Proc. Natl. Acad. Sci. USA
Vol. 74, No. 11, pp. 4986–4990, November 1977
Biophysics

Flexibility of myosin rod, light meromyosin, and myosin subfragment-2 in solution

(myosin fragments/protein dynamics/electro-optics/muscle)

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Contributed by Manuel F. Morales, August 11, 1977

ABSTRACT **Myosin rod was prepared by papain proteolysis of myosin. The components of rod, light meromyosin (LMM) and subfragment-2 (S-2), were prepared by proteolysis of myosin and rod, respectively, using trypsin treated with tosylphenylalanine chloromethyl ketone. S-2, thus prepared, was of greater molecular weight than obtained previously, so that the combined molecular weights of LMM and S-2 were equal to that of rod, and S-2 contained virtually all of the region of the rod susceptible to trypsin. Electro-optical measurements were made on the three fragments in 2 mM sodium pyrophosphate, pH 9.3 at 3°, over a large range of protein concentrations. Analysis of the relaxation of birefringence, at low protein concentration where there was no aggregation, showed that LMM (relaxation time 13.1 μs) behaves as a rigid cylinder. Rod (relaxation time 41.2 μs) and S-2 (relaxation time 6.0 μs) had relaxation rates that were too fast for rigid molecules of their dimensions, and therefore are not straight rods. This implies that myosin rod is flexible in the S-2 portion, presumably in the region susceptible to proteolysis. The implications of rod flexibility for the mechanism of muscle contraction are discussed.**

Current theories of muscle contraction suppose that force is produced by an interaction between the thin and thick filaments (1). More specifically, cross-bridges that project from the thick filament make contact with the thin filament and generate force (2). Studies of the proteolytic degradation of myosin, initiated by Gergely (3), have shown that the myosin molecule may be considered as three functional units (see Fig. 1). Subfragment-1 (S-1) possesses ATPase activity (4) and interacts with actin (5). Light meromyosin (LMM) is the element that enables myosin molecules to aggregate to form filaments (6). Subfragment-2 (S-2) provides a mechanical link between LMM and S-1 (7, 8). S-2 and LMM, collectively, are known as rod. Contraction can occur at different interfilament spacings (9), and S-1 is thought to rotate through perhaps 45° during contraction (see Fig. 1) (10–12). Because S-1 appears to be rigid (13), the S-1/S-2 junction must be able to approach and recede from the myosin filament. Flexible regions at the S-1/S-2 and S-2/LMM junctions would allow such movement (8, 12). The former region has been shown to be be flexible (14–16). Only indirect evidence suggests flexibility of the latter: the myosin molecule is most susceptible to proteolytic attack at the S-1/S-2 junction (by papain) and at the S-2/LMM junction (by trypsin) (17). This proteolytic vulnerability may be associated with flexibility (18). Furthermore, the temperature dependences of the reduced viscosity and helical content of the rod support a flexible structure (19, 20).

We have investigated the flexibility of the S-2/LMM junction directly. The junction was isolated by preparing rod which contains it, but not the S-2/S-1 junction. The electro-optical properties of rod and its constituents, LMM and S-2, have been studied with a view to understanding the dynamics of the rod while it undergoes Brownian rotational motion in solution. In an electro-optical experiment a strong, uniform electric field is applied to a solution of the macromolecules under investigation. The field interacts with the permanent or induced dipole moment of the macromolecules so that the macromolecules become oriented: the solution becomes anisotropic and therefore birefringent. There are many parameters that one can measure in such experiments. Perhaps the simplest to interpret quantitatively is the reciprocal of the rate of decay of birefringence that occurs when the field is abolished and the molecules rotate due to Brownian motion alone. The rate at which

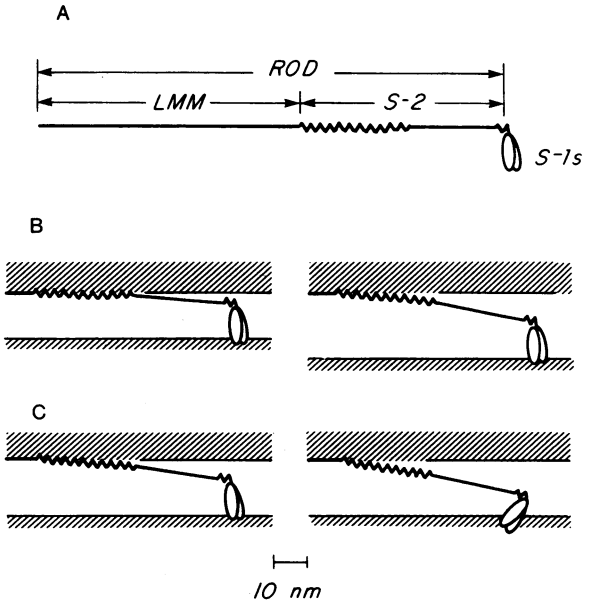

FIG. 1. A scale diagram of cross-bridge conformations during muscle contraction, based on the results from many laboratories. (*A*) The myosin molecule. Two S-1 moieties are attached to the rod, which is a 140-nm coiled-coil of two α-helices. The rod is composed of two sections: LMM and S-2. The zigzag portions are especially susceptible to proteolysis. (*B*) A cross-bridge at different interfilament spacings. Contraction can occur when the interfilament spacing varies between 13.5 (left) and 19.5 nm (right). The hatched portion above represents the thick filament where myosin is anchored by LMM. The hatched portion below represents the thin filament. (*C*) The cross-bridge during contraction. The S-1 portion is thought to change its orientation by 45°. This motion moves the S-1/S-2 junction by about 4 nm away from the thick filament, if the S-1 rotates about its tip. The two S-1s are arbitrarily shown as moving together.

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Abbreviations: S-1, myosin subfragment-1; S-2, myosin subfragment-2; LMM, light meromyosin; $NaDodSO_4$, sodium dodecyl sulfate.

Table 1. Properties of rod and its fragments

Property	Rod	LMM	S-2
Molecular weight	250,000	140,000	116,000
τ, μs	41.2 ± 0.6 (n = 8)	13.1 ± 0.2 (n = 5)	6.0 ± 0.2 (n = 13)
Calculated length, nm	121.9 ± 0.7 (n = 8)	79.1 ± 0.5 (n = 5)	58.7 ± 0.8 (n = 13)
Experimental length, nm	136.0 ± 1.1 (n = 317)	78.5 ± 0.7 (n = 626)	65.0 ± 0.6 (n = 626)

Molecular weights were determined by $NaDodSO_4$/polyacrylamide electrophoresis. The relaxation times (τ) for the decay of birefringence in electro-optical experiments were determined at 3°. The calculated lengths were obtained from τ values using Eq. 3 in the *text*, which assumes rigidity and linearity. The experimental lengths for rod and LMM are electron micrograph data from ref. 40. The experimental length for S-2 was obtained using (molecular $\text{weight}_{S\text{-}2}$/molecular weight_{LMM})(experimental length of LMM). SEM and number of observations (n) are given in the table.

their orientations randomize may be interpreted in terms of the size and flexibility of the molecules.

METHODS

Materials. Rabbit myosin was prepared by the method described by Stone (21). Rod was prepared from myosin by the method of Bálint *et al.* (22) as modified by Goodno *et al.* (20). LMM was prepared from myosin by the method of Lowey *et al.* (5) except that trypsin in the presence of tosylphenylalanine chloromethyl ketone (TPCK) (Worthington Biochemical Co.) was used instead of just trypsin. S-2 was prepared from rod by a modification of the method of Bálint *et al.* (23). In this preparation rod at 10 mg/ml in 0.5 M KCl/0.05 M Na_2HPO_4, pH 6.2 at 25°, is exposed to tosylphenylalanine chloromethyl ketone-treated trypsin (200:1 mole/mole). After 90 sec, the reaction is quenched with a 4-fold molar excess of soybean trypsin inhibitor. The remainder of the preparation is as in Bálint (23).

Molecular weights were determined by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis (24), using markers of known molecular weights between 20,000 and 180,000. Electro-optical measurements were made at 3° on solutions of protein dialyzed to 2 mM $Na_4P_2O_7$, pH 9.3 This solvent was used in order to provide both adequate solubility (25, 26) and the low conductance required for electro-optical experiments. Protein concentrations were determined spectrophotometrically, using an extinction coefficient at 280 nm of 2.00 $\%^{-1}$ cm^{-1}, the measured value for rod (25). Conclusions are not affected by the value chosen for the extinction coefficient.

Electro-Optical Measurements. The optics, instrumentation, and methodology used for electro-optical measurements have been described by Krause, Jost, Pritchard, and O'Konski (27–30). Signals due to the birefringence were detected by a photomultiplier, recorded on a digital transient recorder (Intercomputer Electronics PTR 9200), and stored on magnetic discs. The decay of the birefringence, Δn, with time was analyzed by nonlinear least squares techniques (29) that fit the data to either Eq. 1 or 2.

$$\Delta n = \Delta n_0 \exp(-t/\tau) \quad [1]$$

$$\Delta n = \Delta n_1 \exp(-t/\tau_1) + \Delta n_2 \exp(-t/\tau_2) \quad [2]$$

in which τ, τ_1, and τ_2 are relaxation times. The parameters Δn_0, Δn_1, Δn_2, τ, τ_1, and τ_2 are determined in the curve-fitting procedures. The frequency response of the electro-optical system was tested with propylene carbonate as a sample. This liquid becomes strongly birefringent in an electric field, and the birefringence decays very rapidly when the field is abolished. The measured rate of decay, which depended only on properties of the instrument, had a time constant of less than 0.2 μs (anode resistance in photomultiplier circuit, 1 kΩ). Consequently decay times as small as 2 μs could be measured without distortion. The electric field strength could not be varied over a large range because of the relatively large conductivity of the samples ($\sim 5 \times 10^{-2}$ siemens/m). Typically, electric fields of 0.5 kV/mm were used. The rates of decay of the birefringence were studied as a function of protein concentration. In order to avoid error caused by the presence of oligomers in solutions of LMM and rod, only values of the decay times measured at sufficiently low concentrations were used to evaluate rod kinematics.

Analysis. The rotational diffusion coefficient for a rigid cylinder can be calculated from Broersma's equation (31):

$$\theta = \frac{3kT}{8\pi\eta a^3}\left\{\ln\left(\frac{2a}{b}\right) - 1.57 + 7\left(\frac{1}{\ln\left(\frac{2a}{b}\right)} - 0.28\right)^2\right\} \quad [3]$$

in which a is the half-length and b is the radius of the cylinder, η is the viscosity of the solvent, k is Boltzmann's constant, and T is the absolute temperature. θ is the diffusion coefficient for end-over-end motion of the cylinder. Because a cylinder is circularly symmetric, no other motion is detectable with electro-optical methods. The proteins we have investigated are almost certainly two α-helices in "coiled-coil" conformation (32), so it is reasonable to assume that they have circular symmetry. The fact that the experimental relaxation times were indeed described adequately by single exponentials renders more complex assumptions unnecessary. Thus the relationship between relaxation time and diffusion coefficient derived by O'Konski and Zimm (33) and by Benoit (34) applies:

$$\tau = (6\theta)^{-1}. \quad [4]$$

The presence of flexibility within a molecule is indicated if the measured relaxation time is smaller than that predicted by Eqs. 3 and 4.

RESULTS

Proteins. The molecular weights of the proteins are given in Table 1. The values for rod and LMM (250,000 and 140,000, respectively) are in good agreement with values reported elsewhere (17, 20, 22, 35). The value for S-2 (116,000) is substantially larger than the values reported by Lowey *et al.* (40), Biró *et al.* (36), or Goodno *et al.* (20) (62,000, 74,000, and 86,000, respectively), but trypsin untreated by tosylphenylalanine chloromethyl ketone was used in those preparations. Weeds and Pope (35), using α-chymotrypsin, obtained a value for the molecular weight of S-2 (118,000) equal to ours. Within the accuracy of $NaDodSO_4$-electrophoretic methods (24), the sum of the molecular weights for S-2 and LMM should equal the molecular weight for rod. It does when our value [or that of Weeds and Pope (35)] for S-2 is used rather than the values in the older literature. All our proteins showed one major band on $NaDodSO_4$/polyacrylamide gels, as shown in Fig. 2.

Relaxation Times. A typical trace of the build-up and decay of birefringence is shown in Fig. 3 for a dilute solution of rod. Eq. 1 was fitted to the decay of the birefringence for solutions containing rod between 0.01 and 4 μM. The results for three

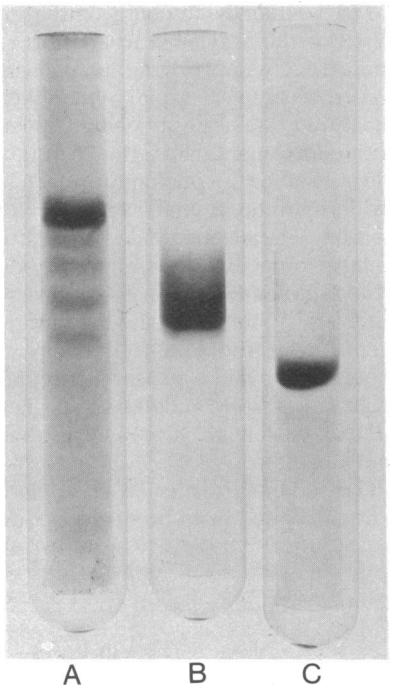

FIG. 2. Rod and fragments. NaDodSO$_4$/4½% polyacrylamide gels of (A) rod, (B) LMM, and (C) S-2 stained with Coomassie blue.

different rod preparations are shown in Fig. 4. The longer decay times measured at higher concentrations were taken to be due to aggregates. The decay time for monomeric rod was calculated using concentrations less than 0.1 μM, at which the decay time became independent of concentration within experimental

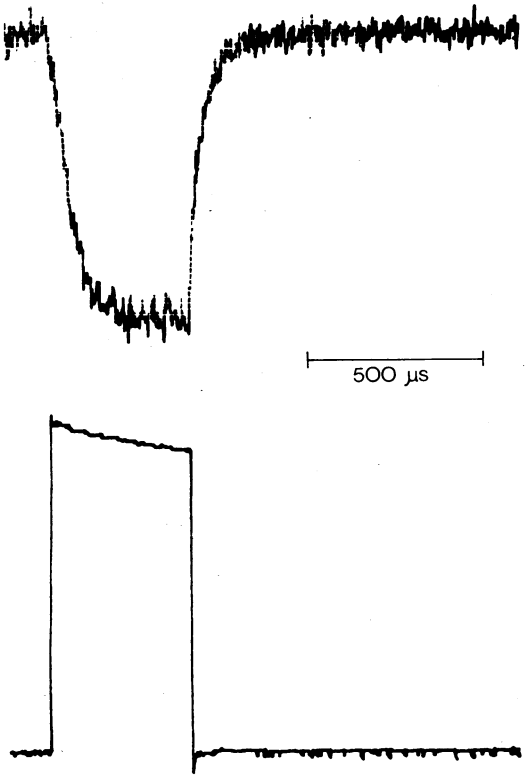

FIG. 3. Electrical birefringence trace. A typical result for 51.6 nM solution of rod at 3° in 2 mM $Na_4P_2O_7$ at pH 9.3. The decay (upwards) on the right was analyzed to obtain the relaxation time, τ. The pulse is shown below the birefringence signal.

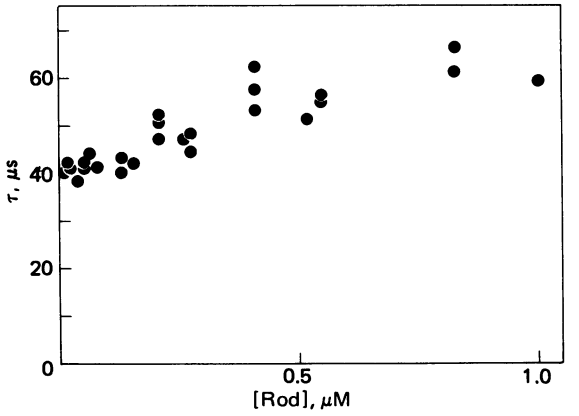

FIG. 4. Concentration dependence of relaxation times for solutions of rod in 2 mM $Na_4P_2O_7$, pH 9.3 at 3°. These are results from three protein preparations.

error. As shown in Table 1, the average relaxation time for rod was 41.2 μs. (The random errors for this and other measurements are shown in Table 1.) Relaxation times for LMM were obtained for solutions containing 0.5–15 μM protein (Fig. 5). The average relaxation time for monomeric LMM, obtained at concentrations less than 2 μM, was 13.1 μs.

Fig. 6 shows the relaxation times obtained for solutions of S-2 between 0.34 and 10 μM. The relaxation times are independent of concentration within experimental error over the entire range, suggesting that there is no aggregation of S-2 under these conditions. The mean of relaxation time measurements on 13 samples was 6.0 μs.

Under conditions where only monomers were thought to exist, the rates of decay for all three proteins were adequately described by single exponentials (Eq. 1). Two exponentials (Eq. 2) must fit the data better. However, erratic values for τ_1, τ_2, Δn_1, and Δn_2 were obtained using Eq. 2, indicating that the data did not justify such a treatment. Accordingly, only single exponential τ values are reported here.

CONCLUSIONS

Our method for preparation of S-2, using trypsin treated with tosylphenylalanine chloromethyl ketone, yields S-2 of greater molecular weight than has been obtained previously with trypsin (17, 20, 36). Thus, S-2 and LMM now account for all of the rod molecule, in agreement with the recent results of Weeds and Pope (35). The large variation in molecular weight with preparative method suggests that S-2 might contain an extended region of the molecule, perhaps 30 nm long, corresponding to the region of S-2 susceptible to proteolytic attack. Indeed,

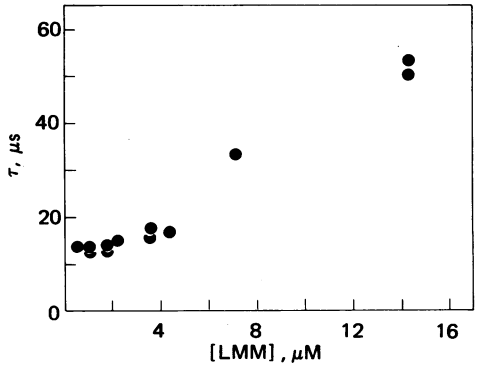

FIG. 5. Concentration dependence of relaxation times for LMM in 2 mM $Na_4P_2O_7$, pH 9.3 at 3° (two preparations).

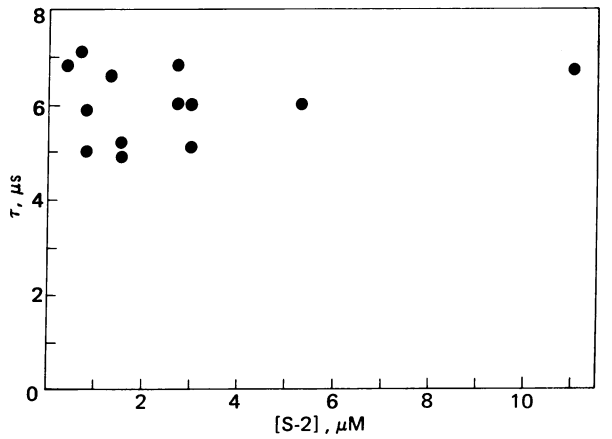

FIG. 6. Concentration dependence of relaxation times for S-2 in 2 mM $Na_4P_2O_7$, pH 9.3 at 3° (three preparations).

studies of the regions of myosin particularly susceptible to tryptic digestion (19, 37) indicate that up to 40 nm of the interior of the rod is susceptible and therefore potentially flexible.

The fact that LMM and S-2 prepared by our methods account for *all* of the rod allows us to test the rod for flexibility by a very direct method. In this method, Eq. 3 is used to calculate equivalent rigid lengths for LMM and S-2 from their relaxation times. These lengths are then added and Eq. 3 is used again, this time to calculate a τ for rod. This calculated τ is what one should observe if the rod is a rigid cylinder composed of S-2 and LMM. The only approximation used is the value for the cylinder radius (b in Eq. 3). Assuming rod is a coiled-coil of α-helices, its dimensions are calculable (38), and the radius of a hydrodynamically equivalent cylinder may be taken as 1 nm. The exact value of b is of little importance because in Eq. 3 θ is insensitive to b. Lengths calculated this way are in Table 1. The calculated τ for rod is 57.3 μs (SD = 1.1 μs). The experimental τ, 41.2 μs, is significantly less ($P < 0.001$). Therefore, the rod cannot be a straight rigid structure having a length that is the sum of the lengths of LMM and S-2. On the basis of this result alone, rod might be a rigid *non*-collinear arrangement of LMM and S-2. It is difficult to imagine what intramolecular forces would produce rigidity in such a structure, and indeed the long, proteolytically vulnerable stretch found in S-2 suggests a much more attractive alternative, namely, that there is a flexibility in the S-2/LMM region. As noted above, such flexibility would explain certain assumptions of cross-bridge mechanics, and would be consistent with Taylor's report (39) that fiber birefringence falls when S-1 moves away radially from the thick filament axis.

When the lengths calculated from the relaxation times are compared to experimental lengths (see Table 1), further details about the flexibility can be obtained. For LMM the length calculated from Eq. 3 and the length actually observed are in excellent agreement (significance of difference, $P \simeq 0.5$). This indicates that LMM is straight and rigid. The calculated length for S-2, however, is less than the experimental value (significance of the difference, $P < 0.001$). This indicates that S-2 is not straight and rigid. Likewise the calculated and experimental lengths for rod indicate flexibility (significance of difference, $P < 0.001$). This flexibility of rod resides in S-2, and probably in the region of the S-2/LMM junction, but not in LMM.

Two complications that could lead one erroneously to conclude that a molecule is flexible must be considered. The first is polydispersity in the form of substantial amounts of fragments smaller than the one being tested. This seems unlikely in the case of LMM and rod, which were prepared by precipitations that leave very small fragments in solution. Furthermore, the $NaDodSO_4$/polyacrylamide gels showed only major bands at appropriate molecular weights and the decay curves (fit by two exponential decays) did not show a systematic fast component. The second complication could be the artifactual introduction of flexibility into the coiled-coil structure by proteolytic cleavage of only one of the two α-helices. We sought to avoid this possible artifact in rod preparation by starting with thick filaments where the trypsin-susceptible area of myosin is presumably less accessible. Again, the $NaDodSO_4$/polyacrylamide gels gave no indication that significant nicking of the rod had occurred.

In conclusion, our data indicate that LMM is a rigid molecule. Rod and S-2, on the other hand, show relaxation times indicating that both of these molecules are probably flexible. These results confirm the prediction made from susceptibilities to proteolysis results (19, 37) and heat denaturation studies (19, 20). Our data do not distinguish between the possibilities that S-2 is flexible along its entire length, or that it possesses a single restricted flexible region. Results from proteolytic degradation support a restricted region of flexibility. One note of caution should be appended to these conclusions. In order to achieve the necessary solubility, we conducted our measurements at pH 9.3; it is conceivable that at physiological pH the flexibility we have found is modified. The degree of flexibility of the rod is also of interest. It is clear that the rod is flexible enough to undergo intramolecular Brownian rotational motion, and if there is any elasticity in the flexible region, it is insufficient to restrain this motion to any large extent in solution.

We wish to thank Profs. Susan Lowey and Bruno Zimm for critical readings of the manuscript. We also thank Dr. David Davore for advice and for his work modernizing the electro-optical apparatus, Ms. Margaret Davenport for technical assistance, and Mr. Dennis Reese and Mr. Ernest Haberkern for computer assistance. S.H. is a U.S. Public Health Service Postdoctoral Fellow. K.M.K. was a fellow of the British Science Research Council and is at present a Visiting Scientist of the American Heart Association, on leave from University College, London. M.F.M. is a Career Investigator of the American Heart Association. This study was supported by U.S. Public Health Service Grant HL-16683, National Science Foundation Grant PCM-75-22698, American Heart Association Grant CI-60, and in part by U.S. Public Health Service Grant CA 12,540.

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