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Thermal Unfolding of Myosin Rod and Light Meromyosin: Circular Dichroism and Tryptophan Fluorescence Studies†

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ABSTRACT: Rabbit skeletal myosin rod, which is the coiled-coil α-helical portion of myosin, contains two tryptophan residues located in the light meromyosin (LMM) portion whose fluorescence contributes 27% to the fluorescence of the entire myosin molecule. The temperature dependence of several fluorescence parameters (quantum yield, spectral position, polarization) of the rod and its LMM portion was compared to the thermal unfolding of the helix measured with circular dichroism. Rod unfolds with three major helix unfolding transitions: at 43, 47, and 53 °C, with the 43 and 53 °C transitions mainly located in the LMM region and the 47 °C transition mainly located in the subfragment 2 region. The fluorescence study showed that the 43 °C transition does not involve the tryptophan-containing region and that the 47 °C transition produces an intermediate with different fluorescence properties from both the completely helical and fully unfolded states. That is, although the fluorescence of the 47 °C intermediate is markedly quenched, the tryptophyl residues do not become appreciably exposed to solvent until the 53 °C transition. It is suggested that although the intermediate that is formed in the 47 °C transition contains an extensive region which is devoid of α-helix, the unfolded region is not appreciably solvated or flexible. It appears to have the properties of a collapsed nonhelical state rather than a classical random coil.

Myosin consists of two polypeptide chains each containing a globular head or subfragment 1 (S1) which contains the actin and ATP binding sites, a coiled-coil α-helical rod with a light meromyosin (LMM) region, used in thick filament formation of muscle and a subfragment 2 (S2) region between the head and LMM (Harrington & Rogers, 1984). The LMM and S2 regions of the rod can be isolated after proteolytic digestion (Margossian & Lowey, 1982). Thermal unfolding studies of the rod and its tryptic fragments have indicated that the rod unfolds in several cooperative transitions involving domains along the molecule (Burke et al., 1973; Goodno et al., 1976; Privalov, 1982; Cross et al., 1984; Stafford, 1985) with indications that the most unstable region involves a portion of the subfragment 2 region of the rod near the light meromyosin (LMM)-S2 junction (Burke et al., 1973; Goodno et al., 1976). It is this "hinge" region (Tsong et al., 1979) which was proposed to be involved with the head in reversible force generation (Harrington, 1979).

In this work, we determined that there are two tryptophan residues located in the LMM portion of our preparations of rod purified from rabbit skeletal back muscle and measured the average fluorescence quantum yield. By comparing the temperature dependence of fluorescence properties of rod and LMM with the helix unfolding profiles, we obtained information about the relationship of the domain containing the tryptophans and the intermediates involved in the thermal unfolding. The results indicate that the rod unfolds in three major helix transitions with the least stable region located in the LMM portion not containing the tryptophan residues. The tryptophan fluorescence is sensitive to the unfolding of the two more stable regions, but the fluorescence parameters indicate that the tryptophan environment of the major partly unfolded intermediate is shielded from solvent in contrast to the tryptophan environment of the fully unfolded state, which is fully exposed to solvent.

Experimental Procedures

Rabbit skeletal myosin was prepared by standard procedures as previously outlined (Sreter et al., 1972), rod and S1 were purified from a chymotryptic digest of myosin (Weeds & Pope, 1977), LMM was purified from a tryptic digestion of rod, and rod and LMM were further purified by chromatography on a Protein Pak glass 200sw gel exclusion column in 0.6 M NaCl,

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1 mM EDTA, 1 mM dithiothreitol, and 50 mM sodium phosphate buffer, pH 7, using an FPLC apparatus (Waters 650, Milford, MA). The purity of the resulting samples were checked by SDS–polyacrylamide gel electrophoresis.

Circular dichroism measurements were made with an Aviv 60DS spectropolarimeter (Lakewood, NJ) containing a Hewlett-Packard 89100A temperature controller which provided programmable sample temperature changes with 0.1 °C resolution. Ellipticity values at 222 nm were obtained from 25 to 70 °C using solutions contained in a stoppered standard 1-cm Spectrosil quartz cuvette into which the temperature probe of the unit was placed. The solution was stirred with a magnetic bar placed below the light path, enabling rapid temperature equilibration to be obtained. Data were collected automatically in 0.2 °C steps using an equilibrium time of 0.3 min and a data averaging time of 10 s at each temperature step. Low protein concentrations (0.1–0.2 mg/mL) in 0.5 M NaCl/50 mM sodium phosphate buffer, pH 7.0, and the use of 1 mM EDTA and 1 mM dithiothreitol avoided possible dimerization (Harrington & Burke, 1972) and production of disulfide cross-links (Lu & Lehrer, 1984), and 50 mM phosphate buffer at pH 7.0 was used because of its low thermal coefficient of pH.

Fluorescence measurements were carried out with a photon counting Spex Fluorlog 2/2/2 spectrofluorometer (Edison, NJ) in the ratio mode with band-passes of 2.25 nm and gratings blazed at 300 nm for both excitation and emission. Spectra were obtained at 3–5 °C intervals after a 15-min equilibration time. The temperature was measured with a calibrated thermocouple. The weighted average fluorescence wavelength, $\langle\lambda\rangle$, was calculated with $\langle\lambda\rangle = \sum F(\lambda)/\sum[F(\lambda)/\lambda]$ (Torgerson et al., 1979) from 310 to 460 nm in 1-nm steps with a program written for the Spex instrument. The relative fluorescence quantum yield at each temperature was calculated as the area under the spectrum yield at each temperature was calculated as the area under the spectrum over the same wavelength range. The fluorescence polarization was determined at 340 nm by using the L-format and excitation at 297 nm with excitation and emission band-passes of 2.25 and 9.0 nm, respectively, with grating and background corrections. The fluorescence anisotropy, r, was calculated from the fluorescence polarization, P, with the equation $r = 2P/(3 - P)$. The tryptophan and tyrosine contents of rod and LMM were determined by absorption spectral methods (Edelhoch, 1967). The tryptophan content was determined by measurements at 288 and 280 nm of proteins in 5 M GdmCl, and tyrosine was determined as tyrosinate at pH 12 at 295 and 300 nm. Protein concentrations were 0.1–0.2 mg/mL for quantum yield and spectral measurements and 1–2 mg/mL for polarization measurements in the same buffer as for CD measurements.

The relative quantum yield of rod and S1 was calculated from the ratio of the total fluorescence intensity to the absorbance at 297 nm compared to the equivalent ratio of an L-tryptophan solution in H_2O obtained from measurements under identical conditions. Absorbance values were <0.1 to avoid inner filter corrections. The band-pass was 0.9 nm for both excitation and emission.

Results

Characterization of Tryptophan Fluorescence. The tryptophan and tyrosine content in rod and LMM was determined by spectrophotometric methods on purified samples of known concentrations as outlined under Experimental Procedures. Values of 2.0 ± 0.2 tryptophans/chain and 5.0 ± 0.2 tyrosines/chain were obtained for both rod and LMM. These values agree with the cDNA-derived sequence recently determined for rabbit skeletal leg muscle LMM, which shows two tryptophans in rod positions 532 and 617 and five tyrosines in positions 508, 621, 649, 1011, and 1037 (Maeda et al., 1987). The same tryptophan and tyrosine contents obtained from rabbit back muscle by direct measurements on the isolated protein and on rabbit leg muscle by cDNA sequence methods indicate that the sequence positions indicated above are probably identical for rabbit leg and back muscle myosin. The same chromophore contents for both rod and LMM clearly indicate that there are no tryptophan or tyrosine residues in the S2 region of the rod, in agreement with previous UV absorption observations in our laboratory.

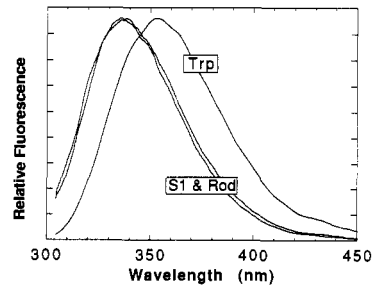

FIGURE 1: Tryptophan fluorescence spectra of rod and S1 compared to L-tryptophan. The rod spectrum is slightly narrower than the S1 spectrum. Rod in 0.6 M NaCl, 50 mM sodium phosphate buffer, and 1 mM EDTA, pH 7.0. The spectrum of LMM is identical with that of rod. S1 in 10 mM HEPES buffer/30 mM NaCl, pH 7.5. Temperature, 25 °C; λ_{exc} = 297 nm; band-pass = 0.9 nm for both excitation and emission.

Fluorescence quantum yield determinations gave values of 0.70 for rod and 0.77 for S1, relative to L-tryptophan in H_2O. A value of 2.7 was obtained for LMM in early studies (Cowgill, 1968). The reason for the discrepancy is not understood. Considering that there are five tryptophans in the S1 portion of each chain (Warrick & Spudich, 1987), the two tryptophans in each rod chain contribute 27% to the total tryptophan fluorescence of myosin. The fluorescence spectra of rod and LMM were identical and almost the same as the S1 spectrum. The weighted average wavelength $\langle\lambda\rangle$ = 345.6 nm for both rod and LMM and $\langle\lambda\rangle$ = 346.3 nm for S1 can be compared to $\langle\lambda\rangle$ = 361 nm for L-tryptophan (Figure 1). The spectra relative to L-tryptophan in H_2O indicated partial exposure to solvent.

Thermal Unfolding. The circular dichroism spectra of rod and LMM were identical, with ellipticity (θ) peaks at 222 and 208 nm characteristic of an α-helix. The magnitude of the ellipticity for LMM indicated an α-helical content of >95%, in agreement with previous studies (Wu & Yang, 1976). Somewhat lower values for ellipticity were obtained for rod at approximately equal concentrations. The temperature dependence of θ_{222nm} of the rod showed three major thermal helix unfolding transitions with midpoints at 43, 47, and 53 °C (Figure 2). For LMM, the 43 °C transition is the most prominent with a much smaller contribution from a 47 °C transition. LMM does show a 47 °C transition, however, as indicated by the fluorescence studies (see below). There is no further unfolding above 60 °C with about 13% of the θ_{222nm} signal remaining for both rod and LMM. The reversibility of thermal unfolding was about 90–95% with the normal heating and cooling rates. Long exposure (hours) at temperatures above 40 °C, particularly at higher temperatures, resulted in losses of reversibility. The slope of the thermal unfolding curves defined the midpoints and the relative contribution of transitions more clearly (Figure 2, lower panel). The transitions associated with the S2 region of the rod can

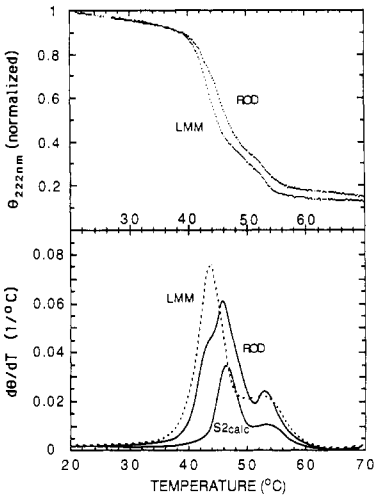

FIGURE 2: (Top) Normalized thermal unfolding profile of rod and LMM helix. (Bottom) Derivative of upper curves. The S2 curve was estimated by subtracting 0.55 × LMM curve from the rod curve. Buffer conditions: 0.6 M NaCl/50 mM sodium phosphate with 1 mM EDTA, pH 7.0, and 1 mM dithiothreitol.

be estimated with the assumption that the unfolding derivative spectrum of the rod is composed of contributions of LMM and S2 proportional to the molecular weights. Subtracting $0.55d\theta(T)/dT$ (LMM) from the rod data [M_r(LMM) ≈ $0.55M_r$(rod)] showed that the major part of the S2 region of rod melts in a broad transition centered at 47 °C.

The unfolding affected the tryptophan environment measured by the relative fluorescence quantum yield, spectral position, and polarization, reflecting changes in quenching by local groups and solvent, exposure to solvent, and mobility, respectively. In the temperature region up to 40 °C, the quantum yield monotonically decreased, indicating thermally activated quenching processes (Figure 3A) without spectral change (Figure 4) and with the depolarization expected for tryptophan rigidly attached (Figure 5A,B). The activation energy for quenching in the unfolded state, obtained from the slope at high temperature of a plot of log $(1/F)$ vs $1/T$ (Figure 3B), was 7 kcal/mol, in reasonable agreement with values obtained for solvent quenching of tryptophan model compounds (6.6–8.5 kcal/mol) (Longworth, 1971) considering the few data points used in the measurement. In contrast, the activation energies for quenching in the folded state, which were estimated from the slope at low temperature, gave 1.5 kcal/mol for rod and 2.5 kcal/mol for LMM, indicating that a different mechanism was involved in the folded state, probably quenching by neighboring protein groups. This double-reciprocal plot made it possible to correct for thermal quenching (Figure 3C) with the simplifying assumption of a two-state unfolding process which affects tryptophan. This correction is only approximate since there are 2 two-state processes involved. A Perrin plot indicated that the limiting anisotropy value was 0.20 for both rod and LMM (Figure 5B). Above 40 °C, two transitions are seen in all three fluorescence parameters, corresponding to the 47 and 53 °C transitions in the helix unfolding profile. The smaller decrease in polarization at $T > 45$ °C for rod suggests some aggregation (Figure 5). The lack of appreciable change in any of the fluorescence parameters corresponding to the 43 °C transition indicates that the region of the LMM that unfolds in the 43 °C transition does not contain the tryptophan residues.

The transition at 47 °C results in a great deal of fluorescence quenching for both rod and LMM with only a slight spectral red shift (2–3 nm) and some depolarization. Thus, intermediates with altered conformation are present which allow increased quenching by local groups without much increased exposure of tryptophan to solvent and with some increase in mobility. In contrast, the transition at 53 °C, which only produced a slight further quenching, resulted in a large spectral

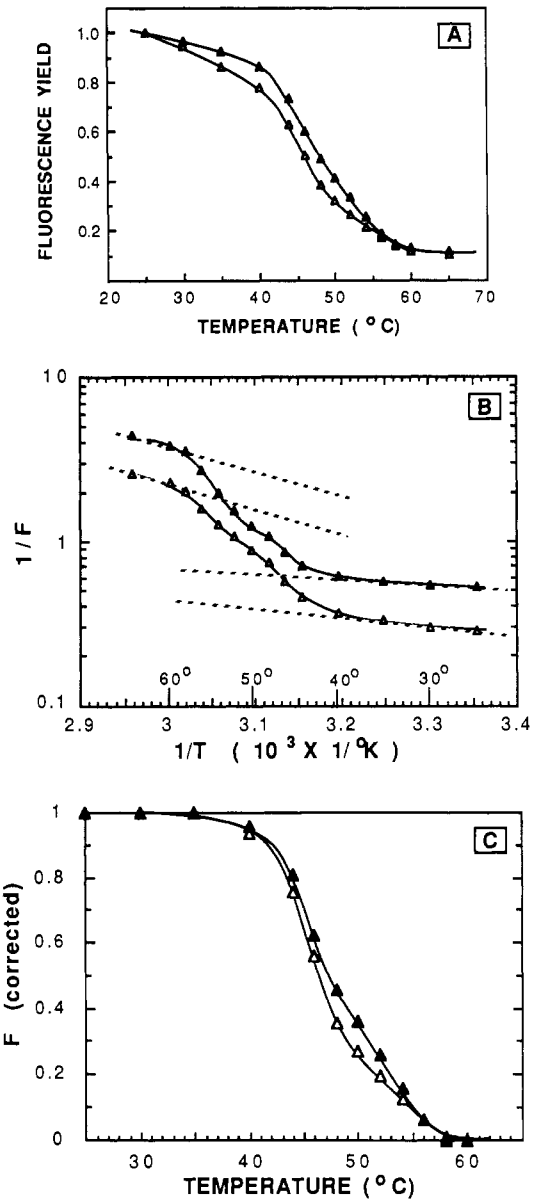

FIGURE 3: Temperature dependence of the tryptophan fluorescence quantum yields of rod (▲) and LMM (△). (A) Normalized data. (B) Arrhenius plot illustrating method of correcting for thermal quenching. (C) Corrected for thermal quenching. Buffer conditions as in Figure 2. λ_{exc} = 297 nm, band-pass = 2.25 nm for both excitation and emission.

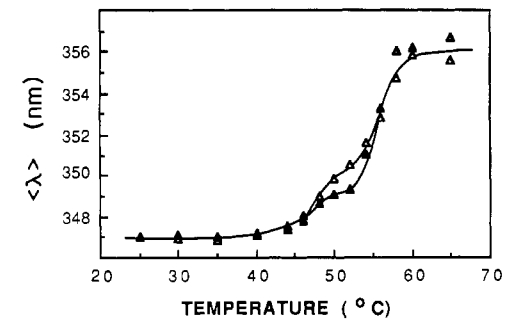

FIGURE 4: Temperature dependence of the spectral change of rod (▲) and LMM (△) calculated as the weighted average tryptophan fluorescence wavelength. Conditions as in Figure 3.

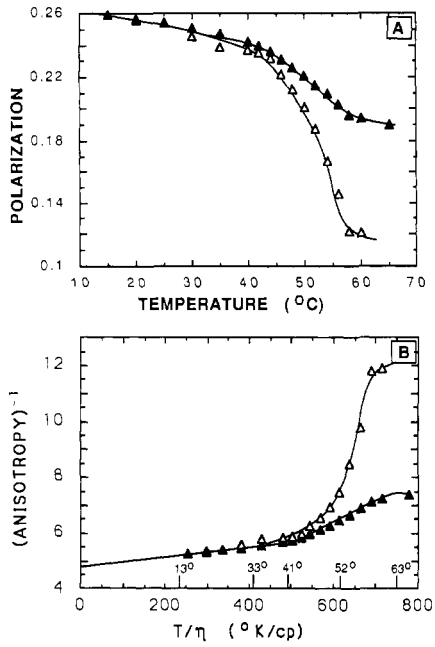

FIGURE 5: (A) Temperature dependence of the tryptophan fluorescence polarization of rod (▲) and LMM (△). (B) Perrin plot of the data in (A). Buffer conditions as in Figure 3.

red shift (7–8 nm) closer to the values expected for an exposed tryptophan residue and to a polarization value expected for tryptophan in a random coil (Weber, 1960).

Discussion

Evidence has been accumulating that the several unfolding transitions that myosin rod undergoes can be associated with domains along the molecule (Burke et al., 1973; Potekhin et al., 1979; Tsong et al., 1979; Cross et al., 1984; Stafford, 1985). Our high-resolution circular dichroism thermal unfolding studies indicated three major unfolding transitions for rod and LMM at 43, 47, and 53 °C. A small transition at 50 °C may also be present. Thus, it appears that we were able to resolve the transition previously reported at 44–45 °C for rod (Burke et al., 1973; Stafford, 1985) into two transitions at 43 and 47 °C. Using differential scanning calorimetry (DSC), Cross et al. (1984) previously noted a 43 °C transition for rod and LMM under similar buffer conditions but did not observe a prominent 47 °C transition. With DSC, Potekhin et al. (1979) obtained transitions at similar temperatures but with different relative contributions than obtained here as well as evidence for several other transitions. This may be consequence of a combination of the different buffer conditions used, the higher protein concentrations which probably resulted in some aggregation and the differences in information expected between calorimetry and circular dichroism (Privalov, 1982).

The observation that the lowest temperature transition of the rod (43 °C transition) is located in LMM and that therefore the main transition of the S2 region of the rod is at 47 °C is in reasonable agreement with studies of Swenson and Ritchie (1980) and Tsong et al. (1979) where major transitions were noted at 45–48 °C for S2 under somewhat similar conditions. In contrast, the studies of Potekhin et al. (1979) indicated that S2 shows a transition only at 51 °C. They also suggest that there is a region in the S2 portion of the rod which is unfolded at low temperatures and would therefore not contribute to the unfolding profile. The observation of a somewhat lower helix content for rod at low temperature appears to be in agreement with this interpretation.

From the fluorescence results, it does not appear that the tryptophans are located in the least stable region of the rod. This is particularly evident for LMM where more than half unfolds in the 43 °C transition whereas the tryptophan fluorescence is not affected until the 47 °C transition. It is reasonable to assume that both tryptophans are located in the same cooperative domain with similar fluorescence properties. This follows because 2 tryptophans are separated by only 84 amino acids, which is about one-eighth the length of the LMM portion of the rod, and are in the same position in the 7-residue repeat of the coiled-coil. Also, preliminary studies on LMM tryptic fragments digested for longer times (Nitray et al., 1983) to mainly contain one tryptophan (617) also showed two similar fluorescence transitions. This indicates that the tryptophans are not in different domains.

The fluorescence properties at 50 °C provide some information about the nature of the intermediate that is partially unfolded in the 47 °C transition. The fluorescence of the intermediate is quenched about 70% (corrected for temperature quenching) relative to the folded state. Associated with this transition is a 3-nm red shift in $\langle\lambda\rangle$ compared to the 10-nm shift seen in the fully unfolded state. The anisotropy of the intermediate decreased by about 20% of the total change from the folded to the fully unfolded state. These data indicate that the tryptophans are not as exposed and mobile as the unfolded state at high temperature. Thus, although the tryptophan fluorescence of the intermediate is almost as completely quenched as in the fully unfolded state, it does not have the properties of a classical random coil, i.e., with mobile and solvated tryptophans. These observations suggest that the 50 °C intermediate may be a kind of collapsed state where hydrophobic amino acid residues such as tryptophan and certain extrinsic fluorescence probes located in the unfolded domain could exhibit appropriate nonsolvated spectra by interacting with a putative hydrophobic core.

Early unfolding studies with tropomyosin, a similar coiled-coil rod, indicated the presence of an intermediate with an unfolded region (Woods, 1976; Lehrer, 1978). Although the nature of the intermediate was not known, evidence was obtained for increased interaction of a dansyl fluorophore at cysteine-190 with a hydrophobic region in the partially unfolded molecule compared to the native folded and denatured states (Betteridge & Lehrer, 1983). These observations on tropomyosin are consistent with the data on LMM and rod which indicate that an unfolded nonhelical region of a coiled-coil molecule, in general, could be in a compact partially flexible domain containing a hydrophobic core rather than in a random-coil configuration. It is interesting to note that compact globules, located in LMM near the S2–LMM junction, were seen in electron micrographs of myosin molecules that were fixed after being subjected to temperatures in the 40–50 °C range (Walker & Trinick, 1986).

Evidence has been presented that the localized unfolding of a portion of the S2 region of the myosin rod (the hinge) to a random-coil configuration (Burke et al., 1973) has a role in the generation of force in muscle contraction (Harrington, 1979), although more recent experimental data indicate that the rod is not required (Hynes et al., 1987). Our data indicate that, under our conditions, the first unfolding transition is not associated with the S2 region although it cannot be ruled out that under physiological conditions, in the filament, the first transition could be shifted to the S2 hinge as a result of local charge or other interactions (Stafford, 1986). The results presented here, which suggest that locally unfolded regions of coiled-coils may be in compact nonhelical configuration rather than a random coil, may still be compatible with the shortening of the rod required in Harrington's theory of force

generation (Harrington, 1979).

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Nucleotide-Induced States of Myosin Subfragment 1 Cross-Linked to Actin†

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ABSTRACT: Actomyosin interactions and the properties of weakly bound states in carbodiimide-cross-linked complexes of actin and myosin subfragment 1 (S-1) were probed in tryptic digestion, fluorescence, and thiol modification experiments. Limited proteolysis showed that the 50/20K junction on S-1 was protected in cross-linked acto–S-1 from trypsin even under high-salt conditions in the presence of MgADP, MgAMPPNP, and $MgPP_i$ (μ = 0.5 M). The same junction was exposed to trypsin by MgATP and MgATPγS but mainly on S-1 cross-linked via its 50K fragment to actin. *p*-Phenylenedimaleimide-bridged S-1, when cross-linked to actin, yielded similar tryptic cleavage patterns to those of cross-linked S-1 in the presence of MgATP. By using *p*-nitrophenylenemaleimide, it was found that the essential thiols of cross-linked S-1 were exposed to labeling in the presence of MgATP and MgATPγS in a state-specific manner. In contrast to this, the reactive thiols were protected from modification in the presence of MgADP, MgAMPPNP, and $MgPP_i$ at μ = 0.5 M. These modifications were compared with similar reactions on isolated S-1. Experiments with pyrene-actin cross-linked to S-1 showed enhancement of fluorescence intensity upon additions of MgATP and MgATPγS, indicating the release of the pyrene probe on actin from the sphere of S-1 influence. The results of this study contrast the "open" structure of weakly bound actomyosin states to the "tight" conformation of rigor complexes.

Muscle contraction is believed to involve cyclic interaction between myosin crossbridges and actin filaments. Such interaction results in the sliding of the two filaments past each other and the generation of force (Huxley & Niedergerke, 1954; Huxley & Hanson, 1954). The widely accepted mechanism of force generation describes two states of myosin crossbridges (Huxley, 1969; Huxley & Simmons, 1971; Huxley & Kress, 1985). Initially, myosin heads would bind to actin at a 90° angle and then rotate to a 45° angle during the power

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