Observation of Two Orientations from Rigor Cross-Bridges in Glycerinated Muscle Fibers†

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Received February 5, 1986; Revised Manuscript Received April 29, 1986

ABSTRACT: The fluorescence polarization from rhodamine labels specifically attached to the fast-reacting thiol of the myosin cross-bridge in glycerinated muscle fibers has been measured to determine the angular distribution of the cross-bridges in different physiological states of the fibers as a function of temperature. To investigate the fibers at temperatures below 0 °C, we have added glycerol to the bathing solution as an antifreezing agent. We find that the fluorescence polarization from the rhodamine probe detects distinct angular distributions of the cross-bridges in isometric-active, rigor, MgADP, and low ionic strength relaxed fibers at 4 °C. We also find that the rigor cross-bridges in the presence of glycerol can maintain at least two distinct orientations relative to the actin filament, one dominant at temperatures $T > 2$ °C and another dominant at $T < -10$ °C. MgADP cross-bridges in the presence of glycerol maintain approximately the same orientation for all temperatures investigated. The rigor cross-bridge orientation at $T < -10$ °C is similar to both the MgADP cross-bridge orientation in the presence of glycerol and the active muscle cross-bridge orientation at 4 °C. These findings show that the rigor cross-bridge in the presence of glycerol has at least two distinct orientations while attached to actin: one of them dominant at high temperature, the other dominant at low temperature or when MgADP is present. The latter orientation resembles that present in isometric-active fibers. These findings suggest that force generation in the activated cross-bridge cycle may occur as a result of an actin-attached cross-bridge transition between these two orientations.

The covalent fluorescent probe (iodoacetamido)tetramethylrhodamine (IATR) specifically modifies the fast-reacting thiol (SH-1) of the myosin cross-bridge in glycerinated rabbit psoas muscle fibers (Borejdo et al., 1979) without altering the fibers' ability to produce calcium-activated tension (Burghardt et al., 1983, 1984). By detecting the polarized fluorescence emission as a function of the excitation light polarization, we can obtain distinctive features of the probe angular distribution and from them infer changes in the cross-bridge angular distribution when the muscle fiber changes its physiological state (Burghardt, 1984).

We have reported previously that the probe angular distribution for IATR-labeled cross-bridges in rigor is unambiguously distinguishable from the distribution from cross-bridges in the presence of MgADP (Borejdo et al., 1982; Burghardt et al., 1983). From these data we concluded that the rigor cross-bridges maintain a different angular distribution from that maintained in the presence of MgADP. This work was performed at room temperature.

We report here data from fluorescence polarization measurements on IATR-labeled cross-bridges in muscle fibers that are used to determine the probe angular distribution as a function of temperature for various physiological states of the fiber. The measurements were obtained from fibers incubated in solutions with 50% glycerol to protect the fibers from freezing. We find that at room temperature the probe angular distribution for cross-bridges in rigor plus glycerol is unambiguously distinguishable from that distribution for cross-bridges in the presence of MgADP (MgADP cross-bridges at this temperature have the same angular distribution of probes whether glycerol is present or not). From this observation we again conclude that the cross-bridge maintains a different angular distribution in rigor plus glycerol compared to that maintained in the presence of MgADP. We also find that, in the presence of glycerol and at low temperature ($T < -10$ °C), the probe angular distribution when the fibers are in rigor is perturbed from its shape at $T > 2$ °C such that the dominant peak is very similar to that maintained in the presence of MgADP.

There is evidence suggesting that in rigor fibers the cross-bridges maintain at least two orientations relative to the fiber axis (Burghardt & Thompson, 1985a; Burghardt & Ajtai, 1986a, 1986b; Taylor et al., 1984). Experiments using NMR on subfragment 1 of myosin (S-1) have also indicated the existence of two different conformations in a temperature-dependent equilibrium (Shriver & Sykes, 1982).

On the basis of these previous observations, the following interpretation of our data is plausible. The observed cross-bridge orientations in rigor fibers are in an equilibrium that can be perturbed by the presence of both low temperature and glycerol. Of the observed cross-bridge orientations in rigor cross-bridges, some of them are very similar to the MgADP cross-bridge orientation since this orientation is nearly achieved by the rigor cross-bridge in the presence of glycerol at $T < -10$ °C.

Analysis, Materials, and Methods

Fluorescence Polarization. For our purposes the information from the fluorescence polarization measurements can be summarized by the ratios $P_\parallel$ and $P_\perp$ such that

$$P_\parallel = (F_{\parallel,\parallel} - F_{\parallel,\perp})/(F_{\parallel,\parallel} + F_{\parallel,\perp}) \quad (1a)$$

$$P_\perp = (F_{\perp,\perp} - F_{\perp,\parallel})/(F_{\perp,\perp} + F_{\perp,\parallel}) \quad (1b)$$

where $\parallel$ and $\perp$ mean parallel and perpendicular, respectively, to the fiber axis and F is the emission light intensity observed at different polarizations of the excitation (first subscript) and

† This work was supported by U.S. Public Health Service Grant HL-16683 and by the Muscular Dystrophy Association.
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0006-2960/86/0425-6203$01.50/0

emission (second subscript) light. A third ratio formed from an independent combination of the fluorescence intensities in eq 1 is redundant because the fiber is known to be unchanged upon rotation about the fiber axis [i.e., to have azimuthal symmetry; see Burghardt et al. (1983)] and because the rhodamine probe has collinear absorption and emission dipoles at the excitation and emission wavelengths used here.

The model-independent treatment of steady-state fluorescence polarization from extrinsic fluorescent probes in biological assemblies leads to a series of equations restricting the probe order parameters (Burghardt, 1984). For IATR-labeled cross-bridges it is easily shown that the quantities $P_\parallel$ and $P_\perp$ are related to the probe order parameters by the equations

$$0 = (2/15)(1 - 2P_\parallel) + \sqrt{8\pi^2/5}a^2_{0,0}\,[(11/21) - (13/21)P_\parallel] + (12/35)\sqrt{8\pi^2/9}a^4_{0,0}\,(1 - P_\parallel/3) \quad (2a)$$

$$0 = -(2/15)P_\perp + \sqrt{8\pi^2/5}a^2_{0,0}\,[-(1/7) + (1/21)P_\perp] + (1/35)\sqrt{8\pi^2/9}a^4_{0,0}\,(5 + 3P_\perp) \quad (2b)$$

where the $a^l_{m,n}$'s are order parameters determining the steady-state angular distribution of probes. If this distribution is given by $N(\beta)$, where β is the polar angle of the probe relative to the fiber axis, then

$$N(\beta) = \sum_{l=0}^{\infty} \sqrt{\frac{2l+1}{8\pi^2}}\, a^l_{0,0} P_l(\beta) \quad (3)$$

where $P_l(\beta)$ is a Legendre polynomial. Using our fluorescence polarization data, we may solve for the order parameters $a^2_{0,0}$ and $a^4_{0,0}$. $a^0_{0,0}$ is determined by the normalization of $N(\beta)$, which we choose to be $\int_0^\pi d\beta \sin\beta\, N(\beta) = 1/(4\pi^2)$ such that $a^0_{0,0} = [1/(8\pi^2)]^{1/2}$. In our plots of $N(\beta)$ referred to later we neglect all order parameters other than $a^0_{0,0}$, $a^2_{0,0}$, and $a^4_{0,0}$.

The fluorescence polarization measurements were made on a SLM 8000 spectrofluorometer (Urbana, IL) equipped with Glan–Thompson polarizers using single fibers mounted on a stainless steel holder that fits in a cuvette as described previously (Burghardt & Thompson, 1985b). The excitation wavelength was 520 nm and emission was collected at 590 nm.

Chemicals. ATP, ADP, P^1,P^5-diadenosine 5′-pentaphosphate (Ap_5A), myokinase, and hexokinase were from Sigma (St. Louis, MO). (Iodoacetamido)tetramethylrhodamine (IATR) was from Research Organics (Cleveland, OH). All chemicals were of analytical grade.

Solutions and Fiber Preparations. Rigor solution was 80 mM potassium chloride, 5 mM magnesium chloride, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 5 mM sodium phosphate, and 0.1 mM dithiothreitol (DTT), pH 7.0.

Relaxing solution had the same composition as rigor solution except that ATP was added at 4 mM concentration.

In activating solution, 0.1 mM calcium chloride replaced the EGTA of the relaxing solution, and 4 mM phosphocreatine and creatine kinase (0.4 mg/mL) were added to regenerate hydrolyzed ATP.

In MgADP or magnesium adenosine 5′-(β,γ-imidotriphosphate) (MgAMP-PNP) containing solution, 4 mM ADP or 2 mM AMP-PNP was added to the rigor solution, together with 10 mM glucose and hexokinase (100 μg/mL) to remove contaminating ATP, and 100 μM Ap_5A was added to inhibit myofibrillar myokinase from converting ADP to ATP.

In low ionic strength relaxing solution, the KCl was excluded from the normal relaxing solution, giving an ionic strength of μ = 58 mM.

Table I: Fluorescence Polarization Ratios ± Standard Deviations for Single Rhodamine-Labeled Fibers at 4 °C[a]

	$P_\parallel$	$P_\perp$
rigor	0.31 ± 0.06 (8)	0.29 ± 0.03 (8)
relaxation	0.44 ± 0.01 (4)	−0.07 ± 0.01 (4)
MgADP	0.68 ± 0.06 (8)	−0.30 ± 0.01 (8)
relaxation (low ionic strength)	0.50 ± 0.02 (4)	−0.15 ± 0.01 (4)
MgAMP-PNP	0.47 ± 0.01 (4)	0.05 ± 0.01 (4)
contraction	0.57 ± 0.03 (8)	−0.13 ± 0.03 (8)
random (theoretical)	0.5	0.0

[a] The numbers in parentheses indicate the number of experiments performed on independently prepared fibers.

Glycerinated rabbit psoas fibers were prepared and labeled according to a procedure described previously (Burghardt et al., 1983). The activated fiber measurements were performed within 1–3 min of the onset of contraction. For experiments done in the presence of glycerol, fibers were incubated first with a solution without glycerol for 10 min at 4 °C and then with the final 50% glycerol-containing solution at the proper temperature for 10 min. The pH of the glycerol-containing solution was adjusted to 7.0.

Results

Order Measurements in the Absence of Glycerol at 4 °C. We have measured $P_\parallel$ and $P_\perp$ from IATR-labeled muscle fibers to determine the angular distribution of IATR. Summarized in Table I are the $P_\parallel$ and $P_\perp$ values for single fibers in various physiological states at 4 °C. These values indicate that the rhodamine probe distinguishes a variety of orientational states of the cross-bridges including the predominant actin-attached state in isometric-active fibers (Burghardt et al., 1983; Burghardt & Ajtai, 1985; Borejdo & Burghardt, 1986). In previous studies using linear dichroism of fluorescence with IATR, we found the dichroism from cross-bridges in the MgADP state to be indistinguishable from that in the isometric-active state at room temperature (Burghardt et al., 1983). The values in Table I indicate that none of the static states at 4 °C are precisely equivalent to the predominant steady-state probe orientation characteristic of the isometric-active cross-bridges.

The theoretical values of $P_\parallel$ and $P_\perp$ for a random distribution of probes are also given in Table I. From them one can deduce that most of the probes in rigor cross-bridges at 4 °C are nearly perpendicular to the fiber axis ($P_\parallel < 0.5$, $P_\perp > 0$), while most of the cross-bridges in the presence of MgADP have probes nearly parallel to the fiber axis ($P_\parallel > 0.5$, $P_\perp < 0$). This is shown graphically in Figure 1 where the probe angular distribution for rigor, MgADP, and active fibers is plotted as a function of cosβ, where β is the probe polar angle. The probe angular distribution $N(\cos\beta)$ was computed by solving the simultaneous equations (2a) and (2b) for $a^2_{0,0}$ and $a^4_{0,0}$ and then using eq 3. A plot of the random distribution is also shown for comparison (each plot occupies the same area). Because of this unmistakable difference between these two static states (i.e., you cannot obtain a "MgADP-like" distribution from a "rigorlike" distribution by adding random probes or vice versa), we refer to other probe distributions as either rigorlike or MgADP-like depending on whether the probe orientation distribution has its dominant peak perpendicular or parallel to the fiber axis, respectively. According to our classification, the active fiber probe angular distribution is MgADP-like.

Order Measurements as a Function of Temperature. The temperature dependence of the cross-bridge orientation was measured between −15 and 25 °C. To make a full-scale investigation between −15 and 25 °C, 50% glycerol was added

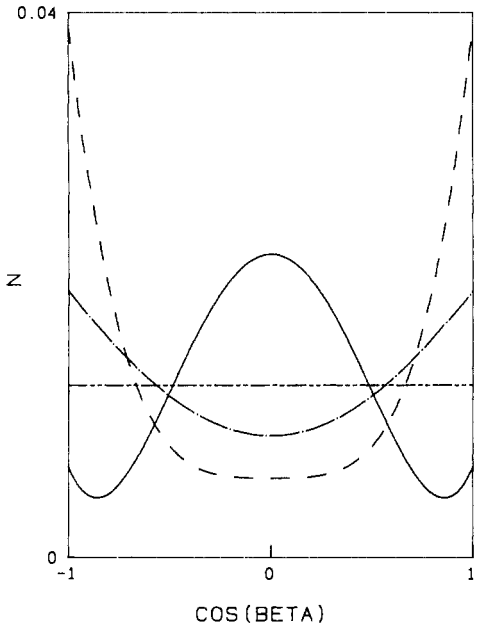

FIGURE 1: Probe angular distribution as a function of cos β, where β is the probe polar angle relative to the fiber axis, for cross-bridges in rigor (—), in the presence of MgADP (– –), and during contraction (–·–). The distribution is computed from values of $P_\parallel$ and $P_\perp$ in the table by using eq 2 and 3. Because of this graph, we specify a rigorlike probe angular distribution as one where the distribution's dominant peak is perpendicular to the fiber axis and a MgADP-like angular distribution as one where the distribution's dominant peak is parallel to the fiber axis. A random distribution (– – –) is also shown for comparison.

to the incubating solutions as a cryoprotective agent.

Shown in parts a and b of Figure 2 are $P_\parallel$ and $P_\perp$, respectively, for fibers in different physiological conditions (rigor, relaxation, and MgADP) plotted as a function of temperature in the presence and absence of glycerol. The presence of glycerol affects the actomyosin bond in rigor but not in the MgADP state, as indicated by comparing the values of $P_\parallel$ and $P_\perp$ in the presence and absence of glycerol at temperatures above 0 °C. Glycerol also affects the relaxed cross-bridges directly since the $P_\parallel$ and $P_\perp$ values in the presence and absence of glycerol for relaxed cross-bridges are different but only if $T > 10$ °C.

Figure 2 shows that the orientation of the rigor cross-bridge is affected by the temperature and that in the presence of glycerol it gradually changes from rigorlike to MgADP-like as the temperature decreases. At −15 °C the rigor cross-bridge has a polarization values ($P_\parallel = 0.56$, $P_\perp = -0.074$) that resemble those of active fibers (i.e., both are MgADP-like) at 4 °C ($P_\parallel = 0.57$, $P_\perp = -0.13$).

The cross-bridge orientation of the relaxed fibers in the presence of glycerol is very near to the random distribution and roughly independent of temperature. In the absence of glycerol, however, lowering the temperature has a marked effect on the relaxed cross-bridge orientation as indicated by changes in $P_\parallel$ and $P_\perp$.

The orientation of the MgADP cross-bridges (in both the presence and absence of glycerol) was not altered or slightly affected by a decrease in temperature, and the probe orientation distribution remains MgADP-like at all temperatures.

The orientation distribution for cross-bridges in rigor plus glycerol is plotted as a function of cos β and temperature in Figure 3. The angular distributions are computed by using eq 2 and 3. Figure 3 illustrates the temperature-dependent change in the probe angular distribution in the fiber bathed in rigor plus glycerol solution. From comparison with Figure

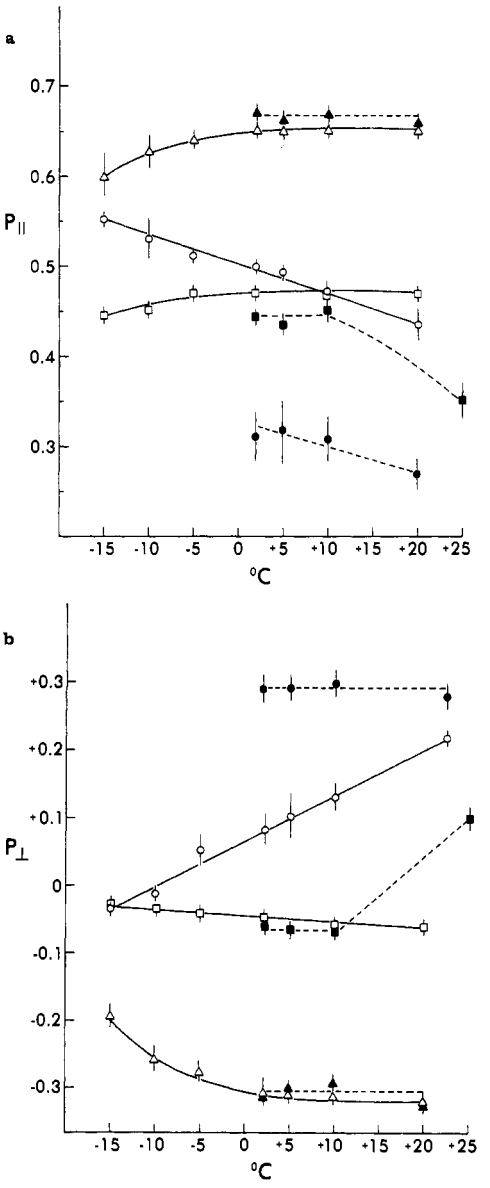

FIGURE 2: Fluorescence polarization ratios, (a) $P_\parallel$ and (b) $P_\perp$, for single rhodamine-labeled fibers plotted as a function of temperature in the presence and absence of glycerol. Three to eight independently prepared fibers were used for each point. Filled symbols with broken lines indicate the absence and open symbols with solid lines indicate the presence of glycerol (○ = rigor, △ = MgADP, □ = relaxation).

1 we see the probe distribution changes from one that is characterized as rigorlike to one characterized as MgADP-like. A plot of the random probe distribution is also shown for comparison.

Discussion

Our interpretation of the temperature dependence of the probe angular distribution of the cross-bridge (in rigor plus glycerol) is that the cross-bridge is in an equilibrium among actin-attached states at different orientations, some of them are rigorlike and the others are MgADP-like. At high temperature ($T > 2$ °C) the equilibrium favors the rigorlike orientation, and as the temperature decreases the equilibrium shifts so that by $T < -10$ °C the MgADP-like orientation is favored. It is particularly noteworthy that the $P_\parallel$ and $P_\perp$ values for the rigor plus glycerol cross-bridges at −15 °C are close to those values observed for active cross-bridges.

There is evidence from electron spin resonance data of SH-1 bound spin probes that fibers in rigor have two predominant cross-bridge orientations (Burghardt & Thompson, 1985a;

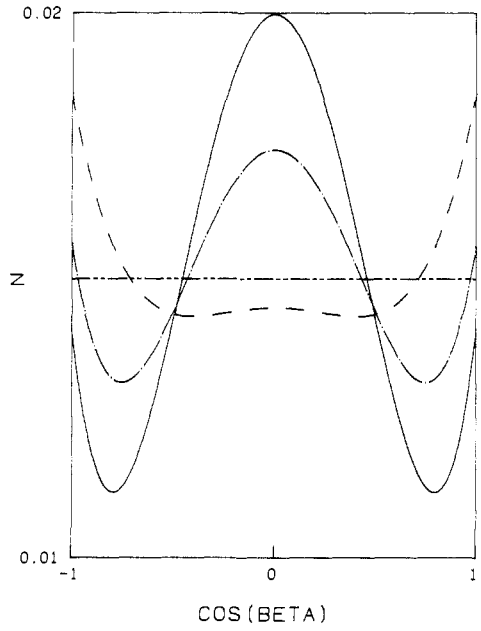

FIGURE 3: Probe angular distribution as a function of cos β for cross-bridges in rigor plus 50% glycerol, as a function of temperature. The distributions are computed by using eq 2 and 3 from values of $P_{\parallel}$ and $P_{\perp}$ appearing in Figure 2. The dominant peak in the distribution is shown to shift from one favoring probes perpendicular to the fiber axis (rigorlike) at 20 °C (—) and 4 °C (-·-) to one favoring probes parallel to the fiber axis (MgADP-like) at $T < -10$ °C (--). A random distribution is also shown for comparison (-···-).

Burghardt & Ajtai, 1986b). A similar result was observed by studying the three-dimensional reconstruction of insect flight muscle in rigor (Taylor et al., 1984). The formation of a nucleotide-bound S-1 conformation by decreasing temperature, in the absence of nucleotide, was shown by the tryptic proteolysis of S-1 (M. J. Redowicz, L. Szilâgyi, and H. Strzelecka-Gołaszewska, personal communication). Furthermore, NMR studies of S-1 (Shriver & Sykes, 1981, 1982), spin-label studies of heavy meromyosin (HMM) (Yamada et al., 1984), and calorimetric measurements of HMM (Kodama, 1981) indicate that S-1 has two fundamental states in a temperature-dependent equilibrium in the absence of nucleotide (Shriver, 1984).

The binding of MgADP to the cross-bridge also shifts the equilibrium of the cross-bridge orientation to favor the MgADP-like orientation. This shift occurs for MgADP cross-bridges in the presence of glycerol for all temperatures investigated here. The slight loss of order in the MgADP cross-bridge as the temperature decreases, as evidenced by $P_{\parallel}$ and $P_{\perp}$ values tendency toward their random values, probably indicates the weakening of the actomyosin bond in the presence of nucleotide. There is evidence of this effect from fibers in the presence of ethylene glycol at low temperature (Johnson, 1985; Marston & Tregear, 1984). At subzero temperatures (in the presence of ethylene glycol) myosin binds nucleotides and has ATPase activity (Marston et al., 1979; Bechet et al., 1979), and the number of attached cross-bridges is not altered (Clarke et al., 1984). The temperature insensitivity of the MgADP cross-bridges in glycerol is probably not the consequence of the high concentration of glycerol used. The same values for $P_{\parallel}$ and $P_{\perp}$ were measured in 30% glycerol for $T > -10$ °C (data not shown).

The rigorlike order of the relaxed cross-bridge at $T > 15$ °C has been observed previously (Burghardt et al., 1983, 1984; Wilson & Mendelson, 1983). The randomization of the relaxed cross-bridge orientation with decreasing temperature has not been reported previously but is similar to the effect of increasing ionic strength (Burghardt et al., 1984). In the earlier publication we attributed the ionic strength effect to an electrostatic interaction within the myosin molecule that was diminished at higher ionic strengths. Whether glycerol has a similar effect needs further study.

We have evidence that the orientational order in the rigor and MgADP cross-bridges in the presence of glycerol is due to their interaction with the actin filament. From experiments on stretched fibers, when there is no overlap between the thick and thin filaments, over the temperature range $-15\ °C \leq T \leq +20\ °C$, the same $P_{\parallel}$ and $P_{\perp}$ values were observed in rigor, relaxation, and MgADP as in the relaxed fibers at full overlap. These $P_{\parallel}$ and $P_{\perp}$ values are characteristic of a nearly random angular distribution.

The effect of temperature on the orientation of the rhodamine probe relative to the cross-bridge was investigated by measuring the rhodamine lifetime, τ, as a function of temperature. τ is a sensitive measure of the dye environment, and motion of the dye relative to the amino acid side chains on the surface of S-1 is likely to be detected as a change in τ. We have measured τ from rhodamine-labeled fibers in rigor in the presence of 50% glycerol for the temperatures $T = -15$, 0, 10, and 20 °C. We find a single lifetime of $\tau = 4.01 \pm 0.1$, 4.0 ± 0.1, 4.0 ± 0.1, and 3.9 ± 0.1 ns (SE, $n = 3$), respectively. These measurements suggest that there is no change in the local environment of the rhodamine probe over this temperature range. Borejdo et al. (1982) have shown that there is no change in τ when nucleotides are bound to the cross-bridge. We find this is also true in the presence of 50% glycerol.

The control experiments strongly suggest that the phenomenon observed does not originate from the independent rotation of the chromophore or from a denaturing of the cross-bridge by glycerol. The glycerol appears to act on the rigor actomyosin bond by shifting the equilibrium among the different orientational states.

Our data can be interpreted as evidence for (at least) two actin-binding orientations of the rigor cross-bridge. One binding orientation, favored at high temperature, is the usual rigorlike orientation as observed previously (Burghardt et al., 1983, 1984). The other orientation, favored at low temperature, is a MgADP-like orientation. The latter orientation is similar to the active cross-bridge orientation. These results are not consistent with the findings using other probe methods. Studies using electron spin resonance (ESR) probes (Cooke et al., 1982) and fluorescent nucleotides (Yanagida, 1981, 1985) suggest that all of the bound states of the cross-bridge have a single polar orientation relative to the actin filament. In the case of the ESR work, a low-resolution, model-dependent analysis of the spectra indicated no difference in the orientation of the rigor cross-bridge compared to that in the presence of MgADP (Thomas et al., 1985). On the other hand, a high-resolution model-independent analysis of the same spectra shows the spin probes maintain distinct probe angular distributions in rigor compared to that in the presence of MgADP. Two separate peaks containing ~70% and ~30% of the spins characterize the rigor probe distribution. Two separate peaks, at angles slightly different from those in rigor, that contain ~80% and ~20% of the spins characterize the probe distribution in the presence of MgADP (Burghardt & Ajtai, 1986b). Similarly, X-ray scattering measurements show the presence of two orientations in the active cross-bridge (Lowy & Poulsen, 1986). We presume the contradiction in the probe findings comes from the fortunate orientation of some of the fluorescent probes that allows a more dramatic difference to be observed in the probe angular distribution

between physiological states. We have recently obtained experimental evidence supporting this view with the fluorescence probe 1,5-IAEDANS attached to SH-1 (Ajtai & Burghardt, 1986).

Our data can also be interpreted in terms of the domain concept of the myosin head. It has been shown that S-1 has at least two actin-binding sites located on the 20K (strong binding site) and on the 50K fragment near the junction with the 20K fragment (weak binding site) (Katoh et al., 1985; Chen et al., 1985; Muhlrad et al., 1986). In rigor both of the actin binding sites are actin attached, while in MgADP only one (probably the strong binding site on 20K) is actin attached (Katoh et al., 1985). The angle of the cross-bridge attachment could be determined by the domains that are involved in the actomyosin interaction. The MgADP-like orientation could be induced by the 20K–actin interaction, while the rigor-like orientation could come from two attachment regions between actin and myosin. The two different rigor orientations may be induced by the weakening of the actomyosin bond at the weak site by the perturbing factors (MgADP or temperature) resulting in the detachment of the weak binding site. This cross-bridge orientation should be (and is observed to be) similar to the MgADP-like one. In view of the finding that lowering the temperature can induce a nucleotidelike conformational change in S-1 (Shriver & Sykes, 1982; M. J. Redowicz, L. Szilágyi, and H. Strzelecka-Gołaszewska, personal communication), the detachment of one of the domains from actin is probably a consequence of a specific conformational change in S-1 induced by binding of a nucleotide or by lowering of the temperature.

In conclusion, our data suggest that the shortening step in the active cross-bridge cycle may be the transition from the MgADP-like orientation to the rigorlike orientation. In the active fiber this transition is induced by the hydrolysis of ATP.

ACKNOWLEDGMENTS

We thank M. F. Morales and J. Borejdo for helpful discussions.

Registry No. MgADP, 7384-99-8; MgAMP-PNP, 69977-25-9; glycerol, 56-81-5.

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