Transients in Orientation of a Fluorescent Cross-bridge Probe Following Photolysis of Caged Nucleotides in Skeletal Muscle Fibres

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In muscle fibres labelled with iodoacetamidotetramethylrhodamine at Cys707 of the myosin heavy chain, the probes have been reported to change orientation when the fibre is activated, relaxed or put into rigor. In order to test whether these motions are indications of the cross-bridge power stroke, we monitored tension and linear dichroism of the probes in single glycerol-extracted fibres of rabbit psoas muscle during mechanical transients initiated by laser pulse photolysis of caged ATP and caged ADP. In rigor dichroism is negative, indicating average probe absorption dipole moments oriented more than 54.7° away from the fibre axis. During activation from rigor induced by photolysis of Caged ATP in the presence of calcium, the dichroism reversed sign promptly (half-time 12.5 ms for 500 μM-ATP) upon release of ATP, but then changed only slightly during tension development 20 to 100 milliseconds later. During the onset of rigor following transfer of the fibre from an ATP-containing relaxing solution to a rigor medium lacking ATP, force generation preceded the change in dichroism. The dichroism change occurred slowly (half-time 47 s), because binding of ADP to sites within the muscle fibre limited its rate of diffusion out of the fibre. When ADP was introduced or removed, the dichroism transient was similar in time course and magnitude to that obtained after the introduction or removal of ATP. Neither adding nor removing ADP produced substantial changes in force. These results demonstrate that orientation of the rhodamine probes on the myosin head reflects mainly structural changes linked to nucleotide binding and release, rather than rotation of the cross-bridge during force generation.

Keywords: muscle; contraction; actomyosin; fluorescent probe; caged ATP

1. Introduction

It is widely accepted that muscle contraction is accomplished by the relative sliding of thick (myosin-containing) and thin (actin-containing) filaments, and that the interaction between the two sets of filaments involves the myosin cross-bridges seen in electron micrographs (H. E. Huxley, 1957). When muscle is activated by calcium, these cross-bridges are believed to act as independent force generators, undergoing cycles of attachment to actin, force production, and detachment, in conjunction with hydrolysis of ATP (A F Huxley, 1974, and references therein). However, the structural changes in actomyosin that produce force have not yet been elucidated at the molecular level.

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Many techniques have yielded evidence of structural changes in cross-bridges. Using electron microscopy and X-ray diffraction of insect flight muscle, Reedy et al. (1965) found that cross-bridges appear to project from the thick filaments at approximately right angles in relaxed muscle but at acute angles in rigor. H. E. Huxley (1969) suggested that, to generate force, the cross-bridges change shape or rotate from the perpendicular relaxed-like angle to the more acute rigor-like angle while attached to actin.

Several investigators have attempted to detect cross-bridge angle changes during the power stroke, using paramagnetic or fluorescent probes attached to the cross bridges (see Thomas, 1987). Although these probes have revealed rotational disorder (Cooke et al., 1982; Fajer et al., 1990) and motion (Barnett & Thomas, 1989; Stein et al., 1990), they have generally not provided evidence of substantial
cross-bridge rotations in conjunction with the power stroke. In fact, in most probe studies, evidence has not been obtained for a population of cross-bridges ordered at an angle substantially different from that of rigor.

One prominent exception is the observation by Borejdo et al. (1982) of the orientation of fluorescent iodoacetamido-tetramethylrhodamine (IATR), attached covalently to SH-I (Cys707 of the myosin heavy chain), the most reactive sulphydryl group in the myosin head. Borejdo et al. measured the linear dichroism, which is proportional to the difference in absorption between axial and perpendicular polarizations of the exciting light. They reported that the dichroism changes markedly when ADP is added to the medium bathing a fibre in rigor (i.e., in the absence of ATP). This result indicates that the probes rotate by a substantial angle (~22°) when ADP binds.

The functional significance of this and other cross-bridge structural changes depends on their correlation with the biochemical and mechanical steps of the cross-bridge cycle. We set out to determine the kinetics of dichroism and force changes during transitions between cross-bridge states. We sought to discern whether orientational changes after photolysis of caged ATP or ADP occur concomitantly with force generation or with other steps in the cycle. Some of our results have been published in abstract form (Tanner et al., 1989; Dantzig et al., 1989).

2. Materials and Methods

(a) Chemicals and solutions

IATR was purchased from Molecular Probes (Eugene, OR; lot 9A). The commercial compound is a mixture of the 5- and 6-iodoacetamide isomers. We initially used IATR from Research Organics, but it contained an impurity that resulted in an ultraviolet absorbance peak, as others have found (Tait & Frieden, 1982). Qualitatively, the results were the same with products from both sources. ADP, obtained from Sigma Chemical Company or Boehringer-Mannheim Biochemicals, was purified as described by Dantzig et al. (1991). Caged ATP and caged ADP were synthesized as described by Walker et al. (1989). All other chemicals were of analytical grade. Solution compositions are shown in Table 1. Experiments were performed at room temperature (18 to 24°C).

(b) Fibre preparation and labelling

Bundles of rabbit psoas muscle fibres were dissected, extracted with glycerol and stored as described (Goldman et al., 1984a). Fibres were labelled with IATR essentially according to the procedure of Burghardt et al. (1983). However, in our experiments, that procedure resulted in a very small fraction (<10%) of labelled heads. Therefore, we increased the labelling concentration and time. First, to wash out the glycerol, bundles were placed for 20 min in a relaxing solution containing 50 mM-KCl, 5 mM-MgCl₂, 2 mM-EGTA, 5 mM-sodium phosphate buffer (pH 7.0), 5 mM-ATP (Borejdo et al., 1982). Next, they were placed for 20 min in the relaxing solution with 0.5%, Triton X-100 to reduce non-specific binding of the label to residual membranes. Following another 20 min in relaxing solution, bundles were incubated for 1 h in relaxing solution containing 0.22 mM-IATR. Excess label was washed out in relaxing solution for 2 to 12 h; the bundles were returned to glycerol storage solution and then stored at -20°C for 1 to 120 days before use. During the labelling procedure, all incubations were in the dark at 4°C.

For experiments, single fibres were dissected and mounted with T-shaped aluminium foil clips onto hooks on a force transducer and a piezoelectric device, as described (Goldman et al., 1984a). Fibres were held isometrically in all experiments described here. Sarcomere lengths and fibre dimensions were determined as described by Goldman & Simmons (1984).

(c) Assessment of labelling

The extent of labelling was assessed from the fractional inhibition of ATPase in the presence of high concentra-
tions of K⁺ and EDTA (Crowder & Cooke, 1984; Stein et al., 1990) on myofibrils prepared from labelled fibre bundles by homogenization. ATPase activity was measured using the malachite green method of Lanzetta et al. (1979) to detect the amount of inorganic phosphate (Pi) produced. The K⁺-EDTA ATPase activity was measured in 600 mM-KCl, 100 mM-Mops, 20 mM-EDTA (pH 7.5-7.5). Protein concentrations were determined by the Bradford (1976) method (BioRad, Richmond, CA) using bovine serum albumin as a protein standard. The fraction of Cys707 residues labelled with IATR, given by the fractional ATPase inhibition, was 37±9. The ratio of total bound dye molecules to heads (the dye-to-protein ratio) was measured spectrophotometrically (Stein et al., 1990). 86(±10)94% of the bound probes were on Cys707.

(d) Dynamic measurement of dichroism

The apparatus provides a signal, r, the experimentally measured absorption anisotropy, at millisecond time resolution. r is an approximation to the theoretical absorption anisotropy, r*, defined as (A₁₋ A₄)/(A₁₊ A₄), where A₁ and A₄ are the intensities of absorption by the probe molecules for light linearly polarized parallel and perpendicular to the fibre axis, respectively. If the probes are cylindrically symmetric about the fibre axis, r* is an order parameter of the orientational distribution of the probe absorption dipole moments given by r* = (2/3)2(β)(θ) - 1, where the symbols denote the average over all probes and θ represents the angle that the absorption dipole of each probe subtends with the fibre axis. This relationship between r* and the distribution of angles θ is derived in Appendix 1. If probes are oriented isotropically or at the magic angle (θ = 54.7°), r* is zero. Negative values of r* indicate probe distributions with average angle greater than 54.7°, and positive values of r* indicate probe distributions with average angle less than 54.7°.

The principle of the method used to detect r was to modulate rapidly the polarization of the light exciting rhodamine fluorescence between orientations parallel and perpendicular to the fibre axis using a photoelastic modulator. The probability of absorption for a particular probe is proportional to cos²(β), where β is the angle between the direction of polarization of the exciting light and the absorption dipole moment of the dye molecule. On the assumption that the quantum yield for fluorescence does not depend on the probe orientation, total fluorescence at any instant is proportional to absorption. Thus, the fluorescence is modulated in a manner related to the distribution of probe angles.

Modulation of the polarization of the fluorescence excitation is implemented by a photoelastic modulator, which acts as a variable retardation plate with principal axis oriented at 45° in the plane of linear polarization of the exciting beam. As shown in Appendix 2, when the phase difference introduced by the photoelastic modulator between the 45° and -45° components of the beam is φ, the intensity of fluorescence is I₁₋ I₄ = 1/2(I₁₊ I₄) cos(φ), where I₁ and I₄ are the fluorescence intensities for exciting light linearly polarized perpendicular and parallel to the fibre axis, respectively. During operation of the photoelastic modulator, φ = 2 sin(2t/fₚ), where t is time in seconds and fₚ is the excitation frequency of the modulator, 42 kHz. Noting the

\[ \cos(2 \sin(\Omega t) - \phi) = 2 \cos(\Omega t) \cos(\phi) \]

where J₁ is the 1st order Bessel function, it follows that the observed fluorescence waveform is composed of an average value plus modulation at even harmonics of 42.1 kHz. The average value is given by L = 1/2(I₁ + I₄) = 1/2J₀(π)(I₁₋ I₄). The amplitude of the 2nd harmonic component, at 84.2 kHz, is D = J₂(π)(I₁₋ I₄).

We define the experimentally measured absorption anisotropy r as (I₁₋ I₄)/(I₁₊ I₄). This quantity was calculated from the measured quantities L and D by:

\[ r = -2(6J₂(\pi)L + (1 - 3J₀(\pi))D) \]

r differs from r*, the theoretical absorption anisotropy, because the experimental apparatus does not collect the entire fluorescence emission from the probes. The effect of the limited collection aperture is discussed in Appendix 3, where it is shown that, at most, a minimal collection aperture causes r to be offset from r* by 0.072, corresponding to about 3° of angular error if the probes are well ordered. The amplitudes and time courses of transients are not significantly affected by the limited collection aperture. The offset is smaller if the probes are disordered or if the numerical aperture of the collection lens is appreciable, as is the case in our experimental set-up. All of the dichroism results are presented quantitatively as r.

(e) Set-up for dichroism measurements

The optical components of the apparatus are shown in Fig. 1. To measure dichroism, fibres were illuminated by a continuous-wave argon ion laser (model 5500A, Ion Laser Technology, Salt Lake City, UT), with output at 514 nm, polarized perpendicular to the fibre axis. A photoelastic modulator (PEM-80 model FSA, Hinds International, Inc., Portland, OR), set to half-wavelength peak retardation and oriented with principal axis 45° from the fibre axis, oscillated the polarization of the beam at 84.2 kHz between orientations parallel and perpendicular to the fibre.

The beam intensity was reduced with an attenuator (model 925B, Newport Research Corporation, Fountain Valley, CA) to avoid photobleaching the dye molecules. The beam was shaped by a ~100 cm focal length spherical meniscus lens and a 30 cm focal length cylindrical lens and directed to the fibre with front surface mirrors. The beam intensity at the fibre was 0.5 to 1 mW. The intensity distribution at the fibre was Gaussian with 1/e² full-widths approximately 3 mm along the fibre axis and 0.5 mm across the fibre. Typically, the entire fibre segment was illuminated.

The fluorescent emission was collected by an aspherical condenser lens 50 cm in diameter, placed 5 cm above the fibre, after spectral filtering by a 1 cm path of 0.1 cm-KCl (O), a 570 nm cut-off filter (Schott Glass type OG570), and a 600 nm interference filter (Melles Griot 03 F1V 046, FWHM 40 nm). The numerical collection aperture was about 0.5, yielding a geometrical collection efficiency of ~13% of the upper hemisphere. The effect of the limited collection angle is discussed in Appendix 3. A small opaque stop blocked the exciting beam from directly entering the fluorescence collection optics, and leakage of 514 nm light scattered by the fibre through the emission filters amounted to less than 0.3% of the typical experimental fluorescence.

Fluorescence emission was detected with a photo-
Figure 1. Experimental set-up for measurement of dichroism during photolysis of caged molecules. The major components are indicated schematically. The muscle fibre is shown end-on in a trough constructed of fused silica windows on the top, bottom and side. The row of components horizontally aligned with the muscle fibre produces an ultraviolet laser pulse for photolysis. The argon ion laser and components through the front surface mirror excite rhodamine fluorescence with modulated polarization. In the actual setup, 3 front surface mirrors direct the excitation beam. The optics and photomultiplier tube above the fibre collect fluorescent emission.

A typical modulated fluorescence signal from the photomultiplier tube is shown in Fig. 2. Increasing fluorescence (trace b) is plotted downward from the thin horizontal line (a), which indicates zero fluorescence. The modulation of fluorescence matches the expected form \( \cos(\pi \sin(2\pi \omega t)) \) as described above and which is plotted with an offset as the lowest trace (c).

The amplitude \( D \) of the 84.2 kHz component of fluorescence modulation, detected with a lock-in amplifier (Ithaco model 3961B, Ithaca, NY), was recorded along with the average intensity \( L \) of the fluorescence. The lock-in amplifier output, which was proportional to the root-mean-square amplitude, was multiplied by \( \sqrt{2} \) to give the peak amplitude \( D \). To verify the calibration of the experimental absorption anisotropy signal, \( r \) values measured using the photoelastic modulator and lock-in amplifier were compared with measurements using static polarizations of the exciting beam. A linear polarizer was used as an analyser in place of the muscle fibre, and the long-pass filters (>570 nm, 580 to 620 nm, and KCr2O4, Fig. 1) were removed to allow detection of the transmitted 514 nm laser light (attenuated to approximately the intensity of fibre fluorescence). With the analyser at 0°, 36° and 90° relative to the usual fibre axis, \( r \) values measured using the photelastic modulator and lock-in amplifier were compared with measurements using static linear polarization of the excitation beam. From the definition of \( r^* \), the theoretical \( r \) values at 0° and 90° analyser positions are 1.0 and -0.5, respectively. These checks indicate that the dynamically measured value of \( r \) approximates the statically measured value to within 7% over the full signal range. No corrections were made to the data for this factor.

The frequency-doubled ruby laser used for photolysis of caged compounds (Fig. 1) was as described (Goldman et al., 1984). Light from the photolysis laser often caused an initial brief transient in the dichroism signal. This transient was probably due to light-scattering, phosphorescence of trough components and saturation of the lock-in amplifier. To avoid this artifact, in some experiments a shutter blocked light to the photomultiplier tube.

Figure 2. Waveform of fluorescence modulation. Increasing fluorescence (b) is plotted downward from the thin horizontal line (a) indicating the dark level. The lowest offset curve (c) is the function \( \cos(\pi \sin(2\pi \omega t)) \), which is the theoretical waveform for the fluorescence signal. Curves (b) and (c) are similar.
for 2 to 3 ms, bracketing the time of the photolysis laser pulse. While light collection was blocked, the direct current and 842 kHz components of the photomultiplier signal were maintained at the lock-in amplifier with an electronic switching circuit. This signal switching caused a 1 to 2 ms delay before the dichroism signal could respond following a laser pulse. The delay can be seen as a flat interval following the laser pulse in the upper trace of Fig. 7. After the 1 to 2 ms dead time, the switching circuit did not affect the dynamic response of the apparatus. The transient response was limited by the output filter of the lock-in amplifier to 3.5 ms, 10 to 90% rise time.

(f) Trough assembly and solution exchange

Solutions were placed in an assembly of stainless steel troughs (Fig. 1) that could conveniently change the medium bathing the fibre. To immerse the fibre in a new solution, the trough assembly was lowered, moved horizontally to position a new trough below the fibre, and then raised; this was accomplished within 3 s by stepper motors. Fibres were immersed in each required solution for at least 2 min. The volume of the troughs was 90 µl except for an optically clear trough which contained 24 µl for photolysis experiments or 90 µl otherwise.

The front and bottom surfaces of the optical trough were made of fused silica, to allow illumination from the ruby and argon lasers, respectively. For fluorescence collection, a fused silica cover was placed on top of the trough to eliminate re-rfraction by a meniscus. The inset in Fig. 1 shows the fused silica trough and, behind it, 1 of 5 solution troughs milled into the stainless steel trough assembly.

(g) Experimental protocols

For studying dichroism changes during the onset of rigor, fibres were transferred from the initial relaxing solution to 0.1 mM-MgATP relaxing solution, and then to rigor solution in the front trough. Optical and mechanical signals were digitized for 5 min at a sampling interval of 300 ms, beginning several seconds before the transfer to rigor solution. The same recording parameters were used for studying dichroism changes during other solution exchanges, as indicated in the text.

The change in dichroism changes upon photorelease of ATP or ADP, fibres were placed in rigor, as described for the slow time base records, and then incubated in the clear trough in rigor solution containing 10 mM-caged ATP or ADP. For experiments with calcium in the photo-lysis medium, an additional wash in calcium rigor solution, and then to rigor medium at 1 mM, a saturating concentration, r changed to +0.046 ± 0.014 (n = 7). The absorption anisotropy in ADP solution corresponds to probe angles of 52.9° if all of the probes are well ordered, or, if half of the probes are completely disordered (isotropic angle distribution) and half are well ordered, 53.4° for the ordered fraction (Burghardt et al., 1983). When ADP was added to the rigor medium at 1 mM, a saturating concentration, r changed to +0.046 ± 0.014 (n = 7). The absorption anisotropy of ADP solution corresponds to probe angles of 52.9° if all of the probes are well ordered, or 53.1° for the ordered fraction if half of the probe molecules are isotropic. Since the anisotropy value changes sign when ADP is added, the change in r cannot be due entirely to a change in the width of the angular distribution or a change in the fraction of ordered probe molecules. Rather, the reversal of the sign of r indicates a change from a probe distribution with average angle greater than 54.7° in rigor without ADP to a distribution with average angle less than 54.7° in the presence of ADP, as reported by Borejdo et al. (1982) and Burghardt et al. (1983). The values of r reported by Burghardt et al. (1983) were 0.209 and 0.119 for the rigor and rigor + ADP conditions, respectively. The values obtained in the present experiments are smaller, due to differences in the preparation or the set-up.

Figure 3 shows tension and the dichroism signal at various ADP concentrations. The upper portion shows a strip-chart recording of r and tension, as the fibre was bathed in rigor solutions with various added concentrations of column-purified ADP.

(h) Data collection and analysis

Mechanical and optical transients were digitized and stored on computer diskettes as previously described (Goldman et al., 1984a). Half-times of the transients were measured from the digitized recordings. Exponential fitting used the Marquardt algorithm (Press et al., 1988). A kinetic model described in Results was fitted to dichroism traces in caged ADP experiments (Fig. 5) using the method of Fletcher & Powell (1963). For comparison of experimentally measured half-times with expectations from the kinetic model, the system of differential equations corresponding to the model was solved numerically using Adams' predictor-corrector algorithm provided within the MLAB mathematical modelling program (Civilized Software, Inc., Bethesda, MD).

3. Results

(a) Steady-state dichroism levels in rigor and with added ADP

The experimentally measured absorption anisotropy, r, in rigor fibres was −0.121 ± 0.013 (mean ± s.e.m., n = 14). This value corresponds to an angle (θ) of 59.8° between the probe absorption dipole moments and the fibre axis if all the probes are well ordered, or, if half of the probes are completely disordered (isotropic angle distribution) and half are well ordered, 53.4° for the ordered fraction (Burghardt et al., 1983). The absorption anisotropy in ADP solution corresponds to probe angles of 52.9° if all of the probes are well ordered, or 53.1° for the ordered fraction if half of the probe molecules are isotropic. Since the anisotropy value changes sign when ADP is added, the change in r cannot be due entirely to a change in the width of the angular distribution or a change in the fraction of ordered probe molecules. Rather, the reversal of the sign of r indicates a change from a probe distribution with average angle greater than 54.7° in rigor without ADP to a distribution with average angle less than 54.7° in the presence of ADP, as reported by Borejdo et al. (1982) and Burghardt et al. (1983). The values of r reported by Burghardt et al. (1983) were 0.209 and 0.119 for the rigor and rigor + ADP conditions, respectively. The values obtained in the present experiments are smaller, due to differences in the preparation or the set-up.

Figure 3 shows tension and the dichroism signal at various ADP concentrations. The upper portion shows a strip-chart recording of r and tension, as the fibre was bathed in rigor solutions with various added concentrations of column-purified ADP.
Dichroism was negative in rigor, and addition of millimolar ADP caused the dichroism to reverse sign. Washing out ADP by subsequently bathing the fibre in plain rigor solution restored the original probe orientation. Dichroism was found to depend on [ADP] hyperbolically (Fig. 3); the average MgADP concentration at half-saturation of the change in dichroism was $10.6(\pm 2.7) \mu M$-ADP (mean $\pm$ S.E.M., $n = 9$).

(b) Tension and dichroism transients upon photorelease of ADP

To study the kinetics of changes in dichroism and rigor tension upon ADP binding, transients were recorded following photolysis of caged ADP. Addition of caged nucleotides to the rigor solution reduced dichroism (see section (g) of Materials and Methods). Tension and dichroism both changed on the millisecond time scale following generation of ADP. The transients in Figure 4 were recorded when $15 \mu M$-ADP (traces labelled (a)), then $85 \mu M$ (traces (b)), and finally $410 \mu M$ (traces (c)) were released successively from caged ADP. The final value of dichroism at approximately $500 \mu M$-ADP released from caged ADP was $0.050(\pm 0.0005)$ ($n = 8$), similar to the value obtained in ADP-rigor without caged ADP.

The tension transients initiated by caged ADP photolysis were typically faster than the dichroism transients, as can be seen in the recordings of Figure 4. When approximately $50 \mu M$-ADP was released from caged ADP and the approach to the steady-state value following the laser pulse was fitted with a single exponential decay, the apparent rate constant obtained for the tension decline was $206(\pm 24) s^{-1}$ (mean $\pm$ S.E.M., $n = 3$), while the rate constant for the dichroism change was $123(\pm 8) s^{-1}$.

The rate at which tension and dichroism approached the new steady-state values increased as [ADP] increased. This behaviour is expected on the basis of the second-order binding kinetics of ADP to actomyosin. Dichroism transients were fitted with transients simulated using the following reaction
Figure 4. Tension and dichroism during photolysis of caged ADP. Top. Strip-chart recording with the fibre starting in rigor. Caged ADP (10 mM) was added at the 1st arrow and the fibre was transferred for the dichroism measurement to the front trough at the subsequent tension spike. The excitation shutter was opened to initiate measurement of dichroism (upper trace) and then 15 μM 85 μM and 940 μM-ADP was released at arrows a, b, c and d. Both tension and dichroism changed on photolysis. At R the ADP and caged ADP were washed out and tension partly recovered. After a control measurement of dichroism the fibre was relaxed at Rel. Bottom. Rapid time base recordings during photolysis trials a, b and c from above. The 3 tension and 3 dichroism traces were recorded during successive laser pulses (arrow) applied successively to the same solution. The thin horizontal lines are zero dichroism (upper) and zero tension (lower). The tension change is faster than the dichroism change.

Scheme 1

\[
\text{Caged ADP} \xrightarrow{k_1} \text{ADP},
\]

\[
\text{ADP} + \text{AM} \xrightarrow{k_{on}} \text{AM} \cdot \text{ADP}.
\]

The rate \(k_1\) of formation of ADP from caged ADP after optical excitation was set to 118 s\(^{-1}\) (Goldman et al., 1984a), and the rates of ADP binding to actomyosin \(k_{on}\) and dissociation \(k_{off}\) were adjusted in the fitting procedure to minimize the squared residuals between the simulated traces and the experimental ones. The rate constants for binding and dissociation of ADP giving the best fit to the traces in Figure 5 were \(k_{on} = 1.74 \times 10^6 \text{ M}^{-1} \text{s}^{-1}\) and \(k_{off} = 84.5 \text{ s}^{-1}\).

Half-times were measured from a series of 17 dichroism traces from nine fibres and compared in Figure 6 (closed symbols) to the half-times expected on the basis of scheme 1. Although the data are scattered, they group in the range of rates corresponding to apparent \(k_{on}\) values of \(3 \times 10^5\) to \(2 \times 10^6\). The open symbols in Figure 6 are from experiments using caged ATP and are discussed later. No difference was found in tension or dichroism traces when caged ADP photolysis was compared in the absence and the presence of 30 μM-free Ca\(^{2+}\) (not shown).

To test whether the transient changes of the dichroism signal following photolysis of caged ADP or caged ATP resulted from binding of liberated nucleotide or from artifactual effects of the ruby laser pulse and/or the by-products of photolysis, we compared photolysis of caged ADP to photolysis of...
caged P_i. The ruby laser pulse and photolysis of caged P_i caused negligible changes in dichroism (Fig. 7). The by-products of photolysis (H^+ and 2-nitroso-acetophenone) are the same with caged ADP, ATP and P_i. Thus the signal deflections on photolysis of caged nucleotides are due specifically to the released nucleotide.

(c) Transients upon photorelease of ATP

To study the changes in probe orientation during cross-bridge detachment and during force development, mechanical and optical transients were recorded following photolysis of caged ATP in the absence or presence of approximately 30 μM-free Ca^{2+}. Upon photorelease of ATP in the presence of Ca^{2+} (Fig. 8), tension initially dropped as cross-bridges detached, and then rose to the active level as cross-bridges reattached and generated force, as reported for unlabelled fibres (Goldman et al.,

Figure 5. Dichroism transients following a series of 5 successive laser pulses (arrow) releasing (a) 14; (b) 70; (c) 70; (d) 160 and (e) 340 μM-ADP from 10 mM-caged ADP. The smooth curves are simulations based on scheme 1 of the text with $k_c = 118 \text{s}^{-1}$, $k_{in} = 1.74 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ and $k_{off} = 84.5 \text{s}^{-1}$. The broken line indicates zero dichroism.

Figure 6. Reciprocal half-times for the change in dichroism following photolysis of caged ADP (filled symbols) or caged ATP (open symbols). The curves show the half-times expected for scheme 1 of the text with $k_c = 118 \text{s}^{-1}$, $k_{eff}/k_{on} = 20 \text{μM}$ and $k_{off}$ set to the value shown next to each curve (in $\text{M}^{-1} \text{s}^{-1}$).

Figure 7. Dichroism transients recorded during photolysis of caged ADP and caged P_i. The arrow indicates the time of the laser pulses. The thin horizontal line indicates zero dichroism. Sarcomere length = 2.66 μm; fibre dimensions = 2.51 mm × 10.700 μm^2.
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Figure 8. Dichroism and tension transients following photolysis of caged ATP. At the arrow 1 mM-ATP was liberated by photolysis of caged ATP. Horizontal lines are baselines for dichroism and tension.

The development of force was appreciably slowed in labelled fibres. This slowing was quantified by measuring the time ($t_{lag}$) between the laser pulse and the inflection point that occurs during tension development and the half-time ($t_{1/2}$) for the remainder of tension development. At approximately 500 μM-released ATP, $t_{lag}$ and $t_{1/2}$ averaged 26.5 (± 4.1) and 365 (± 7.4) milliseconds (mean ± S.E.M., n = 9) in fibres labelled at 60 to 70% of the SH-1 sites according to the decrease in myofibrillar K+-EDTA ATPase activity. The corresponding $t_{lag}$ and $t_{1/2}$ values expected from unlabelled fibres are 16 and 12 milliseconds, respectively (Dantzig et al., 1991). Thus, the labelled fibres activated more slowly than did unlabelled ones. This slowing was variable, however, and did not correlate with the extent of labelling. For instance, a batch of labelled fibres that showed only 18% reduction of myofibrillar K+-EDTA ATPase activity relative to unlabelled fibres, corresponding to 18% of SH-1 sites labelled, gave very long $t_{lag}$ and $t_{1/2}$ values of 580 (± 17.1) and 624 (± 15.3) milliseconds (n = 5), respectively.

Upon photorelease of ATP, the sign of the dichroism promptly reversed, as occurred with ADP. The major change in the dichroism preceded tension development markedly (Fig. 8). The initial response to ATP was similar in magnitude to that following photorelease of ADP (Fig. 9). At low ATP concentrations there was little change in dichroism during the subsequent rise in tension, but when more than approximately 500 μM-ATP was released, dichroism decayed from its maximum value to 86 (± 3)% (n = 11) of the peak (Fig. 10). The level of dichroism in contracting fibres ($r = 0.030 (± 0.005$; n = 6) was intermediate between the values obtained in rigor fibres with ADP and that of relaxation, as reported by others (Borejdo et al., 1982).

The major change in dichroism following ATP release was virtually complete before the onset of tension development. At approximately 500 μM-released ATP, the time for dichroism to reach halfway to the peak signal was 12.5 (± 0.8) milliseconds (n = 14), much faster than $t_{lag}$ or $t_{1/2}$ of the tension traces (Figs 8 to 10). As with ADP, the rate at which dichroism approached the new steady-state values increased as [ATP] increased. The reciprocal half-times for a series of fibres are plotted against released ATP concentration in Figure 6 (open symbols). Comparison with the curves plotted using scheme 1, with ATP replacing ADP, shows that the apparent second-order rate constant of ATP binding to the rigor cross-bridges is $2 \times 10^{5}$ to $5 \times 10^{5}$ M$^{-1}$ s$^{-1}$. Half-times of the dichroism signal were somewhat slower following ATP release than following ADP release at equivalent concentrations (Fig. 6). The faster response to caged ADP photorelease was obtained when a comparison was made in

Figure 9. Dichroism and tension transients following photolysis of caged ATP (a) in the presence and absence of 30 μM-free Ca$^{2+}$ and photolysis of caged ADP ((b) zero Ca$^{2+}$). The amount of photoreleased nucleotide was estimated to be 350 μM in all 3 cases. Horizontal lines are baselines for dichroism and tension. Sarcomere length = 2.92 μm; fibre dimensions = 3.12 mm x 4200 μm$^2$. 
individual fibres and is therefore not due to sample variability. The difference between the two nucleotides may be genuine; it may result from a contribution of the ADP dissociation rate to the observed kinetics; or it may be due to slowing of ATP association caused by weak binding of caged ATP to rigor cross-bridges (Dantzig et al., 1989a).

When ATP was photoreleased in the absence of calcium, the initial change in dichroism was again similar to that upon photorelease of ADP (Fig. 9(a)). With subsequent relaxation, the dichroism decayed toward zero but was maintained at a small positive value (\(+0.011 \pm 0.004; n = 7\) after the fibre relaxed mechanically. This value in the presence of caged ATP is similar to the value (\(+0.018 \pm 0.004; n = 3\) measured in relaxing solution without caged ATP.

(d) Transients following removal of nucleotide

To complement the photolysis experiments in which nucleotides were suddenly introduced into the filament lattice, dichroism transients were studied upon removal of nucleotides. The events took place on a much slower time scale. When a fibre was transferred from a 0.1 mM-MgATP relaxing solution to rigor solution without ATP, development of rigor tension proceeded in several phases. A several second delay was followed by sudden rise to a peak tension and then a partial decay lasting several minutes (Fig. 11). Labelled fibres developed rigor tension more rapidly than did unlabelled fibres. The initial delay phase before the main tension development was curtailed when fibres were labelled.

When ATP was removed, the time course of development of the rigor dichroism signal was markedly slower than the tension increase, with most of the dichroism change occurring after the peak of tension. In standard rigor solution with 1 mM-free Mg\(^{2+}\), the half-time for tension development was 9.8(\(\pm\)0.7) seconds \((n = 18)\), whereas half-time for dichroism change was 46.9(\(\pm\)2.9) seconds.

Since MgADP and MgATP bind to the rigor cross-bridges and the binding might slow diffusion of nucleotides out of the fibre, we tested whether reduction of the free Mg\(^{2+}\) concentration would affect the time course of tension and dichroism when the fibre went into rigor. Both tension development and the change of dichroism upon removal of ATP markedly accelerated when the free [Mg\(^{2+}\)] in the rigor solution was reduced (Fig. 12). Nevertheless, at all free magnesium concentrations tested, tension development preceded the dichroism change (Fig. 12). These results suggest that MgADP formed by hydrolysis of MgATP can exit the fibre more rapidly when it is not bound to magnesium. We confirmed the observation of Borejdo et al. (1982) that magnesium is required for the reversal of the dichroism signal by ADP (not shown).
Figure 11. Dichroism (upper trace) and tension changes when a fibre was transferred at the time indicated by the arrow from relaxing solution to rigor solution in the transparent optical recording trough. Before the transfer, the upper trace is zero because the fluorescence excitation is blocked by the trough changer until the fibre is transferred to the transparent trough. The positive dichroism signal after the time of the arrow is near the average relaxed level. The tension signal changes faster than dichroism. Sarcomere length = 2.57 µm; fibre dimensions = 2.45 mm x 3300 µm².

To test further the hypothesis that the slowness of the dichroism change upon ATP removal is caused by restricted diffusion of MgADP out of the fibre, we compared removal of ATP with removal of ADP. Although the tension changes are very different in these two situations (Fig. 13(a) and (b)), the time course of the dichroism change was similar upon removal of ATP and ADP, except that the initial dichroism was larger in ADP. As with removal of ATP from the bathing medium, the change in dichroism upon removal of ADP was faster when free Mg²⁺ was reduced (Fig. 13(c)). In contrast to the slow dichroism change upon removal of ADP, the change upon diffusing 1 mM-MgADP into the fibre was prompt (Fig. 13(d) and (e)). The dichroism change obtained when the fibre was transferred from rigor solution to rigor solution containing 1 mM-ADP and 1 mM-free Mg²⁺ was typically complete within approximately ten seconds. These results are compatible with the hypothesis that the dichroism signal reports nucleotide binding to myosin heads and that reduction of the nucleotide concentrations within fibres to values well below their dissociation constants requires several minutes, because binding of Mg-nucleotide to the heads slows its diffusion out of the fibre.

4. Discussion
(a) Correlation of mechanical and orientational transients

Recording of orientation of fluorescent probes on the myosin head during rapid activation of muscle fibres initiated by photolysis of caged ATP provides a test for the temporal correlation between rotations of the myosin head and force development. Borejdo et al. (1982) and Burghardt et al. (1983) previously reported that the orientation of IATR bound by an acetamide linkage to the fast-reacting sulphydryl group on the myosin heavy chain (i.e. SH-1 or Cys707) varies between relaxed, active and rigor states and that binding of ADP to the rigor cross-bridge causes a substantial rotation. The same probe was used here in conjunction with photolysis of caged ATP and caged ADP to determine the kinetics of the motions. Although our steady-state r values are smaller, our results confirm the previous reports for steady-state conditions that the values for r are related as rrigor < ractive < rrelaxed. However, in transient situations, either using laser photolysis of caged nucleotides, or else when ATP and ADP were allowed to diffuse into or out of the fibres, our results show that the major changes in probe angle do not occur during force-generating cross-bridge transitions. Rather, the angle changes were mainly correlated with binding or desorption of nucleotides.

Following photogeneration of ATP, the dichroism change preceded force production (Figs 8 to 10), whereas, upon removal of ATP, the dichroism change lagged behind force production (Figs 11 to 13). In both cases, the major probe motions were temporally separated from the development of force. The change in dichroism following photolysis of caged ATP was similar in amplitude (Fig. 9) and time course (Fig. 6) to that following photolysis of
Figure 12. (a) Dichroism and tension changes when a fibre was transferred from relaxing solution with 1 mM-free Mg$^{2+}$ to rigor solution with various free Mg$^{2+}$ concentrations. Sarcomere length = 2.57 μm; fibre dimensions = 245 mm x 3300 μm$^2$. (b) Half-times for the changes in dichroism (filled symbols) and tension (open symbols) from the records shown in (a) and other similar experiments. For [Mg$^{2+}$] = 0.01 and 1, average half-times measured for 5 and 18 trials, respectively, are plotted, along with error bars showing S.E.M. for dichroism half-times; for tension half-times, error bars are smaller than the symbols. For other Mg$^{2+}$ concentrations, individual half-times are plotted, with different symbol shapes for different fibres.

caged ADP. Likewise, the change in dichroism during the onset of rigor from relaxation (Figs 11 to 13) was similar to that during washout of ADP (Fig. 13). Addition or removal of ADP in rigor produced only small changes in force (~5% of active tension). These results indicate that the dichroism change can occur either before or after a force transient, and when there is little change in force. In each case, the major angle change of the probe occurred as the ATP or ADP bound or dissociated. Therefore, orientation of the rhodamine probes on SH-1 appears to report mostly structural changes linked to nucleotide binding and release rather than to the force-generating transition.

(b) Nucleotide-dependent structural changes in myosin heads

Three-dimensional reconstructions of thin filaments decorated with myosin subfragment-1 (S1)
Figure 13. (a) and (b) Dichroism and tension changes following (a) transfer of a fibre from relaxing solution to rigor solution and (b) transfer of a fibre from a rigor solution containing MgADP to a rigor solution lacking ADP. Free [Mg$^{2+}$] was 1 mM throughout (a) and (b). (c) Dichroism and tension changes following transfer of a fibre from a rigor solution containing MgADP and 1 mM-free Mg$^{2+}$ to a rigor solution lacking ADP with or without 1 mM-free Mg$^{2+}$. (d) and (e) Dichroism and tension changes following transfer of a fibre from (d) a rigor solution containing MgADP to a rigor solution lacking ADP or from (e) a rigor solution lacking ADP to one with 1 mM-ADP. (a), (b), (d) and (e) Sarcomere length = 2.81 μm; fibre dimensions = 302 μm x 3200 μm$^2$; (c) sarcomere length = 2.92 μm; fibre dimensions = 2.71 mm x 4600 μm$^2$.

Biotinylated at Cys707 have shown that Cys707 (SH-1) is very near the actin binding site of myosin (Toyoshima & Wakabayashi, 1985). Peptide fragments corresponding to the region of the myosin heavy chain near Cys707 cause inhibition of actomyosin ATPase activity and influence force development by fibres, suggesting that the region near Cys707 participates in actin binding (Suzuki et al., 1990; Keane et al., 1990). The small amplitude of the probe motion during force generation may be
due to the proximity of Cys707 to actin. Probes at myosin sites more distal to actin could then detect substantial motions correlated with force development.

The nucleotide binding site of myosin is on the opposite side of the myosin head, approximately 14 nm from the actin binding site and 4 to 5 nm from Cys707 (Tokunaga et al., 1987). Cys707 is approximately 7 nm from the tip of the head (Sutoh et al., 1984) and the nucleotide site is approximately 5 nm from the tip (Tokunaga et al., 1987). Since nucleotide binding has a small influence on actin binding, it is not surprising that binding of nucleotides is communicated across the 4 to 5 nm distance to Cys705. An iodocetamide spin label on Cys707 has been shown to change its mobility when ADP or ATP binds to myosin in solution (Seidel et al., 1970; Barnett & Thomas, 1987). Batts et al. (1989) have reviewed the many other studies showing nucleotide-dependent changes of intersite distances in S1, such as Cys707 to Cys697 (SH-2).

A number of fluorescent and spin label probes attached to Cys707 have been found to change orientation by smaller amounts (1 to 5') than the 7.2°22 shift obtained with IATR when ADP is added to a rigor fibre (for a review, see Fajer et al., 1980a). Miyashiki & Borejdo (1989) observed ADP-dependent rhodamine orientation when the probe was bound to Cys707, but not when the probe was bound to the nearby residue Cys697. Other physical techniques have generally not identified large cross-bridge structural changes associated with ADP binding to rigor cross-bridges. A small, and partly reversible, tension decline on addition of MgADP to rigor fibres was estimated to correspond to approximately 0.32 nm of filament sliding (Dantzig et al., 1991). If this were to be caused by a rigid axial rotation of the 19 nm myosin head, the angular change would be only 0.6°. Similarly, optical birefringence (Ohnora & Irving, 1989) and X-ray diffraction (Rodger & Tregear, 1974), techniques that should be sensitive to global changes in head orientation and shape, did not detect substantial motions of the head when ADP bound in rigor. These results are consistent with an ADP-induced local, but not global, change of orientation within the cross-bridge near Cys707. Indeed, Yeung and colleagues (Wells et al., 1980; Huston et al., 1988) using agents to cross-link Cys697 and Cys707, demonstrated that the region containing these thiol groups can move at least 1 nm under the influence of nucleotide binding.

(c) Kinetics of the dichroism changes

Upon photolysis of caged ADP, tension changed about five milliseconds earlier than dichroism (Fig. 4). A possible explanation of this finding may be that rhodamine reports a molecular motion that occurs following the binding of ADP and the tension decrease. Alternatively, the ADP binding rate might be strain-dependent and, within the distribution of strains among the rigor cross-bridges, those at high strain, which contribute most heavily to tension, are the ones that bind ADP the most rapidly. Finally, it is possible that labelled cross-bridges bind nucleotides more slowly than do unlabelled ones, so the tension change, indicating the overall nucleotide binding rate, is more rapid than the optical signal. There are several findings that make a slow slowing of nucleotide binding to labelled heads unlikely. The apparent second-order rate for binding of ATP and ADP (2 × 10⁻⁵ to 5 × 10⁻⁵ s⁻¹ and 10⁻⁵ s⁻¹, respectively. Fig. 6) are in the range previously found for nucleotide binding from mechanical measurements with caged nucleotides in unlabelled fibres (Goldman et al., 1984a,b; Dantzig et al., 1991). Borejdo et al. (1985) directly compared ATP-induced dissociation of unlabelled S1 from actin with that of S1 labelled with IATR at Cys707. They reported that labelling does not alter the rate of ATP-induced dissociation.

The amplitude of the dichroism transient observed on photolysis of caged ADP was very similar to that obtained with ATP release in either the presence or the absence of Ca²⁺ (Fig. 9). The similar kinetics and amplitude suggest that the structural changes leading to the dichroism transient are similar with the two nucleotides. This conclusion is compatible with the symmetrical ATPase cycle proposed by Rosenfeld & Taylor (1984) based on the kinetics of interaction of acto-subfragment 1 and ATP and ADP. In that model, the structural changes accompanying substrate binding are reversed on product release.

When relaxed muscles are activated electrically, the stiffness of the muscle and parts of the X-ray diffraction pattern change earlier than tension development (Ford et al., 1986; Kress et al., 1986). The early events are interpreted by most investigators to indicate the shift from detached to attached populations of cross-bridge before the force-generating transition. The time scale of the transition into the force-generating state, estimated from the delay between stiffness and tension increase, is approximately 15 milliseconds in electrically stimulated intact frog muscle fibres at 0 to 2°C and approximately five milliseconds in experiments with rabbit psoas fibres activated from relaxation at 0°C by photolysis of caged Ca²⁺ (Barsotti et al., 1989). Since the dichroism signal changes before tension development in the caged ATP experiments, we should consider whether the dichroism signal is reporting a population of the same initial attached intermediate that forms upon activation from the relaxed condition.

Several characteristics distinguish activation from rigor and from relaxation. In the caged ATP experiment, the cross-bridges are already attached and Ca²⁺ is present before the photolysis pulse, whereas in electrical activation, the muscle is relaxed and nucleotide is bound before stimulation. With rabbit psoas fibres at 0°C, when caged ATP photolysis from rigor was directly compared with
eaged Ca\(^{2+}\) photolysis from relaxation, the initial
tension development was 10 to 20 milliseconds
slower in the eaged ATP experiment (Barsotti et al.,
1989), with a lag phase presumably due to the time
required for binding and hydrolysis of ATP
(Goldman et al., 1984b; Dantzig et al., 1991). The
reversal of the dichroism signal is essentially
complete during this lag period, and the delay
between the half-completion points of the dichroism
and tension signals was approximately 50 milli-
seconds (Figs 8 to 10). Thus, the change in
dichroism upon binding of ADP or ATP does not
seem to signal attachment of cross-bridges into pre-
force configurations that become force-generating
intermediates on the 5 to 15 millisecond time scale.

Though the structural change reported by rhodamine
dichroism occurs prior to force generation in the
reaction scheme, this change may nevertheless
be an important prerequisite for force generation.
Botts et al. (1984) have suggested that Cys707 may
be a site along the route of communicating struc-
tural changes from the nucleotide binding site to
actin.

(d) Transients following onset of rigor or removal
of ADP

When ATP was removed from the bathing
medium, the onset of rigor tension was strikingly
faster than the change of dichroism. Several
minutes of incubation time were required to achieve
the final dichroism level when ATP or ADP was
removed from the medium (Figs 11 to 13). Three
possible explanations should be considered: (1) slow
release of the hydrolysis product ADP from
the cross-bridges; (2) slow diffusion of ADP out of the
fibre. (3) slow rearrangement (annealing) of some
rigor cross-bridges from the actin-binding sites
that initially occupied to a final, lower energy,
distribution. This possible annealing process
would explain the decline in tension that occurs for
several minutes following attainment of the peak rigor
tension (Figs 11 to 13). However, dichroism also
changed slowly on the several minute time scale
when ADP was washed out of a rigor fibre, a situation
not accompanied by annealing. Thus, explanation
(3) for the slow time course of dichroism change can be eliminated. Removing Mg\(^{2+}\) accelerates the
dichroism change when either ATP or ADP is
removed from the medium, ruling out possibility
(1).

On the basis of this evidence, it appears that, at
normal Mg\(^{2+}\) concentrations, ADP diffuses out of
the fibre slowly because it binds to, and dissociates
from, cross-bridges during the diffusion. The
restricted diffusion hypothesis (explanation (2))
would predict that the dichroism change when ADP
diffuses into a nucleotide-free fibre would be faster
than that when ADP is washed out of a fibre, as
found experimentally (Fig. 13(d) and (e)).

(e) Lack of concerted probe rotation during
force generation

Following photolysis of eaged ATP, the dichroism
signal reached a peak positive value and then
decayed somewhat toward zero. This decay was
more prominent in the absence of Ca\(^{2+}\) than in its
presence (Fig. 9(a)) and was more prominent at
higher ATP concentrations (Fig. 10). These results
suggest that the secondary decay of dichroism is
caused by cross-bridge detachment, since myosin
heads are highly disordered when detached (Thomas
& Cooke, 1980), and isotropic components of the
orientational distribution would contribute zero
dichroism. The time course of the dichroism during
relaxation in the absence of Ca\(^{2+}\) is similar to the
final tension relaxation, consistent with assignment
of the decay of dichroism to cross-bridge detach-
ment. In the presence of Ca\(^{2+}\), however, cross-
bridge detachment is nearly complete and tension
development is proceeding while most of the decay
occurs in dichroism.

At high ATP concentrations (>500 \(\mu M\)), the
decay amounted to a change in the anisotropy, \(r\), of
10 to 20\(^{\circ}\) of the signal observed on binding ATP.
This change in dichroism during the onset of tension
development at high ATP concentrations would
be expected to correspond to an angle change of the
probe absorption dipole moments of \(\leq 2^{\circ}\) relative to the fibre axis if all the probes are well
ordered, or, if half of the probes are completely
disordered, \(\angle 4^{\circ}\) for the ordered fraction. At lower ATP concentrations the
apparent change in dichroism and probe angles is
less after the early reversal (Fig. 10).

The model that force is produced by a rotation of
myosin heads has generated considerable contro-
versy. Although there is evidence for conformation-
tional changes in actomyosin during ATPase
activity, no concerted rotation of the head has been
demonstrated during the force generating transition
(Cooke et al., 1984; Cooke, 1986; Thomas, 1987). The
dichroism signal is sensitive enough to detect axial
angle changes expected from the rotating cross-
bridge model. However, the change in dichroism
during force development triggered by photolysis of
eaged ATP is smaller than what would be expected on
the basis of a power stroke comprising a concerted
tilting motion of rigid myosin heads. This result is
compatible with models in which force generation is
not associated with cross-bridge rotation.

On the other hand, there are several possible
explanations for this finding that are compatible
with the tilting cross-bridge model. It is possible
that labelling the cross-bridge at Cys707 prevents
them from undergoing normal structural changes.
This possibility might be tested rigorously by label-
ing other sites. Modification of Cys707 has been
shown to partially inhibit actin-activated ATPase
activity of myosin heads (Svensson & Thomas,
1986; Titus et al., 1989; Root et al., 1991). However,
fibres with up to 75\(^{\circ}\) cross-bridges labelled have
been reported to generate normal tension (Crowder
& Cooke, 1984).
The probe might be placed in an unfavourable position or orientation to detect the crucial change in angle. An attached, pre-force state might be so short-lived during the onset of activation in our experiment that it does not contribute substantially to the observed signal. Faster activation at higher ATP concentrations or using aged Ca++ would address this point.

Another possible explanation for the lack of substantial probe rotation during force development is that cross-bridges may become disordered upon ATP binding, obscuring any changes in orientation during force generation. Both electron paramagnetic resonance (Cooke et al., 1982; Barnett & Thomas, 1989; Fajer et al., 1990a) and phosphorescence anisotropy studies (Stein et al., 1990) of fibres labelled at Cys707 have shown a high degree of dynamic orientational disorder among cross-bridges attached during contraction. Disorder is consistent with the small value of dichroism in relaxing solutions and the somewhat greater value in contraction. Although some of the disorder in contracting fibres could arise from detached cross-bridges (Barnett & Thomas, 1984), studies on spin-labelled S1 in solution at high actin concentration have shown that S1 undergoes microsecond rotational motions bound to actin in the presence of ATP (Berger et al., 1989). Whether such attached and mobile cross-bridges are capable of generating force or are in a pre-force state is not known. Nevertheless, the angular range of submillisecond motions detected by phosphorescence anisotropy of probes on attached heads during contraction is 40 to 50° (Stein et al., 1990), a value compatible with the tilting cross-bridge model. Rapid rotations among multiple attachment angles over this range might help to explain not only the spectroscopic probe data but also the rapid force recovery observed in quick length-step experiments (Huxley & Simmons, 1971). Although our results strongly indicate that the major angle change of rhodamine on Cys707 is spectroscopic probe rotati- the absorption dipole angles. All signals nucleotide binding rather than the force-generating transition of the cross-bridge cycle. The absorption anisotropy is proportional to $\cos^2(\theta)$, while $A_\perp$ is proportional to $\frac{1}{2}\sin^2(\theta) = \frac{1}{2} - \frac{1}{2}\cos^2(\theta)$. Given any normalized distribution density for probe dipole angles, $n(\theta)$, and taking into account that the area in spherical co-ordinates occupied by an incremental angle $d\theta$ is $\sin(\theta)\ d\theta$, the total absorptions will be:

$$A_\parallel = A_0 \int_0^{\pi} n(\theta) \cos^2(\theta) \sin(\theta) \ d\theta,$$

and:

$$A_\perp = A_0 \int_0^{\pi} n(\theta)\frac{1}{2} - \frac{1}{2}\cos^2(\theta) \sin(\theta) \ d\theta,$$

where $A_0$ is a constant taking into account absorptivities, probe concentration and intensity of the excitation. The absorption anisotropy $r^*$, defined as $(A_\parallel - A_\perp)/(A_\parallel + 2A_\perp)$, is then:

$$r^* = \frac{-1}{1 + \frac{3}{2}\cos^2(\theta)}.$$  

where the $\langle \rangle$ notation denotes the average over all probes.

### Appendix 2

Here, we describe the variation of probe absorption and total fluorescence as the photoelastic modulator introduces a phase difference between two orthogonally polarized components of incident light. A dichroic sample is illuminated with a light beam, initially polarized perpendicular to the sample’s primary axis, passing through a photoelastic modulator oriented at 45°. Let $\mathbf{i}$ and $\mathbf{j}$ denote the directions perpendicular and parallel to the sample’s primary axis, respectively, and let $A_\parallel$ and $A_\perp$ be the absorptions by the sample of incident light polarized in those respective directions. The unit vector representing the initial electric vector of the light can be decomposed into components parallel and perpendicular to the photoelastic modulator’s axis:

$$i \sin(\omega t) = \left[\left(\mathbf{i} + \mathbf{j}\right) + \frac{1}{2}\left(\mathbf{i} + \mathbf{j}\right)\sin(\omega t)\right].$$

The photoelastic modulator acts as a retardation plate, introducing a phase difference $\phi$ into the latter of these components, so that the beam emerging from it has unit electric vector:

$$\left\{ \frac{1}{2}\sin(\omega t) + \frac{1}{2}\sin(\omega t + \phi) \right\}.$$
The unit electric vector has two components out of perpendicular to the sample axis and the other of product of the electric vector with the absorption dipole moment. Absorption is proportional to the square of dot magnitude $\sin(\phi/2)$ parallel to the sample axis. The fluorescence is $I_{11} + I_{1} = I_{11} - I_{11}$ and $I_{11} + I_{11} = I_{11} + I_{11}$ respectively, where, following customary notation, the angles, with the limiting case in which only the optical axis (the same direction as the excitation) is collected. The actual experimental set-up, with appreciable collection aperture, is between those extremes. For the case of strictly on-axis collection, the fluorescence intensities for parallel and perpendicular excitation are $I_{11} = I_{11} = I_{11}$ and $I_{11} = I_{11} = I_{11}$ (eqns (5) and (6) of Tregear & Mendelson (1975)). Thus, for an array of probes containing a disordered component, the corrections would be even smaller than those given in the previous paragraph for the case of one probe angle. Likewise, if a probe distribution has some width, the correction for limited collection aperture is smaller than for a single angle. For instance, with a Gaussian distribution of probe angles with $10^\circ$ width, the difference between $r^*(\theta)$ and $r(\theta)$ is at most $0.045$, compared to $0.045$ for a single angle.

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Appendix 3

Here, we discuss the effect of the limited light collection aperture on the measured dichroism signal. We compare the ideal dichroism measurement, in which the fluorescence is collected from all angles, with the limiting case in which only the fluorescence propagating along the detector’s optical axis (the same direction as the excitation) is collected. The actual experimental set-up, with appreciable collection aperture, is between those extremes. For the case of strictly on-axis collection, the fluorescence intensities for parallel and perpendicular excitation are $I_{11} = I_{11} = I_{11}$ and $I_{11} = I_{11} = I_{11}$ respectively, where, following customary notation, the first and second subscripts denote the directions of polarization of excitation and emission, respectively. Assuming that the absorption and emission dipoles are parallel and that the probes are immobile during the excited lifetime so the absorption and emission dipoles are colinear at angle $\theta$ from the fibre axis, equations (1) to (4) of Tregear & Mendelson (1975) show that $r_1(\theta) = r^*(\theta)$ and $r_1(\theta) = r^*(\theta)$. The detected “absorption anisotropy” at limiting low collection aperture, defined as:

$$r_1(\theta) = \frac{\cos^2(\theta) - \frac{1}{2} \sin^2(\theta)}{\cos^2(\theta) + \frac{1}{2} \cos^2(\theta) \sin^2(\theta) + \frac{1}{2} \sin^4(\theta)}.$$


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