes diminished, and a corresponding increase was observed in the peaks corresponding to probes at the rigor orientation (data not shown). Presumably, this treatment dissolves the thick filaments and allows myosin molecules to diffuse out of the non-overlap region of
stretched fibers to find contacts with actin and form bridges. These data, in conjunction with the results obtained by Thomas & Cooke (1980) using IASL-labeled S-1 and heavy meromyosin, confirm that myosin head orientation in rigor is dependent on the bond between the myosin head and actin.

(c) Rotational motion (saturation transfer e.p.r.)

Saturation transfer e.p.r. spectra were obtained for actomyosin (5 actin/myosin) as well as each of the samples discussed above. When the saturation transfer e.p.r. spectra for actomyosin or fibers in rigor at full overlap (Fig. 4, top two spectra) are compared to reference spectra (Fig. 2), spectral parameters ($L^*/L$; Thomas et al., 1976; T. C. Squier & D. D. Thomas, unpublished work) indicate that there is essentially no motion on the sub-millisecond time-scale. This is also the case for rigor myofibrils and S-1 immobilized by crosslinking to glass beads (Thomas et al., 1980). However, saturation transfer e.p.r. spectra of synthetic myosin filaments (Fig. 4, bottom spectrum) (Thomas et al., 1975, 1980) and relaxed myofibrils (Thomas et al., 1980) show clear evidence of rotational motion, with effective correlation times on the order of one to ten microseconds. These observations

![Graph showing saturation transfer e.p.r. spectra of spin-labeled pea fibers at varying percentages of overlap. Top to bottom: rabbit actomyosin (5 actin/myosin); 100% overlap; 33-6% overlap; 9-3% overlap; synthetic myosin filaments. At 100% overlap as in actomyosin, there is no rotational motion on the sub-millisecond time scale. As the percentage overlap is decreased, the spectra lose intensity and exhibit spectral changes which correspond to faster motion, much like that seen in synthetic myosin filaments. Each spectrum has been normalized to represent the same number of spins.](image-url)
have been employed to demonstrate that the immobilization of the myosin head
requires the actomyosin bond (Thomas et al., 1980).

However, the question remains whether the rotational mobility of detached
heads in native thick filaments in the muscle fibers is the same as in synthetic
myosin filaments, i.e. whether the orientational disorder observed in stretched
fibers (Fig. 3) is dynamic or static. The saturation transfer, e.p.r. spectra obtained
from stretched fibers (Fig. 4) answer this question. The spectra indicate increasing
rotational mobility as the percentage of heads in the overlap zone decreases,
strongly implying that the non-overlap heads have considerable microsecond
rotational mobility. When these spectra are compared directly with hemoglobin
reference spectra (Fig. 2), the effective correlation times are \( \geq 700 \mu s \) for 100% 
overlap, 200 \( \mu s \) for 36\% overlap, and 80 \( \mu s \) for 9\% overlap. However, this
comparison assumes that each spectrum corresponds to a homogeneous
population of probes, in contrast to the indications of at least two components in
the \( V_1 \) spectra. Therefore, these effective correlation times are not an accurate
characterization of the motions in stretched fibers. In the following section,
computer subtraction of spectra is used to resolve the individual spectral
components in both the \( V_1 \) and \( V_2 \) spectra.

(d) Difference spectra

(i) Orientation (conventional e.p.r.)

The fraction of myosin heads that form crossbridges to actin in the absence of
ATP is identical to the fraction of heads in the overlap zone between the thick
and thin filaments, as calculated from the sarcomere length (see Materials and
Methods, section (a)), as shown previously by e.p.r. (Thomas & Cooke, 1980) and
Therefore, we used the sarcomere length as a guide in computer subtractions of
digitized e.p.r. spectra in order to obtain spectra corresponding only to heads not
in the overlap zone, hence not bound to actin. Figure 5 (unbroken curves) shows
difference spectra generated from the spectra in Figures 3 and 4 that correspond
to 9\% and 100\% overlap.

The unbroken curves on the left side of Figure 5 are both the \( V_1 \) difference
spectrum generated by subtracting 9\% of the full overlap spectrum (Fig. 3, top)
from the spectrum corresponding to 9\% overlap (Fig. 3). The upper spectrum is
overlaid with a computer simulation corresponding to \( \theta_0 = 68^\circ \), with \( \Delta \theta = 90^\circ \)
(i.e. no change in mean angle of orientation but increased width of the Gaussian
distribution), while the lower spectrum is overlaid with the \( V_1 \) spectrum of
myosin filaments. The \( V_1 \) difference spectrum is fitted much more closely by a
spectrum corresponding to a completely random distribution, whether produced
by computer simulation (Fig. 1, bottom row) or observed experimentally (e.g.
myosin filaments: Fig. 3, bottom; Fig. 5(a), bottom dotted line). Indeed, the
difference spectrum is virtually indistinguishable from that due to a random
distribution. Figure 1 shows that the sensitivity to large \( \Delta \theta \) values depends on the
value of \( \theta_0 \), but that the spectrum corresponding to \( \Delta \theta = 90^\circ \) can always be
Fig. 5. Comparison of difference spectra, generated by computer subtraction, with reference spectra. (a) top: overlay of \( V_1 \) difference spectrum corresponding to non-overlap heads (unbroken line, produced as described in section Results). (b) top: computer-simulated spectrum assuming a spherically weighted Gaussian orientation distribution of probes relative to the fiber axes, with a mean angle \( \theta_0 \) in eqn (1) of 68° and an angular range \( \Delta \theta \) in eqn (1)) of 90°. (a) bottom: same \( V_1 \) difference spectrum as above (unbroken line) overlayed with the \( V_1 \) spectrum of a randomly oriented dispersion of synthetic myosin filaments (dotted line). (b) top: \( V_2 \) difference spectrum from same sample (unbroken line, produced as described in Results section) overlayed with the spectrum of spin-labeled hemoglobin which matches its rotational correlation time (dotted line, 90% glycerol at 5°C, \( \tau_r = 38 \mu s \)). (b) bottom: (unbroken line) overlayed with \( V_2 \) spectrum of myosin filaments (dotted line, \( \tau_r = 13 \mu s \)). The overlaid spectra above have been normalized to represent the same number of spins as the difference spectrum.

distinguished from the spectrum corresponding to a random distribution. After varying both \( \theta_0 \) and \( \Delta \theta \) and comparing the resulting simulations with the difference spectrum, we find that (1) there is too much disorder to permit an estimate of \( \theta_0 \) and (2) \( \Delta \theta \) must be greater than 90°. Therefore, the full width of the orientation distribution of the non-overlap myosin heads must be greater than 90°.

A complementary subtraction, done by removing 90-7% of the myosin filaments spectrum from the spectrum corresponding to 93% overlap, resulted in a \( V_1 \) line shape which resembles closely the full overlap spectrum (data not shown). Similarly a good fit to any of the spectra of stretched fibers is obtained by computer addition of appropriate fractions of the single component spectra of full overlap fibers and myosin filaments. The percentage of overlap of the thick and thin filaments can be estimated without knowledge of the sarcomere length by this kind of spectral titration, assuming a two-component \( V_1 \) spectrum. By using either the full overlap (oriented) or the myosin filament (disoriented) spectrum.
and subtracting by increment until the difference spectrum contains only one component and contains no abnormalities in line shape, the percentage overlap was found to match that predicted by sarcomere length to within 5%.

(ii) Rotational motion (saturation transfer e.p.r.)

Deconvolution of spectral components is more ambiguous with saturation transfer e.p.r. ($V_2'$) spectra than with conventional e.p.r. ($V_1$) spectra. The resonance positions for mobile and immobile populations of spins overlap one another completely (Fig. 2) and therefore provide no clear indications of the number of populations present, or their relative proportions. However, since the $V_1$ spectra show that the probes appear to be in two populations as anticipated from the sarcomere length, we have analyzed the saturation transfer e.p.r. spectra by a subtraction procedure similar to that used on the conventional spectra. That is, a fraction of the spectrum of the fibers at full overlap was subtracted from that of stretched fibers, this fraction being equal to the remaining fraction of overlap, as determined by sarcomere length. For each stretched fiber sample, the resulting difference spectrum, which presumably corresponds to the heads not in the overlap zone, shows increased rotational mobility. Figure 5 (b), top shows one of these difference spectra, compared with a hemoglobin reference spectrum.

In Figure 5 (b), the upper pair of spectra shows the difference spectrum obtained when 9·3% of the $V_2'$ spectrum of full overlap fibers was subtracted from the $V_2'$ spectrum of fibers stretched to 9·3% overlap (unbroken line), overlayed with the hemoglobin spectrum which has the same shape in the wings of the spectrum, and hence the same effective correlation time (dotted line, $\tau_c = 38$ μs). The fit of the lineshape and the intensity is reasonably good in the wings of the spectra, indicating that the motion of the residual (non-overlap) population may be approximately described by the effective rotational correlation time of the hemoglobin standard. The lower pair of spectra shows the overlay of the same saturation transfer e.p.r. difference spectrum (unbroken line) and the $V_2'$ spectrum of synthetic myosin filaments (dotted line). The line shapes and intensities of these spectra are poorly matched at all points in the spectra. The effective rotational correlation time determined from the peak ratio $L'/L$ (Fig. 2) of the myosin filaments spectrum (Fig. 4) is 13 μs, three times shorter than that of the non-overlap component of the stretched fibers spectrum. Therefore, the heads corresponding to this difference spectrum, which are presumably non-overlap heads, have considerable microsecond rotational mobility, but have less rotational mobility than heads in synthetic myosin filaments.

4. Discussion

(a) Summary of results

In the present study, we have used conventional ($V_1$) and saturation transfer ($V_2'$) e.p.r. in the study of spin-labeled muscle fibers to monitor both the orientation and rotational mobility of probes rigidly fixed to myosin heads, as a
function of sarcomere length. This was done in order to correlate orientations and molecular motions of myosin heads in the intact contractile system. The data show that spin-labeled fibers, at full overlap, yield spectra which exhibit a narrow orientation distribution and little or no rotational motion on the sub-millisecond time scale. As fibers are stretched toward 0% overlap, the orientation distribution (as detected by the $V_1$ spectrum) changes to include a component indicating a highly disoriented distribution. It is also apparent that fibers at full overlap yield $V_2'$ spectra indicating slow rotational motion, while the stretched fibers whose $V_1$ spectra contain a disoriented component give rise to $V_2'$ spectra that indicate increased rotational motion in the microsecond time range. Computer subtraction of digitized $V_1$ spectra permitted the resolution of spectra into two components, with the percentage of orientationally disordered heads equal to the percentage of heads in the non-overlap zone. The orientational disorder of these (presumably) non-overlap heads covers at least a full width of 90°. Through comparison with reference spectra obtained from 100% overlap fibers, synthetic myosin filaments, and computer simulations, we have determined that these non-overlap heads undergo Brownian rotational diffusion through a large angular range, with an effective rotational correlation time of about 40 μs. From these data we conclude that myosin heads undergo microsecond rotational motion with large angular displacements when heads are denied contact with actin, even in the absence of ATP.

(b) Interpretation of results

(i) Probe motion with respect to myosin

Since previous work has shown that these probes are immobile with respect to the framework of isolated immobilized myosin heads (Thomas et al., 1980), we assume in the above conclusions and in the following discussion that the orientational disorder and motion of the heads is accurately reflected by the probe spectra. Nevertheless, the possibility cannot be rigorously ruled out that internal motion within the myosin head contributes to the detected motion in stretched fibers. Future experiments on probes at other sites on the myosin head could reduce this uncertainty.

(ii) Number of probe populations

Another possibility which must be considered is that there may exist more than two distinct populations of heads in stretched fibers. Myosin heads in thick filaments close to the termini of the thin filaments could conceivably be in a different environment from that of heads far removed from thin filaments. However, the difference spectra obtained from $V_1$ spectra of stretched fibers indicate that any population of heads having a preferred orientation other than the rigor orientation ($\theta_0 = 68°, \Delta\theta = 15°$) can only represent a small fraction (less than 5%) of the total heads. Therefore in the discussion which follows, the possible contribution of such a fraction of heads is disregarded.
(iii) Non-overlap myosin heads

The primary purpose of the present study is to extend the conventional e.p.r. studies of spin-labeled psoas fibers (Thomas & Cooke, 1980) to include saturation transfer e.p.r. studies, in order to determine whether the orientational disorder evident in conventional e.p.r. spectra is dynamic (as in synthetic myosin filaments) or static on the microsecond time scale. At full overlap, the disorder is small but significant (Fig. 3, $\Delta \theta = 15^\circ$). The $V_2'$ spectrum (Fig. 4) indicates no significant sub-millisecond rotational mobility, as predicted from previous results on purified actomyosin and myosin filaments (Thomas et al., 1975, 1980), implying that this disorder is static and that the rigor bond is truly rigid on the microsecond time scale. The disorder evident in the $V_1$ spectrum of non-overlap heads is large ($\Delta \theta > 90^\circ$, as shown in Fig. 5(a)) and microsecond mobility is clearly evident in the $V_2'$ spectrum (Fig. 5(b)), implying that the disorder in non-overlap heads is dynamic. The mobility is not as great as in synthetic myosin filaments (Fig. 5(b), bottom); the effective correlation time is about three times longer, suggesting that synthetic myosin filaments are not an exact analog of native thick filaments. An alternative, but less plausible, explanation is that the mobility of non-overlap heads in myosin filaments is reduced allosterically due to the attachment of the small fraction of heads that are in the overlap zone.

(iv) Theoretical analysis of saturation transfer e.p.r. spectra

A decrease in rotational mobility, as evidenced by an increase in the effective correlation time of saturation transfer e.p.r. spectra, can correspond to a decrease in the rate of motion or the angular amplitude or both, as shown by computer simulation of saturation transfer e.p.r. spectra (K. Lindahl & D. D. Thomas, unpublished work). For example, the observed decreased mobility (3-fold increase in effective correlation time) of detached heads in fibers compared with synthetic myosin filaments could correspond to a threelfold decrease in the rate of motion, a two to threefold decrease in the amplitude, or an intermediate change in both (K. Lindahl & D. D. Thomas, unpublished work). This ambiguity is greatly decreased by the fact that we have recorded, from the same sample, both $V_2'$ and $V_1$ spectra, the latter with the sample's symmetry axis oriented parallel to the magnetic field. The $V_1$ spectrum thus gives us a direct measurement of the angular range of orientations, i.e. the amplitude of rotational motions, and greatly reduces the uncertainty of the rate of motion. The fact that the saturation transfer e.p.r. spectrum of non-overlap heads does not match precisely all parts of the hemoglobin spectrum (Fig. 5(b), top) suggests that the motion is not isotropic, i.e. that there is some restriction in amplitude. However, the large range in orientations ($> 90^\circ$) indicated by the $V_1$ spectrum (Fig. 5(a)) suggests that this restriction is not great, that the effective correlation time (38 $\mu$s) therefore remains an accurate measure of the actual time scale of motion (Lindahl & Thomas, unpublished work), and that the difference between the motions in synthetic and native filaments reflect primarily a difference in the rates (frequencies) of motion. We note that the effectiveness of the combined use of $V_2'$ and oriented $V_1$ spectra is not limited to the study of muscle fibers but can be
applied to any system whose symmetry axis can be oriented relative to the 
magnetic field, e.g. fibrous proteins, DNA, or stacks of membranes.

The large orientational disorder detected from the $V_1$ spectra of non-overlap 
heads (Fig. 5(a)) is entirely due to disorder in the orientation of probes relative 
to the fiber axis, described by the axial angle $\theta$, and is independent of azimuthal 
disorder. Thus, it is likely that most of the motion observed from the $V_2'$ spectra 
corresponds to axial probe rotations. Previous work has shown that the probe is 
probably aligned approximately parallel to the long axis of the head (Thomas et 
al., 1975), therefore the motions probably correspond to axial head rotations. 
Since the probe may not be precisely aligned with the head, other degrees of 
freedom of head rotation (e.g. torsional motions) may be contributing. However, 
even if the worse case is assumed, in which the head can have complete torsional 
freedom and the probe can have any orientation with respect to the head, our 
e.p.r. spectra require that the full width of the axial head rotations must be at 
least on the order of 50° (Mendelson & Wilson, 1982).

(v) Comparison to results from triplet anisotropy

Further insight into questions about amplitudes and rates of motion can be 
provided by a comparison of the present e.p.r. results with those of triplet 
anisotropy. Like saturation transfer e.p.r., this technique is sensitive to rotational 
motions in the time range of nanoseconds to milliseconds, whereas singlet methods 
(e.g. fluorescence) are only sensitive to nanosecond motions. Unlike the steady-
state e.p.r. methods used here, triplet anisotropy measurements can be performed 
with time resolution, permitting a more direct analysis in terms of the number of 
correlation times and the amplitudes of the motions. Workers in this laboratory 
have used time-resolved triplet anisotropy to study the rotational motion of 
phosphorescent dyes attached to SH$_1$ on myosin heads in synthetic myosin 
That study has shown that myosin heads undergo slightly restricted rotational 
motion, characterized by two distinct correlation times in the microsecond time 
range. The full angular range of these motions is on the order of 100°. This result 
is consistent with the finding in the present study that the angular range of 
microsecond motions for non-overlap heads is greater than 90°.

(c) Relationship to other work

(i) Rigor

While conclusions about the precise orientation of myosin heads depend on the 
measurement technique and the model assumed for head shape, there is a general 
agreement with the present result that most myosin heads are rigidly attached 
and highly ordered in the rigor state at full overlap, in rabbit psoas muscle 
(Thomas & Cooke, 1980; Yanagida, 1981; Borejdo et al., 1982), frog sartorius 
muscle (Hasegrovve, 1975), and insect flight muscle (Reedy, 1967; Thomas et al., 
1983a), among others.
(ii) *Non-overlap heads in the absence of ATP*

An important technique used to study the static (time-averaged) structure of stretched fibers in the absence of ATP has been X-ray diffraction (Haselgrove, 1975; Poulsen & Lowy, 1983). In both these studies, on frog muscle, the data were interpreted to be consistent with considerable disorder in the arrangement of non-overlap myosin heads in rigor. Poulsen & Lowy (1983) quantitatively studied the central disk of diffuse scatter observed in the diffraction pattern, and concluded that the data were consistent with myosin heads having essentially total orientational disorder. Conventional, e.p.r. through its superior site-specificity and orientational sensitivity, has more directly and quantitatively measured this disorder (Thomas & Cooke, 1980; this study). In the present study we have used saturation transfer e.p.r. to show further that this disorder is dynamic on the microsecond time scale.

(iii) *ATP effects*

An important question that we have not addressed here is whether the orientation and motion of detached heads depends on the presence of ATP. That is, how does the behavior of non-overlap heads in the absence of ATP compare with that of heads in relaxed fibers? Previous e.p.r. results, obtained with a maleimide spin-label (MSL) attached to myosin heads, indicated that the heads behave very similarly in these two cases: saturation transfer e.p.r. spectra of relaxed myofibrils (Thomas et al., 1980) show considerable microsecond rotational mobility (similar to that observed for synthetic myosin filaments) and conventional e.p.r. spectra of relaxed fibers (Thomas & Cooke, 1980) show considerable rotational disorder (similar to that observed for non-overlap heads). These results were consistent with previous studies, which showed that fluorescence polarization was the same in relaxed fibers as in non-overlap fibers (in the presence or absence of ATP), measured either from intrinsic tryptophan residues (dos Remedios et al., 1972) or from dyes attached to SH$_1$ (Nihei et al., 1974). These results are also consistent with the high level of disorder detected from the dichroism of dyes attached to myosin heads at SH$_1$ in relaxed fibers (Borejdo et al., 1982) or detected from the polarization of fluorescent nucleotides bound to myosin heads in relaxed fibers (Yanagida, 1981).

However, X-ray diffraction patterns of relaxed muscle show distinct layer-lines, which can be assigned to the thick filaments, for frog (Huxley & Brown, 1967; Haselgrove, 1975,1980; Poulsen & Lowy, 1983), rabbit (Rome, 1972; Lymn, 1975), insect (Miller & Tregear, 1972), and crab (Wakabayashi & Namba, 1981). Although it has been reported that layer-lines are less distinct in relaxed glycinated rabbit psoas fibers than in living fibers, layer-lines are clearly visible out to the sixth order in preparations similar to those used in the present study, suggesting considerable helical order of crossbridges (Rome, 1972). In contrast, the X-ray diffraction patterns obtained from rigor fibers stretched the non-overlap to show little or no evidence of myosin-based layer-lines (Haselgrove, 1975; Poulsen & Lowy, 1983). These results have been interpreted to indicate that helical order of crossbridges in relaxed fibers requires not only detachment from
actin but also the presence of ATP. These data are not necessarily in conflict with
the probe results, which indicate that probes on myosin heads have equally high
axial orientational disorder in relaxed muscle fibers and in non-overlap rigor
(Nihei et al., 1974; Thomas & Cooke, 1980). One possible explanation is that
internal rotational mobility within myosin disorders the probed region (in which
SH is located). However, control experiments in the probe studies suggest that
the probe and the myosin head rotate together as a rigid unit. A second, more
plausible explanation comes from the fact that X-ray diffraction is not primarily
sensitive to orientational order but to translational order, whereas the probes
detect axial orientation directly. A high degree of axial rotational mobility within
the crossbridge is quite consistent with a relatively high degree of translational
order of the crossbridge centers of mass in a helical array (Thomas & Cooke,
1980). Poulsen & Lowy (1983) have pointed out that there is considerably more
diffuse scatter in X-ray diffraction patterns of relaxed frog muscle than in those of
rigor muscle, suggesting substantial myosin head disorder even when myosin
layer-lines are present. The complex decay of triplet anisotropy observed for
eosin-labeled myosin (at SH) in synthetic myosin filaments has been interpreted
to indicate that at least two modes of microsecond rotational motion, possibly
corresponding to two sites of crossbridge flexibility, are available for myosin
heads detached from actin (Eads, T. M., Austin, R. H. & Thomas, D. D.,
unpublished results). As a result, models have been proposed in which either the
distribution of orientations (Poulsen & Lowy, 1983) or the range of rotational
motions (Eads T. M., Austin, R. H. & Thomas, D. D., unpublished results)
available for each head would be governed by the flexibility within the
crossbridge, at the two putative hinges at either end of S-2 and/or within S-2. An
appropriate degree of flexibility could allow the translational and rotational
motions of a crossbridge to become nearly independent of one another and
provide a mechanism by which the observed X-ray data (implying some
translational order) and probe data (implying rotational disorder) could be
produced.

(d) Future work

This question about the effect of ATP on detached heads can not be resolved by
the present study, in which ATP was not used during spectral acquisition. IASL
has been shown to become mobile while attached to myosin in the presence of
ATP (Seidel & Gergely, 1973), therefore its choice as a probe precludes studies of
myosin head motion in relaxed fibers. IASL was used in the present study, rather
than MSL (which remains rigidly fixed to the myosin heads in the presence of
ATP), because IASL shows superior labeling specificity with fewer manipulations of
fibers (Thomas et al., 1980). Future e.p.r. studies on MSL fibers will be required to
determine directly the effect of ATP on the orientation and rotational motions of
detached (non-overlap) myosin heads. To resolve possible discrepancies between
X-ray diffraction and e.p.r. data, e.p.r. and X-ray measurements should be
performed on the same samples. The other major question which requires
resolution is whether the rotational motion of non-overlap myosin heads is
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restricted in amplitude. Time-resolved triplet anisotropy studies (Eads, T. M., Austin, R. H. & Thomas, D. D., unpublished results) on intact fibers at varying degrees of overlap should yield, the information necessary to resolve that issue quantitatively.

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