

Myosin heads have a broad orientational distribution during isometric muscle contraction: Time-resolved EPR studies using caged ATP

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ABSTRACT To study the orientation of spin-labeled myosin heads in the first few seconds after the production of saturating ATP, we have used a laser flash to photolyze caged ATP during EPR data acquisition. Rabbit psoas muscle fibers were labeled with maleimide spin label, modifying 60% of the myosin heads without impairing muscle fiber biochemical and physiological activity (ATPase and force). The muscle bundles were incubated for 30 min with 5 mM caged ATP prior to the UV flash. The flash, from an excimer laser, liberated 2–3 mM ATP, generating maximum force in the presence of Ca^{2+} and relaxing fully in the absence of Ca^{2+} . Control experiments, using fibers decorated with labeled myosin subfragment, showed that the flash liberates sufficient ATP to saturate myosin active sites in all regions of the muscle bundles. To increase the time resolution, and to minimize the time of the contraction, we followed in time the intensity at a single spectral position (P_2), which is associated with the high degree of orientational order in rigor. ATP liberation produced a rapid decrease of P_2 with liberation of ATP, indicating a large decrease in orientational order in both relaxation and contraction. This transient was absent when caged AMP was used, ruling out nonspecific effects of the UV flash and subsequent photochemistry. The steady-state level of P_2 during contraction was almost as low as that reached in relaxation, although the duration of the steady state was much more brief in contraction. Upon depletion of ATP in contraction, the P_2 intensity reverted to the original rigor level, accompanied by development of rigor tension. The steady-state results obtained in the brief contractions induced by caged ATP are quantitatively consistent with those obtained in longer contractions by continuously perfusing fibers with ATP. In isometric contraction, most ($88\% \pm 4\%$) of the heads are in a population characterized by a high degree of axial disorder, comparable to that observed for all heads in relaxation. Since the stiffness of these fibers in contraction is 80% of the stiffness in rigor, it is likely that most of the heads in this highly disoriented population are attached to actin in contraction and that most actin-attached heads in contraction are in this disoriented population.

Electron microscopic observation of different orientations of muscle cross-bridges with respect to the muscle fiber axis in rigor and relaxation spawned the widely discussed “rotating head” hypothesis of muscle contraction (1). It has been postulated that the myosin head forms a cross-bridge by binding to the actin filament at a 90° angle; it then undergoes a conformational change that results in a pivoting motion of the head to a 45° orientation (2). Strain in the cross-bridge is then relieved by a sliding motion of actin and myosin filaments (2, 3). A rigorous confirmation of this hypothesis requires direct evidence for a large ($\approx 45^\circ$) tilting motion of the entire actin-attached myosin head with respect to the muscle

fiber axis. Despite a wide range of studies in which x-ray diffraction, electron microscopy, and spectroscopy were used, this evidence has not been conclusively obtained (reviewed in refs. 4 and 5).

Previous EPR studies of contraction, involving the continuous perfusion of muscle fiber bundles with ATP-containing solutions, revealed two populations of spin-labeled heads, a minor population ($\approx 20\%$ of heads) similar to rigor in its high degree of orientation and rigidity, and another ($\approx 80\%$) similar to relaxation in its orientational disorder and microsecond rotational mobility (6, 7). The principal question remaining from that work is the following: How are these orientational and motional populations related to the cross-bridge cycle?

Much of the uncertainty comes from the complexity of a steady-state experiment on contracting cross-bridges, making it difficult to assign a spectroscopic signal component to specific states in the biochemical cycle or even to determine which components come from actin-attached myosin heads.

We (and others) have taken several approaches to resolving this question. First, we have attempted to “trap” possible intermediates in the cycle by using nucleotide analogues or low ionic strength conditions. We have observed attached myosin heads that were (i) mobile and disordered [low ionic strength relaxation (8)]; (ii) forming a single-headed cross-bridge with one head strongly attached at a rigor angle and the other free to move [AMP-PNP (9), PP_i (10)]; (iii) firmly attached at an angle slightly different from the rigor angle [ADP (11, 12)].

Another approach, initiated in the present study, is to resolve the cross-bridge cycle biochemically by observing the transient phase of cross-bridge action. Such transient biochemical experiments in muscle fibers have been made possible by the development of caged ATP [the P^3 -1-(2-nitrophenyl)ethyl ester of ATP] and other caged compounds, which can be used to cause sudden and large changes in substrate (e.g., ATP) concentration by flash photolysis with a pulsed laser (13). Although most studies in which this technique is used in muscle have involved measurements of force and stiffness during the transient phase, a promising approach is to obtain information about molecular structure and dynamics during this phase by monitoring x-ray diffraction or spectroscopic signals (reviewed in ref. 14). In the present study, we have used caged ATP technology to obtain the EPR spectrum from spin-labeled myosin heads in skinned muscle fibers milliseconds after the ATP is released by flash photolysis. This permits us to saturate all ATP-binding sites in the fiber quickly, to observe the steady-state phase of a

Abbreviations: S1, myosin subfragment 1; MSL, 4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy; caged ATP, the P^3 -1-(2-nitrophenyl)ethyl ester of ATP.

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very brief contraction, and to establish the technology in future transient-phase studies designed to correlate the time course of cross-bridge motions with the time courses of biochemical and mechanical events.

METHODS

Sample Preparation and Assays. Detailed procedures have been published (9). We have used glycerinated rabbit psoas muscle labeled with maleimide spin label (MSL; 4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy). ATPase assays were described in full by Fajer *et al.* (9). As estimated from the fractional inhibition of K^+ -ATPase, 59% of the heads have been labeled (Table 1) without impairment of tension generation and Ca^{2+} regulation of tension (9, 15)—although SH-directed spin labels inhibit the myosin ATPase at high ionic strength—the V_{max} of actomyosin subfragment 1 ATPase in solution (16), and calcium regulation of actomyosin ATPase in solution (17). The different effects in fibers and in solution may be related to different rate-limiting processes. The fibers were not treated with $K_3Fe(CN)_6$ to remove background signal, which constitutes 60–75% of the total integrated signal (and $\approx 20\%$ at the diagnostic P_2 position), since that treatment can impair the mechanical properties of fibers (9). Importantly, this background signal, which arises from sites other than SH1 of the myosin head, is not sensitive to nucleotide addition (8).

Fiber relaxation was achieved by incubation in 126 mM KPr/20 mM Mops/2 mM $MgCl_2$ /1 mM EGTA/5 mM MgATP for flow experiments or 10 mM caged ATP/5 mM $MgCl_2$ for transient experiments. Contraction was induced by adding 1.5 mM $CaCl_2$ to the relaxation solution.

EPR. EPR experiments were performed with a Bruker ER-200D spectrometer. Spectra were acquired, averaged, and analyzed with a Zenith 158 microcomputer interfaced to the EPR spectrometer as described (9). Thin (diameter, 0.2 mm) muscle bundles were used for all EPR experiments. During acquisition of entire spectra in the presence of ATP, the appropriate solution was continuously flowed through a capillary containing the isometric fiber bundle (length, 20 mm), mounted parallel to the magnetic field in a modified TM_{101} cavity, as described (6, 9). These are referred to below as EPR flow experiments. For transient EPR measurements, fiber bundles were placed in a specially designed EPR flat cell providing isometric conditions and orientation of the fibers parallel to the magnetic field, using a TE_{102} cavity with an optical port. The EPR transients were recorded by locking the magnetic field on the desired field position with a dwell time of 24 ms per point. The order of solutions was varied. A typical set of experiments consisted of relaxation–contraction–relaxation or contraction–relaxation–contraction. The conclusions of the present study are all based on data in which there is no significant difference between the first and last spectrum in a set, and there is no dependence on the order of the solution.

Table 1. Fiber characterization (25°C)

	Control \pm SEM	Labeled \pm SEM
Heads labeled, %	0	59 \pm 3
ATPase, units		
– Ca^{2+}	0.031 \pm 0.002	0.032 \pm 0.005
+ Ca^{2+}	0.31 \pm 0.02	0.17 \pm 0.02
Tension, kg/cm ²		
– Ca^{2+}	0.097 \pm 0.010	0.119 \pm 0.007
+ Ca^{2+}	2.56 \pm 0.1	2.48 \pm 0.1
Stiffness, % rigor		
– Ca^{2+}	3	7 \pm 1
+ Ca^{2+}	76	82 \pm 2

Six different fiber preparations were used.

Optics. A gas excimer laser (Lambda Physik EMG 53 MSC, Göttingen, F.R.G.), operating with XeCl (308 nm), was used to deliver a 2-s burst of 20 light pulses (10–20 mJ each), photolyzing 2–3 mM ATP from the 10 mM caged precursor. Due to the light absorption of photolysis products and the low extent of photolysis at 308 nm, compared with 347 nm (14), a longer wavelength might be an advantage in future studies. The light intensity distribution was monitored with a 0.5-mm fiber optic probe attached to a power meter and/or UV-sensitive photographic film, and it was found to be uniform within 10% throughout the length of the sample.

Mechanics. Fiber stiffness was measured as the force generated in response to a series of small length perturbations (<0.1 – 0.5% of fiber length) as described in ref. 15. Single fibers (6–10 mm long) were glued (with Duco cement) at one end to a force transducer (400A; Cambridge Technology, Cambridge, MA) and at the other end to a galvanometer coil (Servo amplifier 125; Cambridge Technology). The force was recorded 1 ms after beginning the length change, and the stiffness was taken as the slope of force versus length change and normalized to a value obtained in rigor for the same muscle fiber. Tension transients were measured on EPR fiber bundles placed in quartz capillaries and attached to a strain gauge (AE 801; SensoNor, Horten, Norway) with a silk thread. The active tension produced by fiber bundles was the same as that measured for single fibers.

RESULTS

ATP Saturation. To determine whether caged ATP photolysis produces a saturating concentration of ATP throughout the fiber bundles, an unlabeled fiber bundle was decorated with MSL–myosin subfragment (S1), mounted in the EPR flat cell, and studied by the same procedure used to obtain transient EPR spectra from labeled fibers. The spectrum of S1 infused into a fiber (see figure 4 in ref. 9) consists of a superposition of two signals, one from S1 bound to actin filaments at a well-defined angle, giving rise to a peak at P_2 , and another from excess free and randomly oriented S1, giving rise to a peak at a different spectral position, P_1 (9). Thus, the detachment of the S1 heads can be measured by the loss of P_2 intensity.

In the presence of saturating ATP, bound S1 is dissociated from the fiber, and its EPR spectrum is the same as that of free S1 (figure 4 in ref. 9). The magnetic field was locked at the P_2 position. The intensity was monitored as a function of time before, during, and after the photolyzing flash. The intensity of P_2 decreased precisely to the level characteristic of S1 in solution when the spectra were normalized to the same intensity by double integration, indicating complete disorientation and hence saturation with ATP throughout the fiber bundle. As the ATP is hydrolyzed, S1 binds back to the fiber and the intensity reverts to the original value.

The fraction of disoriented heads was estimated from the decrease of P_2 after the flash, interpolating linearly between the levels found for free and bound S1 (after normalization to the concentration of total, infused S1). Complete disorientation was observed (Table 2), implying full saturation by ATP. The effect is observed not only at 5°C in the absence of Ca^{2+} , when the ATPase activity is very low, but also at 22°C or in

Table 2. Disorientation of spin-labeled S1

Solution	t , °C	Fraction disoriented	SEM
Relaxation	5	0.99	0.01
Relaxation	22	0.99	0.03
Contraction	5	1.0	—

Fraction disoriented was calculated from $(P_2^{rigor} - P_2^{after\ flash}) / (P_2^{rigor} - P_2^{free})$.

the presence of Ca^{2+} , when the ATPase of the fiber is 10 times higher (Table 2).

Tension Transients. To test whether the fiber bundles are capable of mechanical relaxation and contraction utilizing photolyzed caged ATP, tension was measured on the samples under the conditions of EPR flow experiments in quartz capillaries (wall thickness, 1 mm). At the beginning of each test, rigor tension was induced by allowing ATP to be depleted. Starting in the absence of Ca^{2+} , rigor tension was relieved after the photolysis of caged ATP (Fig. 1 Upper), as expected for relaxed muscle. In the presence of Ca^{2+} , the tension that developed after photolysis of caged ATP was the same as the tension using ATP (Fig. 1 Lower). Moreover, repeated ATP contractions and relaxations were reproducible, thus implying no gross mechanical damage due to the photolyzing UV pulse and the products of photolysis even though no radical scavengers (dithiothreitol or glutathione) were present.

EPR Transients on Labeled Fibers. Having established that the laser flash is saturating fiber bundles with ATP as demonstrated by infused S1 detachment and mechanical relaxation, we have performed transient EPR experiments on MSL-labeled fibers by monitoring the intensity at P_2 before and after the laser flash (Fig. 2). In the absence of Ca^{2+} , P_2 decreases after photolysis of caged ATP, indicating a rapid disordering of the myosin heads upon muscle relaxation (Fig. 2 Upper). The intensity reaches a steady-state plateau [Fig. 2 Upper (Inset)] that persists about as long as the ATP is expected to be present at a saturating concentration based on the fiber ATPase activity.

The value of the steady-state level is higher than in the case of free S1, due to the higher background signal originating from nonspecific labeling in fibers. No significant transient was observed when caged AMP was used in place of caged ATP, although a slight positive deviation was sometimes observed [Fig. 2 Upper (Inset)].

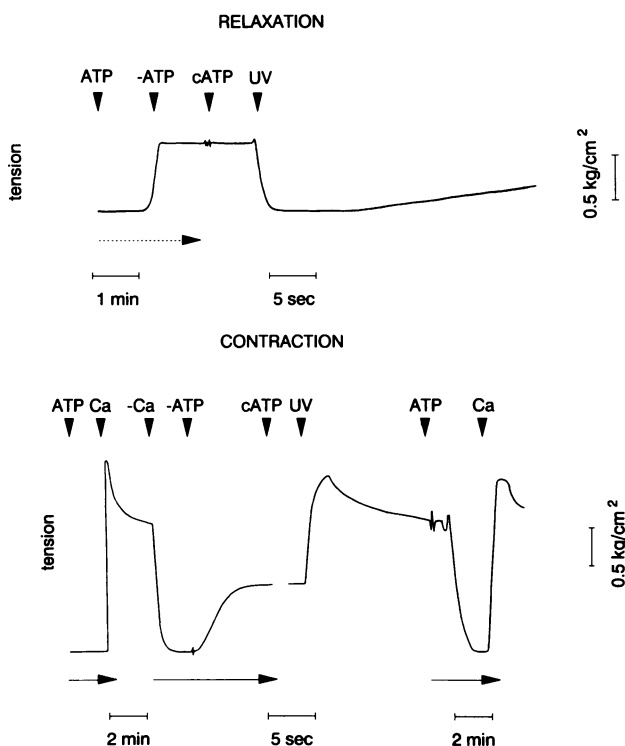


FIG. 1. Tension development by MSL-labeled fiber bundles using caged ATP at 20°C. Horizontal lines indicate when the solution was flowing. (Upper) Relaxation of the rigor tension. (Lower) Contraction from rigor using ATP (first and third contraction) or photolysis of caged ATP (cATP).

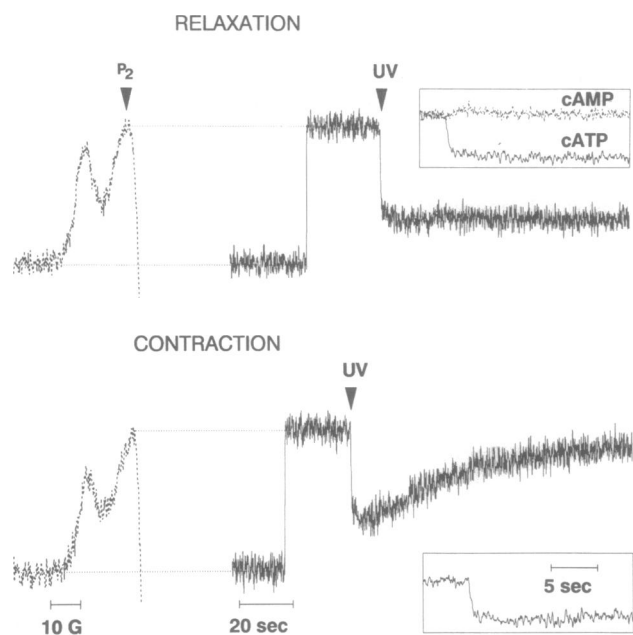


FIG. 2. Transient EPR of MSL-labeled fibers in the absence of Ca^{2+} (Upper) and in the presence of Ca^{2+} (Lower) at 5°C. (Left) The low-field region of the EPR spectrum before caged ATP photolysis. The arrowhead shows the P_2 position, and horizontal dotted lines are drawn to the right to show the baseline and the level of P_2 in rigor. (Right) Time-resolved EPR intensity. The trace initially shows the baseline level, and then the intensity at P_2 is monitored before and after the UV flash (arrowhead). (Insets) Expanded time base for caged ATP (cATP) (bottom traces) and caged AMP (cAMP) (top trace).

In the presence of Ca^{2+} (isometric contraction), a similar transient was observed, except that (i) the P_2 level during the steady-state plateau is slightly higher and (ii) the plateau is much more brief (Fig. 2), as expected due to the higher ATPase activity in the presence of Ca^{2+} .

Similar traces, with rapid initial transients followed by clear steady-state plateaus and recoveries, were observed at 22°C, for both relaxation and contraction, except that the duration of the plateau was decreased relative to 5°C. Table 3 summarizes the fraction of heads with rigor-like orientation present in contraction at 5°C and at 22°C, as measured by the P_2 intensity, assuming that the signal from heads in contraction is a linear combination of rigor and relaxation signals, as suggested (6).

As shown in Fig. 2 (Insets), we observed consistently (in the presence and absence of calcium) that the P_2 level in transients drops to within the noise level of the steady-state level within 1 s, less than the width of the laser burst. A 2-fold decrease in the number of laser pulses (at constant energy per pulse) did not affect the steady-state level. The duration of the steady state was always at least several times as long as the approach to the steady state, and the recovery time was always at least an order of magnitude longer than the onset time, demonstrating that a true biochemical steady state is reached, during which the myosin active sites must be saturated with ATP.

Table 3. Fraction of rigor-like orientation in contraction

t , °C	Caged ATP		Flow	
	Fraction \pm SEM	n	Fraction \pm SEM	n
5	0.05 \pm 0.01	15	0.09 \pm 0.01	3
22	0.11 \pm 0.03	3	0.13 \pm 0.01	6

Fraction was calculated from $(P_2^{\text{contraction}} - P_2^{\text{relaxation}}) / P_2^{\text{rigor}} - P_2^{\text{relaxation}}$. n , Number of determinations.

Continuous Flow EPR. Steady-state EPR spectra were recorded in rigor, relaxation, and contraction under conditions of continuous flow, following the procedure described in ref. 6 (Fig. 3).

The small lineshape difference between the spectra in Figs. 2 and 3 is due to (i) variability between different fiber preparations and (ii) slight differences in the field distribution in the two different cavities used. These factors do not affect the fraction of the rigor-like component in the contraction spectrum because it is calculated from rigor and relaxation spectra of each particular preparation. To compare these results (Fig. 3) with the caged ATP experiments (Fig. 2), we measured the intensities at P_2 and determined the apparent mole fraction of the rigor-like component, assuming a linear combination of rigor and relaxation spectra. The results are in excellent agreement between flow and caged ATP experiments (Table 3). To test more rigorously the linear combination assumption, we constructed a composite spectrum from the rigor and relaxation spectra using the molar fractions indicated from Table 3. When this composite spectrum was subtracted from the actual experimental spectrum in rigor, the residual was not flat, indicating that the spectrum in contraction is not strictly a linear combination of those in rigor and relaxation (Fig. 3). Small but significantly nonzero residuals were observed consistently at both 5°C and 22°C. This result implies that either (i) the background signal is sensitive to ATP and/or Ca^{2+} , which is unlikely (8), or (ii) the orientational distribution of heads in contraction is distinct from those in rigor and relaxation. Although the spectra in relaxation and contraction clearly show large orientational disorder, they are significantly different from the spectra of randomly oriented myofibrils (data not shown; see ref. 12), indicating that the orientational distributions are not completely random in either relaxation or contraction. This is consistent with previous reports and conclusions from this laboratory (18, 19), despite citations that interpret our data as implying random distribution in relaxation (20).

DISCUSSION

Interpretation of Results. In this work, we have developed the technology to observe the transient orientation of spin-labeled myosin heads following the photolysis of caged ATP. The time resolution is 24 ms, with a good signal/noise ratio, but the weakness of the laser pulse required a 2-s burst of pulses to obtain sufficient energy for caged ATP photolysis throughout the sample. Therefore, the quantitative conclusions of the present study focus on the brief steady-state phase. A more powerful laser should make it possible to extend this analysis to the transient phase in the near future. We have shown that we can uniformly liberate 1–3 mM ATP

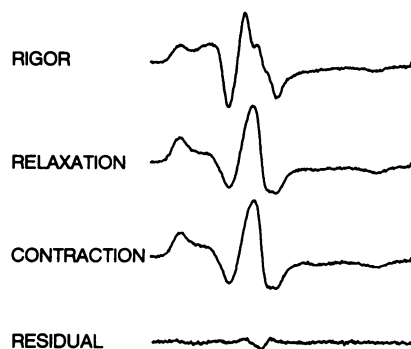


FIG. 3. The continuous flow EPR spectra in rigor, relaxation, and contraction. The residual spectrum at the bottom was obtained by subtracting a composite spectrum (9% of the rigor spectrum + 91% of the relaxation spectrum) from the contraction spectrum.

within a fiber bundle. Under these conditions, all infused myosin heads (MSL-S1) are totally disoriented (Table 2), implying complete saturation with ATP in fiber bundles. Under conditions essentially the same as those of EPR measurements, caged ATP photolysis produced normal mechanical relaxation in the absence of Ca^{2+} and full isometric tension in the presence of Ca^{2+} (Fig. 1).

The EPR signal during the contraction transient (Fig. 2) reached nearly the same level as in relaxation (Fig. 2), indicating a large decrease in orientational order in both cases. To a first approximation, the small difference between relaxation and contraction could be a small fraction of heads with a rigor-like orientation in contraction but not in relaxation (Table 3), as shown previously (6). This fraction is <10% at 5°C. Increasing the temperature to 22°C causes a small increase of this fraction (to 11–13%), but this increase is much smaller than the increases in active tension (3-fold) or ATPase (5-fold). The instantaneous stiffness of these MSL-labeled fibers during contraction is 75–85% of the rigor stiffness, as previously reported for unlabeled fibers (21). If the small fraction of heads with rigor-like orientation is responsible for this high stiffness, they must have much higher stiffness than in rigor (10). However, if we assume that the fraction of rigor stiffness (75–85%) is the fraction of attached heads, then at most 17% (13%/75%) of the attached heads exhibit rigor-like orientation, since all the heads are bound in rigor (22, 23). This upper bound could increase to 34% if all attached cross-bridges are single-headed in active muscle, since single-headed cross-bridges have been shown to be as stiff as double-headed cross-bridges under some conditions (9, 10).

It is an oversimplification to regard the P_2 intensity in contraction as arising solely from a linear combination of spectra in rigor and relaxation, since the residual spectrum in Fig. 3 is not flat. Therefore, either (i) the disordered (relaxation-like) population in contraction is slightly different in orientation from that in relaxation, (ii) the ordered (rigor-like) population in contraction is slightly different from that in rigor, or (iii) there is a third population, present only in contraction, that either represents a small fraction of the heads or has disorientation similar to that of relaxation.

In principle, analysis of the whole EPR spectrum can provide detailed information about the orientational distribution of the spin-labeled heads (24). However, we have not attempted to determine the detailed orientational distribution of the heads in relaxation and contraction because of (i) the presence of background signal due to labels on sites other than S1 (25) and (ii) the difficulty in finding a unique solution to a spectrum characterized by a broad orientational distribution (18, 26). Nevertheless, some quantitative conclusions can be drawn. For the disordered population in either relaxation or contraction, the principal axis of the nitroxide spin label has an axial orientational disorder (full width at half maximum $\geq 90^\circ$) (18). This implies that the full axial disorder of the head is on the order of 50° or greater (26). The significant difference between the contraction and relaxation spectra indicates that some redistribution of the head orientation takes place upon activation. If this change is in the disordered component, it could correspond to a change in the average orientation of as much as 15° (26). A newly developed optimization procedure for estimating the orientational distribution might help us in directly addressing this question in the future (12, 24).

In the flow experiments (Fig. 3), contraction is initiated from relaxation, while in the transient experiments (Fig. 2) contraction starts from rigor, but the agreement of the P_2 values between the two experiments (Table 3) indicates that the steady-state results are the same. This agreement is expected, since the ATP turnover in isometric fibers is 1–5

s⁻¹, so the steady-state condition is achieved after 1 s in the EPR transients.

Relationship to Previous EPR Studies on Contracting Fibers.

The results of the present study are generally consistent with previous EPR studies of contracting MSL fibers (6, 19). The present study confirms that most of the spin labels are highly disoriented in contraction, with the spectrum resembling that of relaxation much more than that of rigor (6, 7, 19). Control experiments have consistently indicated that MSL is rigidly fixed to the myosin head, even in the presence of ATP (8, 25, 27), so the probe disorder indicates head disorder, but we cannot rigorously rule out the possibility that some of the disorder observed in contraction is due to ATP-dependent disorder within the head. Saturation transfer EPR measurements have shown that the dynamically disordered heads are not statically disordered but are rotationally mobile on the microsecond time scale (7), and caged ATP photolysis during saturation transfer EPR has shown that myosin heads undergo ATP-dependent microsecond rotational motions while attached to actin in solution (27). Cooke *et al.* (6) suggested that the heads can be divided into two populations—a major population that is highly disoriented (similar to relaxation) and a minor population that is highly oriented (similar to rigor). The estimate of the fraction of heads in the rigor-like fraction at 22°C, obtained in the present study (0.12 ± 0.04 ; Table 3), is smaller than previously found— 0.19 ± 0.03 (6, 19) and 0.17 ± 0.05 (7). This possibly significant difference may be due to the difference in preparations (no ferricyanide treatment, smaller fiber bundles) or to improved methodology (verification of ATP saturation by using caged ATP). In addition, the quantitative analysis in the present study (Fig. 3) shows that the orientational distribution in contraction is not strictly a linear combination of those in rigor and relaxation, as discussed above.

Conclusions. Most of the spin-labeled myosin heads in contracting fibers, probably including a majority of the heads that are in actin-attached cross-bridges, are highly disordered, apparently undergoing large-amplitude rotational motions. The orientational distribution of these probes has not yet been quantitatively analyzed, but the distribution is clearly not completely random. These rotationally disordered attached heads could correspond primarily to an early weakly attached phase of the cross-bridge cycle (28) or to a distribution of orientations corresponding to several steps in the power stroke (3). The precise correlation of these rotationally mobile heads with the biochemical and mechanical cross-bridge cycles will have to await further studies. The technique developed in the present study should make it possible to obtain the kind of time-resolved information about cross-bridge orientation, during transient biochemical and mechanical conditions, that will be essential in achieving this goal.

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