Orientational disorder and motion of weakly attached cross-bridges

Piotr G. Fajer,* Elizabeth A. Fajer,* Mark Schoenberg,‡ and David D. Thomas*
*Department of Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota; and ‡Laboratory of Physical Physiology, National Institutes of Health, Bethesda, Maryland 20892 USA

ABSTRACT In a relaxed muscle fiber at low ionic strength, the cross-bridges may well be in states comparable to the one that precedes the cross-bridge power stroke (Schoenberg, M. 1988. *Adv. Exp. Med. Biol.* 226:189–202). Using electron paramagnetic resonance (EPR) and (saturation transfer) electron paramagnetic resonance (ST-EPR) techniques on fibers labeled with maleimide spin label, under low ionic strength conditions designed to produce a majority of weakly-attached heads, we have established that (*a*) relaxed labeled fibers show a speed dependence of chord stiffness identical to that of unlabeled, relaxed fibers, suggesting similar rapid dissociation and reassociation of cross-bridges; (*b*) the attached relaxed heads at low ionic strength are nearly as disordered as in relaxation at physiological ionic strength where most of the heads are detached from actin; and (c) the microsecond rotational mobility of the relaxed heads was only slightly restricted compared to normal ionic strength, implying great motional freedom despite attachment. The differences in head mobility between low and normal ionic strength scale with filament overlap and are thus due to acto-myosin interactions.

The spectra can be modeled in terms of two populations: one identical to relaxed heads at normal ionic strength (83%), the other representing a more oriented population of heads (17%). The spectrum of the latter is centered at approximately the same angle as the spectrum in rigor but exhibits larger (40°) axial probe disorder with respect to the fiber axis. Alternatively, assuming that the chord stiffness is proportional to the fraction of attached crossbridges, the attached fraction must be even more disordered than 40°, with rotational mobility nearly as great as for detached cross-bridges.

INTRODUCTION

A major effort in understanding muscular contraction is identification of the molecular change in the acto-myosin interactions responsible for force generation. After original electron microscopy observations of two different orientations of the myosin head (Reedy et al., 1965), the rotating cross-bridge theory has been proposed, in which the pivoting motion of a myosin head attached to actin produces strain which is then relieved by the sliding motion of the myosin and actin filaments, resulting in muscle contraction (Huxley, 1969; Huxley and Simmons, 1971). Thus, the rotating head is expected to be mobile and capable of attachment at angles different from rigor, which is assumed to represent the end of the power stroke. The evidence for orientational change associated with the power stroke is indirect (see reviews by Cooke, 1986, and Thomas, 1987). In contracting muscle, most of the spin-labeled heads were found to be nearly as disoriented as in relaxation at physiological ionic strength, where most heads are detached from actin (Cooke et al., 1982). More recently, we have concluded that the high stiffness of contracting fibers indicates that some of the disoriented heads are attached to actin (Fajer et al., 1990*c*). Using nucleotide analogues, we have attempted to identify the stage of the biochemical cycle when head reorientation takes place. The MgADP-bound cross-bridge is a poor candidate, because the head orientation in the presence of ADP is nearly identical to that in rigor (Fajer et al., 1990*b*). The nonhydrolyzable ATP analogues, adenosine-imido-triphosphate (AMPPNP) and pyrophosphate (PP_i) induce head orientation by dissociating heads from actin, resulting in a single-headed cross-bridge under some conditions, but not changing the orientation of the attached head (Fajer et al., 1988; Pate and Cooke, 1988).

According to the model of Eisenberg and Greene (1980), a transition from "weakly binding" to "strongly binding" cross-bridge states is associated with a change of head orientation. Mechanical (Brenner et al., 1982; Yanagida et al., 1982) and structural (Yu and Brenner, 1989) studies have led to the proposal that the weakly binding state can be modeled by relaxation at low ionic strength and low temperature, and that a similar state is produced in solution when S1 binds to actin in the presence of ATP at low ionic strength (reviewed by Brenner, 1985, 1987). The increase of stiffness with the speed of stretch indicates that the weakly-binding state is characterized by a rapid (submillisecond) dissociation

Address correspondence to Dr. P. G. Fajer or Dr. David D. Thomas. Drs. P.G. Fajer and E. A. Fajer's present address is Institute of Molecular Biophysics, B-301, Florida State University, Tallahassee, FL 32306.

0006-3495/91/09/642/08 $2.00

and reassociation of cross-bridges (Schoenberg, 1985). The states induced by PP_i or by AMPPNP serve as a model for the strongly-binding state. It has been suggested that binding of the relaxed cross-bridge at low ionic strength represents initial electrostatic docking of the myosin head which subsequently is reinforced by binding hydrophobically to another site (Mornet et al., 1981; and Podolsky and Arata, 1988).

Electron microscopy on cross-linked acto-S1 complexes during steady-state ATPase activity, in which weakly-bound states should predominate, showed a very disordered distribution of head orientations, rather than a single discrete angle (Craig et al., 1985; Applegate and Flicker, 1987). Saturation-transfer EPR (ST-EPR) studies under similar conditions showed that ATP induces microsecond rotational motion of S1 cross-linked to actin (Svensson and Thomas, 1986), and subsequent work using caged ATP in the absence of cross-linking confirmed this result (Berger et al., 1989). However, (*a*) head orientation relative to actin cannot be measured in solution, and (*b*) the relationship between an acto-S1 solution and the muscle-fiber lattice is not entirely clear. Therefore, in the present work, to determine the head orientation and dynamics in the early parts of the cycle, we have performed EPR experiments on spin-labeled heads in muscle fibers at low ionic strength, low temperature (5°C) relaxing conditions (LIS), which are thought to represent the initial stages of cross-bridge attachment (reviewed by Brenner, 1985). As the EPR cannot resolve between attached and detached heads we have used a combination of stiffness measurements (to estimate attachment), conventional EPR (to measure orientation) and saturation transfer EPR (rotational mobility).

METHODS

Sample preparation

Detailed experimental procedures were published previously (Fajer et al., 1988). We used glycerinated rabbit psoas muscle labeled with maleimide spin label (MSL). As estimated from the fractional inhibition of K^+-ATPase, 59%± 8% of the heads were labeled at the reactive sulfhydryls. There was no impairment in the ability of the fiber to relax or contract. The low values of myofibrillar ATPase (3.81 nmol P_i/mg/min) and tension (0.08 kg/cm^2) observed in relaxation at low ionic strength, low temperature (5°C), are in agreement with the values measured previously for unlabeled fibers under similar conditions (Yanagida et al., 1982). At normal ionic strength at 5°C, the corresponding values for enzymatic activity and tension were 2.27 nmol P_i/mg/min and 0.04 kg/cm^2 in the absence of Ca^{2+} (relaxation) and 11.5 nmol P_i/mg/min and 0.70 kg/cm^2 in the presence of Ca^{2+} (contraction).

Solutions

Rigor solution (RS) contained 141 mM KPr, 10 mM MOPS, 1 mM EGTA, 2 mM $MgCl_2$, pH 7.0. Normal ionic strength relaxing solution (ionic strength μ = 0.17 M), designated NIS, contained 5 mM MgATP in RS and low ionic strength relaxing solution (designated LIS) was like NIS but with no KPr, resulting in μ = 0.03 M.

Electron paramagnetic resonance

Thin, 0.5-mm muscle bundles were placed in a specially designed EPR flat cell allowing (*a*) isometric conditions and (*b*) parallel orientation of the fiber to the external magnetic field. EPR experiments were performed on a spectrometer (ER-200D, Bruker Instruments, Billerica, MA) using a TM_{101} cavity modified to accept samples parallel to the magnetic field or in a TE_{102} cavity with the fiber axis perpendicular to the field. Fibers stretched out-of-overlap were contained in capillaries in which the silk thread holding the ends of the fiber bundle was glued in place after stretching. Spectra were acquired, averaged and analyzed using a Zenith 158 microcomputer interfaced to the EPR spectrometer as described before (Fajer et al., 1988). Computer simulations of EPR spectra were described previously (Fajer et al., 1990*a*).

Stiffness

To aid the assignment of EPR signals to detached and attached heads, we have used mechanical stiffness to estimate the fraction of heads attached to thin filaments. Chord stiffness measurements were performed as described by Schoenberg (1988*a*). The total length of the stretch, as measured by optical diffraction, was 6 nm/half-sarcomere. Three data points were obtained from each stretch by calculating the cord stiffness 1, 2, and 4 nm into the stretch.

RESULTS

Stiffness

As shown in Fig. 1, the chord stiffness of unlabeled fibers measured with the briefest duration stretches ($\sim 10^{-4}$ s) in the presence of ATP at physiological ionic strength (NIS) was <10% of rigor stiffness and did not change with labeling. In LIS, stiffness was comparable to that at

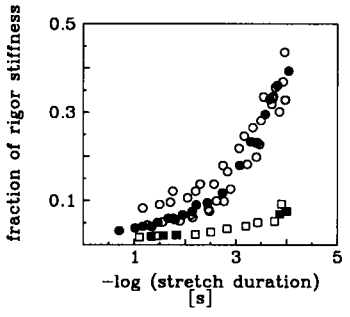

FIGURE 1 Dependence of the chord stiffness (relative to that of the same fiber in rigor) on the duration of stretch, in LIS relaxation (μ = 0.03 M) for unlabeled fibers (*filled circles*) and labeled fibers (*open circles*), and in NIS relaxation (μ = 0.17 M) for unlabeled (*open squares*) and labelled fibers (*filled squares*). The data are from two labelled and one unlabelled fibers. The temperature was 5°C.

NIS when the duration of stretch was long (1 s), but increased to 50% of the rigor stiffness with the fastest stretches possible. This is similar to the behavior described previously for unlabeled fibers (Brenner et al., 1982; Schoenberg, 1988*a*; Dantzig et al., 1988).

IASL blocking

In the absence of $K_3Fe(CN)_6$, there is a background signal arising from nonspecific labeling with MSL (Fajer et al., 1988). To ascertain that the changes observed in the EPR spectra do not come from this background, we initially labeled the fibers with IASL, which is known to label very specifically the SH1 thiols of the myosin head (Thomas et al., 1980; Thomas and Cooke, 1980). The reaction was carried out for 4 h following the protocol of Crowder and Cooke (1984). The nitroxide group of IASL was then reduced for 5 min with a 10-mM solution of ascorbate, which abolishes the EPR signal, and the fibers were then labeled as before with MSL. These preblocked fibers exhibit an EPR signal which originates from the background alone. The EPR spectra are shown in Fig. 2.

In rigor (Fig. 2, *top*), the sharp peak at P_2, characteristic of spin labels with their principal axes oriented at ~80° relative to the fiber axis, (Fig. 3, *top*) is gone, and the spectrum resembles that of highly disoriented probes,

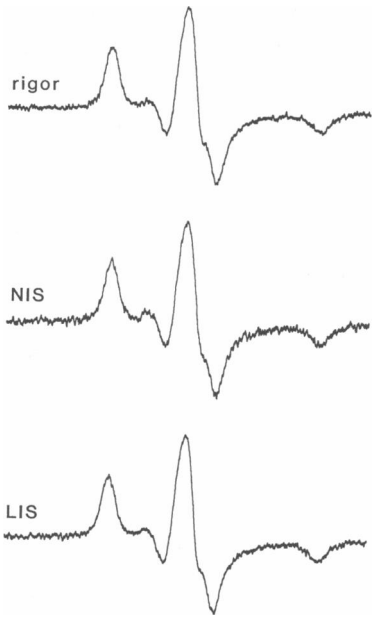

FIGURE 2 Conventional EPR spectra of MSL fibers preblocked with IASL. Rigor (no ATP, *top*), NIS (relaxation at physiological ionic strength, *middle*) and LIS (relaxation at low ionic strength, *bottom*). The temperature was 5°C.

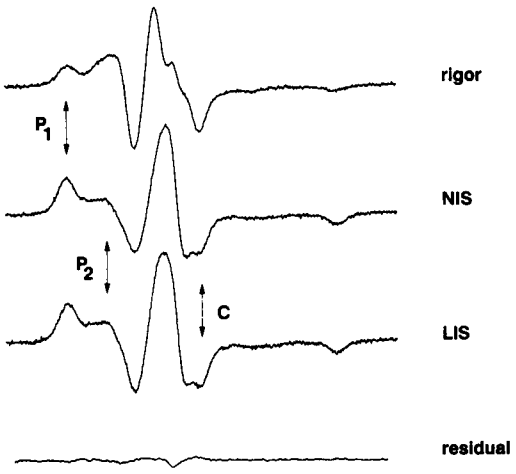

FIGURE 3 Conventional EPR spectra of fibers in rigor, relaxation at normal ionic strength (NIS) and relaxation at low ionic strength (LIS). The residual spectrum was obtained by subtracting the LIS spectrum from a composite spectrum (83% NIS plus 17% rigor). The temperature was 5°C.

as normally observed during relaxation. Detailed lineshape analysis establishes however, that the spectrum of the background is not identical to that of randomly oriented spin labels or the spectrum in relaxation. Aside from this, the background spectrum does not change upon ATP addition (relaxation) at either NIS (Fig. 2, *middle*) or at LIS (Fig. 2, *bottom*). Therefore, any changes in relaxation reported below are due to the SH1-bound labels. The blocking experiment does not address possible changes in the so called 'weakly' immobilized component, as this component gets blocked with IASL. This is however, less of a problem than might be anticipated because: (*a*) the splitting (2T) of this signal is 33 Gauss, and thus it is well resolved from the ordered component (2T = 17-18 Gauss) and from the disordered component (2T = 69 Gauss); (*b*) the component does not contribute more than 5% of total intensity; (*c*) control experiments, in which the weakly immobilized component was eliminated by $K_3Fe(CN)_6$ treatment, resulted in the same spectral changes as the untreated fibers. There is virtually no difference in the STEPR spectra between NIS and LIS relaxation at full overlap (Fig. 7) and stretched out-of-overlap (Fig. 8). Thus, the blocking experiment is not an essential control for the STEPR spectra.

Conventional EPR

As previously described for 20°C, at low temperature the spins oriented in rigor (P_2 in Fig. 3, *top*) become disordered in NIS relaxation and contribute to the

intensity at P_1 (Fig. 3, NIS). Lowering the ionic strength to 0.03 M in relaxation (Fig. 3, LIS) results in a small but significant change in the spectrum, notably at P_2 and in the center of the spectrum at the position denoted C (Fig. 3). The change in the EPR spectrum cannot be accounted for by a linear combination of the spectra in rigor and NIS, as shown by the nonzero residual (Fig. 3).

Difference spectra

If we assume that at LIS there are two populations of heads, one with spectra identical to those in relaxation at NIS and the other more ordered, then we can obtain the spectrum of the latter by digitally subtracting a fraction of the spectrum at NIS from that at LIS. The endpoint of such a subtraction is decided by nulling the intensity at the P_1 position (see Fig. 3), which is characteristic of disordered spin labels. Subtraction of this kind removes the contribution from the nonspecific binding of MSL (see Fig. 2). The remaining spectrum (Fig. 4) is similar to that observed for S1 decorated fibers in rigor (Fajer et al., 1988) or the rigor difference spectrum obtained in a similar way to correct for the background (Fig. 4 *right*) but the LIS spectrum is significantly broader. The similar splitting of the EPR resonances in rigor and LIS implies that the heads are making a similar average angle with respect to the filament axis, but the larger width (and smaller ratios of low-field to center-field resonances, L/C, and high- to center-field, H/C) of each of these resonances implies larger orientational disorder in LIS (Table 1).

The comparison of difference spectra with computer-simulated spectra (Fig. 4), reveals that the disorder of the principal axis of the spin label (θ) is ~3 times larger for LIS difference spectra ($\Delta\theta = 40°$) than for the heads in rigor ($\Delta\theta = 15°$).

TABLE 1 **Spectral parameters of rigor and LIS difference spectra**

	2T (Gauss)	S.E.M.*	L/C	S.E.M.*	H/C*	S.E.M.*
rigor	17.20	0.04	0.82	0.01	0.49	0.01
LIS relaxation	17.90	0.03	0.47	0.02	0.30	0.01

*Number of determinations was 14 for rigor spectra and 39 for LIS difference spectra.

The subtraction factor used to null the P_1 intensity in LIS gives the molar fraction of the oriented component to be 0.17 ± 0.05, which is substantially lower than 0.5–0.6, the fraction of attached cross-bridges estimated from rapid stiffness experiments (Fig. 1 and Schoenberg [1988*a*]). Because the molar fraction of the oriented component did not change significantly with ATP concentration between 0.5 and 5 mM, the observed effect does not appear to be due to lack of ATP saturation (Fig. 5).

One way of obtaining the signal arising from myosin heads bound to actin is to assume, from stiffness and x-ray diffraction measurements (Schoenberg, 1988*a*; Yu and Brenner, 1989), that the fraction of attached cross-bridges in relaxation at ionic strength μ = 0.03 M, is 0.5–0.75. From these numbers we can calculate the fraction of detached cross-bridge heads at 0.03 M, depending upon whether the cross-bridges bind with one or two heads. It is unclear whether stiffness measures the number of attached cross-bridges or the number of attached heads. Usually, it is assumed that: (*a*) stiffness of any attached head is the same as it is in rigor; (*b*) all the heads are bound in rigor. Hence, the fraction of rigor stiffness was taken as a measure of the head attachment. Our own study of 'strongly' bound heads in presence of AMPPNP (Fajer et al., 1988) suggested however, that the fibers maintain the same stiffness as in rigor although half of the heads are

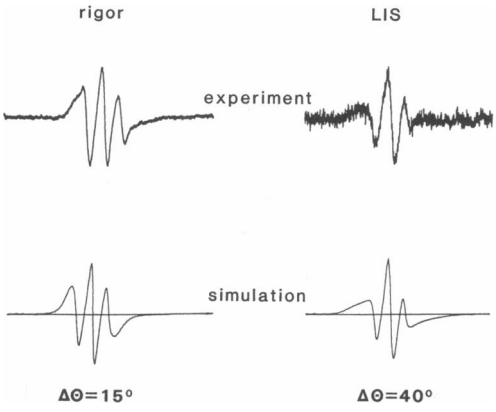

FIGURE 4 *Top:* Difference spectra of the oriented population, obtained by subtracting the spectrum in NIS from the spectrum in LIS (*right*) and rigor (*left*), in order to null the signal at P_1. (*Bottom*) Simulated spectra corresponding to a Gaussian distribution of principal axis orientation centered at $\theta_0 = 82°$, with disorders ($\Delta\theta$) of 40° and 15°, fitting LIS and rigor spectra.

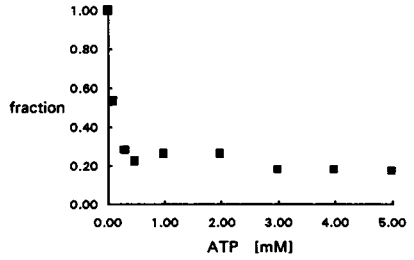

FIGURE 5 Dependence of the oriented fraction of spin labels in LIS, obtained from difference spectra (as in Fig. 4) as a function of ATP concentration.

detached. We have concluded that 'single-headed' cross-bridges display the same stiffness as a 'double-headed' cross-bridge. Whether the same is true for 'weakly' attached cross-bridges is unclear and therefore we consider below two types of cross-bridges. For the double-headed cross-bridge, the fraction of attached heads corresponds to the fraction of rigor stiffness; for the 'single-headed' attachment, the attached fraction is 50% of stiffness.

If we then assume that the spectra of detached heads at LIS is the same as that of detached heads at NIS, it is relatively straightforward to subtract from the LIS spectrum that portion of the NIS spectrum corresponding to the detached heads. The resulting difference should then correspond to the signal from the attached myosin heads. This is shown in Fig. 6 both for the case where the M.ATP crossbridge binds with one head, and for the case where it binds with both heads. For the lowest estimate of attached myosin heads (*top right*), $\Delta\theta$ = 40–50°; for the highest estimate (*top left*), $\Delta\theta$ = 80–100°.

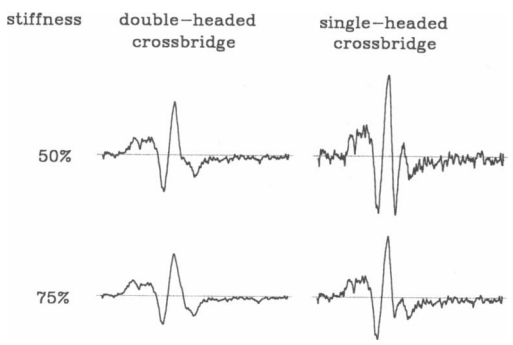

FIGURE 6 EPR difference spectra corresponding to the fraction (f) of attached myosin heads in LIS, obtained by subtracting a fraction (1–f) of the NIS spectrum from the LIS spectrum. For the assumption of double-headed cross-bridge attachment (*left column*), f was equal to the fraction of attached cross-bridges as estimated from the stiffness and x-ray data, as given in the left column. For the assumption of single-headed cross-bridges (*right column*), f was half the number of attached cross-bridges as estimated from the stiffness and x-ray data. Spectra were corrected for the background signal before subtractions.

STEPR

Saturation transfer EPR was used to study the dynamics of the myosin heads. This measurement is complementary to the conventional EPR (Figs. 3–6), which detects orientational disorder but cannot distinguish static from dynamic disorder. The STEPR spectra of fibers in rigor and in relaxation at normal and low ionic strength are shown in Fig. 7. The spectrum in rigor has a much higher (200%) integrated intensity than the spectrum in relaxation at LIS, indicating less rotational motion in the

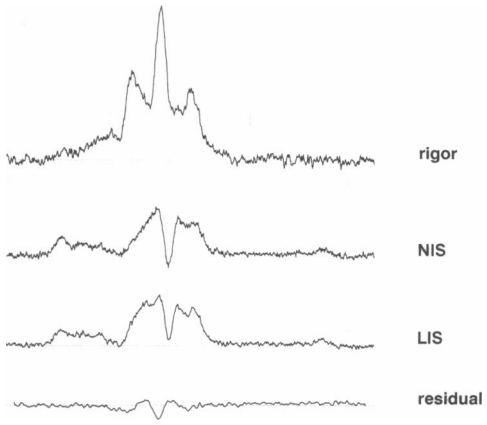

FIGURE 7 Saturation transfer EPR spectra of MSL fibers in rigor, relaxation at normal ionic strength (NIS), and relaxation at low ionic strength (LIS). The residual spectrum was obtained by subtracting the LIS spectrum from a composite spectrum (83% NIS plus 17% rigor). The temperature was 5°C.

microsecond time range. The spectrum of relaxed fibers at LIS is of slightly greater (4%) overall intensity than in relaxation at NIS, with the largest difference apparent in the center of the spectrum.

Because the above spectra contain both orientational and motional information and the motion is likely to be highly anisotropic, we cannot use the lineheight ratios of Thomas et al. (1976) to analyze the rotational motion independently. We can, however, determine effective rotational correlation times from the integrated intensities of the STEPR spectra normalized to the number of spins (Evans, 1981; Horvath and Marsh, 1983). The effective rotational correlation times are >2 ms (rigor), 7 ± 3 μs (NIS), and 9 ± 3 μs (LIS). These are probably upper bounds for the actual correlation times, because (*a*) head motions in the fiber are likely to be highly anisotropic whereas the calibrations were obtained from freely diffusing globular proteins, and (*b*) the correlation time of the background signal is difficult to estimate but the background is likely to increase the values of the apparent correlation time. Nevertheless, the conclusion from Fig. 7 is quite clear: the disorder observed in both NIS and LIS (Fig. 3) is dynamic, on the sub-msec time scale, and there is no substantial difference between the motion occuring in NIS and LIS.

Stretched fibers

It is important to show that the observed effects on the EPR spectra are the result of actin-myosin interactions rather than direct effects of ionic strength on the myosin heads. The latter possibility can be investigated by stretching the muscle fiber out-of-overlap, so that no

heads are capable of attachment to thin filaments. Fig. 8 shows EPR and STEPR spectra of fibers with sarcomere lengths of 4.1 ± 0.1 μm. There are no significant differences among the three spectra, indicating that any effects of ATP and ionic strength are dependent on the myosin-actin interactions and do not reflect local changes in the myosin head alone. Note that the experimental geometry of the fibers in the STEPR spectra of Fig. 8 and Fig. 7 is different and they should not be compared. The main conclusion from STEPR spectra of Fig. 8 is the absence of changes in mobility of the heads which do not interact with thin filaments and this conclusion is not affected by the orientation of the fibers.

DISCUSSION

Skinned fibers are regulated by Ca at low ionic strength. Isometric tension and shortening velocity are comparable to the values observed at normal ionic strength (Brenner et al., 1982; Yanagida et al., 1982). The cross-bridge state at LIS is predominantly the myosin.ATP or myosin.ADP.Pi state (Schoenberg, 1988*b*), a state which, from its position in the biochemical ATPase cycle, has been postulated to correspond to the beginning of the cross-bridge power stroke (Eisenberg and Greene, 1980).

In relaxed fibers at low ionic strength, a significant fraction of myosin heads is attached to actin, giving rise to stiffness in response to submillisecond stretches Brenner et al., 1982; Yanagida et al., 1982). The value depends strongly on speed of stretch (Brenner et al., 1982; Schoenberg et al., 1984; Schoenberg, 1988*a*), suggesting rapid equilibrium attachment and detachment of the cross-bridges (Schoenberg, 1985). Recently, Schoenberg (1988*a*) using the 1985 model of the equilibrium crossbridge, extracted attachment and detachment rate constants for the myosin.ATP crossbridge heads. These showed a lifetime for the heads of longer than 20 μs, two orders of magnitude longer than the time scale of the conventional EPR experiment. Thus, the spectrum of the attached head should not be averaged with the spectrum of a detached head. If the attached head and detached states have distinct orientations, their spectra should be resolved from each other.

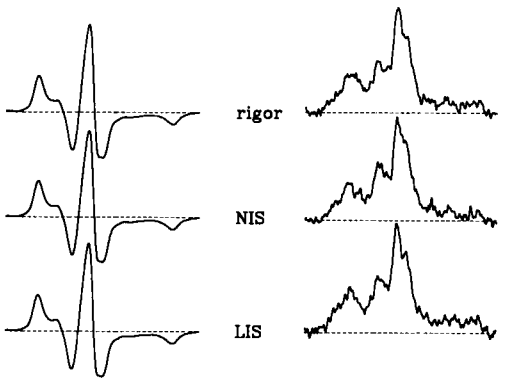

FIGURE 8 Conventional (*left*) and STEPR (*right*) spectra of MSL fibers stretched out-of-overlap (sarcomere length of 4.1 μm). Rigor, relaxation at normal ionic strength (NIS) and relaxation at low ionic strength (LIS). STEPR spectra recorded with the magnetic field perpendicular to the fiber axis. The temperature was 5°C.

It is important to consider possible perturbations of the fibers due to labeling, because MSL is known to affect the ATPase properties of myosin (Svensson and Thomas, 1986; Titus et al., 1989). SH blocking with other probes has been shown to perturb the equilibrium between myosin.ATP and myosin.ADP.P (Barnett and Thomas, 1987), which may be related to increased crossbridge disorder in relaxation as judged from electron microscopy of scallop muscle (Vibert and Castellani, 1989). However, labeling does not affect the mechanical properties of fibers at LIS (Fig. 1). In the absence of Ca^{2+}, no resting tension is developed either at normal or at low ionic strength. Both labeled and unlabeled fibers similarly show the typical speed dependence of chord stiffness that is characteristic of equilibrium crossbridges (Schoenberg, 1985, 1988*a*) as shown in Fig. 1.

As always, when the changes are small and the background is substantial, it is important to show that the changes detected do not originate from the background, as demonstrated in Fig. 2. In our case, labeling by MSL is rather less specific than with other probes, with 60–70% of the total signal originating from sites other than SH1 or SH2. However, blocking experiments using IASL (Fig. 2) or IAA (data not shown) correlated the magnitude of changes with the amount of specific labeling. This suggests strongly that the changes in the EPR lineshape come from the labels bound to SH1.

The main conclusion of this work is that myosin heads are almost as disordered at LIS, when stiffness suggests that a large fraction is weakly attached to actin , as at NIS, when the heads are probably detached (Fig. 3). Although we have not analyzed the distribution quantitatively due to difficulties in analyzing broad EPR lineshapes (Fajer et al., 1990*c*), this result implies many orientations at which the myosin head is attached to actin. Equally important is the finding of microsecond mobility of these heads (Fig. 4). If the LIS state represents the beginning of the power stroke, then these results are not consistent with a simple two-state model in which all heads have one orientation (e.g., 90°) at the beginning of their power stroke and another (e.g., 45°) at the end, with no intermediate orientations. The results are more consistent with a model having many orienta-

tional states with rapid transitions between them, (Huxley and Simmons, 1971), and are perhaps most consistent with a model in which the initial attached state has virtually no orientational preference.

In the absence of spectral resolution of the signals coming from attached and detached heads, it is impossible to determine unambiguously the spectrum of attached heads. However, we can assume that the spectrum of the detached heads at LIS is identical to that at NIS and use the latter for digital subtraction (Fig. 4). This difference spectrum shows the same average orientation as the heads in rigor but with a significantly broader orientational distribution (full width of 40° versus 15° in rigor). The fraction (17%) estimated from the spectral subtractions is in good agreement with the 20% fraction of an oriented fluorescent ATP analogue (epsilon-2-aza-ATP) under similar conditions (Nagano and Yanagida, 1984). Alternatively, we can assume that the fraction of attached crossbridges is given by the fractional stiffness (compared with rigor) at high speeds of stretch (50–75%, Fig. 1). As discussed above, the resulting spectrum implies even greater orientational disorder for attached heads, depending on whether single- or double-headed attachment is assumed (Fig. 6). The combination of stiffness and EPR data favors the second alternative and we propose that LIS heads are mobile and disordered while transiently attached.

It is important to note that the small spectral changes in LIS are due to thick-thin filament interactions. When the fibers were stretched out-of-overlap (sarcomere length of 4.1 μm) the difference between LIS and NIS EPR and STEPR spectra vanished (Fig. 8), ruling out the possibility of some nonspecific changes in spin label environment brought about by changes in polarity.

Relationship to other work

Reconstruction of electron density maps from the first five equatorial reflections suggest 60% attachment of heads based on increased mass in the vicinity of actin filaments and assuming the same axial projections of rigor and relaxed heads (Yu and Brenner, 1989). However, this is only a rough estimate because, as stressed by the authors, the two modes of attachment are vastly different. The authors concluded that the transition from NIS to LIS is qualitatively different from the transition from NIS to rigor: (*a*) the effective diameter of the actin filaments did not increase as much in LIS as in rigor; and (*b*) mass was lost from the space between the filaments rather than from the myosin filament. This suggested that in comparison to rigor heads, a smaller portion of the LIS head was immobilized and ordered by attachment, with more freedom of motion in the remaining part of the head (Yu and Brenner, 1989) which is in agreement with this work. Our conclusion that the orientational disorder in LIS is similar to that of NIS but quite different from that of rigor is consistent with observations of x-ray diffraction layer lines and meridional reflections (Matsuda and Podolsky, 1984). The high degree of orientational disorder in both LIS and NIS might seem inconsistent with the myosin-based layer lines that are often seen in relaxed muscle, but (*a*) EPR sees orientational order while x-ray diffraction sees translational order, (*b*) the myosin-based layer lines are very weak in rabbit muscle at 5°C (Wray et al., 1988), and (*c*) SH blocking may decrease cross-bridge order in relaxation (Vibert and Castellani, 1989). The latter possibility, along with the possibility that the myosin head may itself be flexible (Cooke, 1986), raises the importance of future complementary studies with probes at other sites on the myosin head and coordinated EPR and x-ray diffraction study on labeled fibers.

In conclusion, we have shown that in the early stages of the contractile cycle, as mimicked by low ionic strength relaxation, the myosin heads are attached to actin at a variety of angles and they show microsecond rotational mobility. This is consistent with a modified rotating cross-bridge model that includes dynamically attached cross-bridges.

We thank Leepo Yu and Bernhard Brenner for helpful discussions; Chris Wendt, Eric Svensson, and Nancy Brunsvold for technical assistance. Piotr Fajer was a Muscular Dystrophy Association fellow.

This work was supported by National Institutes of Health grants AR32961 and RR4300.

Received for publication 2 August, 1990 and in final form 4 April 1991.

REFERENCES

Applegate, D., and P. Flicker. 1987. New states of actomyosin. *J. Biol. Chem.* 262:6856–6863.

Barnett, V. A., and D. D. Thomas. 1987. Resolution of conformational states of spin-labeled myosin during steady-state ATP hydrolysis. *Biochemistry.* 26:314–323.

Berger, C. L., E. C. Svensson, and D. D. Thomas. 1989. Photolysis of a photolabile precursor of ATP (caged ATP) induces microsecond rotational motions of myosin heads bound to actin. *Proc. Natl. Acad. Sci. USA.* 86:8753–8757.

Brenner, B. 1985. Mechanical approaches to the elucidation of the crossbridge cycle. *J. Muscle Res. Cell. Motil.* 6:659–668.

Brenner, B. 1987. Mechanical and structural approaches to correlation of cross-bridge action in muscle with actomyosin ATPase in solution. *Annu. Rev. Physiol.* 49:655–672.

Brenner, B., M. Schoenberg, J. M. Chalovich, L. E. Greene, and E. Eisenberg. 1982. Evidence for cross-bridge attachment in relaxed muscle at low ionic strength. *Proc. Natl. Acad. Sci. USA.* 79:7288–7291.

Cooke, R. 1986. The mechanism of muscle contraction. *CRC Crit. Rev. Biochem.* 21:53–118.

Cooke, R., M. S. Crowder, and D. D. Thomas. 1982. Orientation of spin labels attached to cross-bridges in contracting muscle fibers. *Nature (Lond.)* 300:776–778.

Craig, R., L. E. Greene, and E. Eisenberg. 1985. Structure of the actin-myosin complex in the presence of ATP. *Proc. Natl. Acad. Sci. USA.* 82:3247–3251.

Crowder, M. S., and R. Cooke. 1984. The effect of myosin sulphydryl modification on the mechanics of fiber contraction. *J. Muscle Res. Cell. Motil.* 5:131–146.

Dantzig, J. A., J. W. Walker, D. R. Trentham, and Y. E. Goldman. 1988. Relaxation of muscle fibers with adenosine 5′-[gamma-thio]triphosphate (ATP[gammaS]) and by laser photolysis of caged ATP[gamma S]: evidence for Ca2+-dependent affinity of rapidly detaching zero-force cross-bridges. *Proc. Natl. Acad. Sci. USA.* 85:6716–6720.

Eisenberg, E., and L. E. Greene. 1980. The relation of muscle biochemistry to muscle physiology. *Annu. Rev. Physiol.* 42:293–309.

Evans, C. A. 1981. Use of the integral of saturation transfer electron paramagnetic resonance spectra to determine molecular rotational correlation times of slowly tumbling spin labels in the presence of rapidly tumbling spin labels. *J. Magn. Reson.* 44:109–116.

Fajer, P. G., E. A. Fajer, N. J. Brunsvold, and D. D. Thomas. 1988. Effects of AMPPNP on the orientation and rotational dynamics of spin-labeled muscle cross-bridges. *Biophys. J.* 53:513–524.

Fajer, P. G., B. Bennett, C. F. Polnaszek, E. A. Fajer, and D. D. Thomas. 1990*a*. High resolution analysis of EPR spectra using simplex algorithm. *J. Magn. Reson.* 85:111–125.

Fajer, P. G., E. A. Fajer, J. M. Matta, and D. D. Thomas. 1990*b*. Orientational distribution of crossbridges in muscle fibers in rigor and ADP. *Biochemistry.* 29:5865–5871.

Fajer, P. G., E. A. Fajer, and D. D. Thomas. 1990*c*. Time resolved studies of crossbridge orientation during muscle contraction using photolysable ATP precursor. *Proc. Natl. Acad. Sci. USA.* 5538–5542.

Horvath, L. I., and D. Marsh. 1983. Analysis of multicomponent saturation transfer EPR spectra using the integral method: application to membrane systems. *J. Magn. Reson.* 54:363–373.

Huxley, A. F., and R. M. Simmons. 1971. Proposed mechanism of force generation in striated muscle. *Nature (Lond.).* 233:533–538.

Huxley, H. E. 1969. The mechanism of muscular contraction. *Science (Wash. DC).* 164:1356–1365.

Matsuda, T., and R. J. Podolsky. 1984. X-Ray evidence for two structural states of the actomyosin cross-bridge in muscle fibers. *Proc. Natl. Acad. Sci. USA.* 81:2364–2368.

Mornet, D., R. Bertrand, P. Pantel, E. Audemard, and R. Kassab. 1981. Structure of the actin-myosin interface, *Nature (Lond.)* 292:301–306.

Nagano, H., and T. Yanagida. 1984. Predominant attached state of myosin cross-bridges during contraction and relaxation at low ionic strength. *J. Mol. Biol.* 177:769–785.

Pate, E., and R. Cooke. 1988. Energetics of the actomyosin bond in the filament array of muscle fibers, *Biophys. J.* 53:561–573.

Podolsky, R. J., and T. Arata. 1988. Force generating mechanisms in striated muscle. *Adv. Exp. Med. Biol.* 226:319–330.

Reedy, M. K., K. C. Holmes, and R. T. Tregear. 1965. Induced changes in orientation of the cross-bridges of glycerinated insect flight muscle. *Nature (Lond.)* 207:1276–1280.

Schoenberg, M. 1985. Equilibrium muscle cross-bridge behavior. Theoretical considerations. *Biophys. J.* 48:467–475.

Schoenberg, M. 1988*a*. Characterization of the myosin adenosine triphosphate (M.ATP) crossbridge in rabbit and frog skeletal muscle fibers. *Biophys. J.* 54:135–148.

Schoenberg, M. 1988*b*. The kinetics of weakly- and strongly-binding crossbridges: implications for contraction and relaxation. *Adv. Exp. Med. Biol.* 226:189–202.

Schoenberg, M., B. Brenner, J. M. Chalovich, L. E. Greene, and E. Eisenberg. 1984. Cross-bridge attachment in relaxed muscle. *Adv. Exp. Med. Biol.* 170:269–284.

Svensson, E. C., and D. D. Thomas. 1986. ATP induces microsecond rotational motions of myosin heads crosslinked to actin. *Biophys. J.* 50:999–1002.

Thomas, D. D. 1987. Spectroscopic probes of muscle cross-bridge rotation. *Annu. Rev. Physiol.* 49:691–709.

Thomas, D. D., L. R. Dalton, and J. S. Hyde. 1976. Rotational diffusion studied by passage saturation transfer electron paramagnetic resonance. *J. Chem. Phys.* 65:3006–3024.

Thomas, D. D., and R. Cooke. 1980. Orientation of spin-labeled myosin heads in glycerinated muscle fibers. *Biophys. J.* 32:891–906.

Thomas, D. D., S. Ishiwata, J. C. Seidel, and J. Gergely. 1980. Submillisecond rotational dynamics of spin-labeled myosin heads in myofibrils. *Biophys. J.* 32:873–889.

Titus, M. A., G. Ashiba, and A. G. Szent-Gyorgyi. 1989. SH-1 modification of rabbit myosin interferes with calcium regulation. *J. Muscle Res. Cell Motil.* 10:25–33.

Vibert, P., and L. Castellani. 1989. Substructure and accessory proteins in scallop myosin filaments. *J. Cell Biol.* 109:539–547.

Wray, J. S., R. S. Goody, and K. C. Holmes. 1988. Towards a molecular mechanism for the crossbridge cycle. *Adv. Exp. Med. Biol.* 226:49–59.

Yanagida, T., I. Kuranaga, and A. Inoue. 1982. Interaction of myosin with thin filaments during contraction and relaxation: effect of ionic strength. *J. Biochem.* 92:407–412.

Yu, L. C., and B. Brenner. 1989. Structures of actomyosin crossbridges in relaxed and rigor muscle fibers. *Biophys. J.* 55:441–453.