Fluorescence Depolarization Studies on the Flexibility of Myosin Rod†

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ABSTRACT: The single photon counting method has been used to measure the decay of fluorescence polarization anisotropy of myosin rods labeled with extrinsic fluorophores. Rods labeled with 8-anilino-1-naphthalenesulfonate (ANS) or 5-dimethylaminonaphthalene-1-sulfonyl chloride (DNS-Cl) exhibit negative rotational correlation times; the anisotropy increases with time. Possible artifactual causes for the negative decay times are ruled out. It is shown that such curves are to be expected for rigid rods when the fluorophore is bound so that the absorption and emission dipoles each make a small angle with the long axis of the molecule and lie on opposite sides of the rod. At pH 4 and below, rapid decay of the anisotropy (positive correlation times) indicates the presence of a freely bending region in the rod. This is probably the proteolytically sensitive region between light meromyosin and heavy meromyosin subfragment 2. At pH 8, no such free bending is observed, even at temperatures as high as 50 °C. From this observation and other physical properties of the rod, we conclude that, at pH 8, the hinge region has considerable resistance to bending. It is more like a spring than a free hinge. The rotational diffusion about the rod axis is faster than would be predicted for a rigid, smooth molecule.

In the sliding filament model of muscle contraction (Huxley, 1969), the thick filament is formed by the aggregation of the tails of myosin molecules, while the myosin heads interact with the actin filament (Figure 1). Because the interfilament distance is about the same as the length of myosin S1, part of the myosin rod may extend out from the thick filament (Lowey, 1971).

Myosin has two regions which are easily digested by a variety of proteolytic enzymes. Trypsin digestion of myosin produces LMM[1] and HMM (Gergely, 1953; Szent-Györgyi, 1953), while papain digestion of HMM gives S1 and S2 (Lowey et al., 1969). Papain may also be used to digest myosin, to produce S1 and rod (Lowey et al., 1969). The papain-sensitive region at the base of S1 has often been suggested to possess some flexibility, allowing the myosin head to pivot while attached to actin as the filaments slide past one another. This flexibility has been demonstrated experimentally by nanosecond fluorescence depolarization measurements (Mendelson et al., 1972, 1973).

In addition to the hinge at the base of S1, it is possible that the trypsin-sensitive region between LMM and S2 may possess some flexibility. Pepe (1967) and Huxley (1969) pointed out that flexibility at that site would enable the entire HMM region to move toward and away from the thin filament at successive steps in the force-generating cycle. It is known that both LMM and S2 are rigid, coiled coil rods (Lowey et al., 1969; Harrington and Burke, 1972), so that it should be possible to find experimental means of detecting flexibility between these fragments, if it exists. In an early review, Lowey (1971) concluded that there was no strong evidence for a hinge. In later work, however, Burke et al. (1973) combined results from viscosity measurements and optical rotatory dispersion measurements, finding evidence for the presence of a region of low thermal stability between S2 and LMM. They concluded that the rod does bend when the temperature is raised to 35 °C or the pH is lowered to 2, even though there is no significant loss of helical content under those conditions.

Since the shape of the myosin rod is known (Lowey et al., 1969; Harrington and Burke, 1972), theoretical curves can be generated which will predict the time course of the decay of the fluorescence polarization anisotropy when labeled rods are excited with a pulse of polarized light. These curves, based on the assumption of rod rigidity, can be compared with experimental curves. If the rod is not rigid, the anisotropy will decay more rapidly than if it is rigid. This technique has previously been used to show flexibility of immunoglobulin G (Yguerabide et al., 1970) and of the region of myosin between S1 and the rod (Mendelson et al., 1972, 1973).

In the present study we have made fluorescence depolarization measurements using the single photon technique (Yguerabide, 1972) to look for flexibility in myosin rods. This method has the advantage that, unlike viscosity measurements, the Brownian motion of the macromolecule is observed without the imposition of an external stress. This is particularly important in cases where only limited flexibility exists, for even small flow stresses may produce bending in such cases. We have found that the rod does bend quite freely at pH 2 but that at pH 8 no intrinsic bending occurs below 50 °C. From the comparison of our results with the work of Burke et al. (1973), we conclude that, at neutral pH, the rod is probably like a spring in the hinge region between S2 and LMM; it can be bent, but there is an appreciable restoring force opposing the bending.

Materials and Methods

Proteins. Myosin rods were prepared from rabbit skeletal muscle myosin by the method of Harrison et al. (1971). A solution of myosin, at a concentration near 10 mg/mL, was precipitated by dialysis at 4 °C against 0.2 M ammonium acetate, pH 7.0. Papain was dissolved at a concentration of 2 mg/mL in 5 mM cysteine (freshly prepared), 2 mM EDTA, pH 6.0. The suspension of precipitated myosin was brought to room temperature, the papain solution was added to give a papain concentration of 0.08 mg/mL, and the digestion was allowed to proceed for 10 min, with stirring. Digestion was stopped by adding iodoacetic acid to a concentration of 1 mM,

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[1] Abbreviations: HMM, heavy meromyosin; LMM, light meromyosin; S1 and S2, myosin subfragments 1 and 2, respectively; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; ANS, 8-anilino-1-naphthalenesulfonate; 1,5-IAEDANS, *N*-iodoacetyl-*N'*-(1-sulfonyl-5-naphthyl)ethylenediamine; DNS-Cl, 5-dimethylaminonaphthalene-1-sulfonyl chloride.

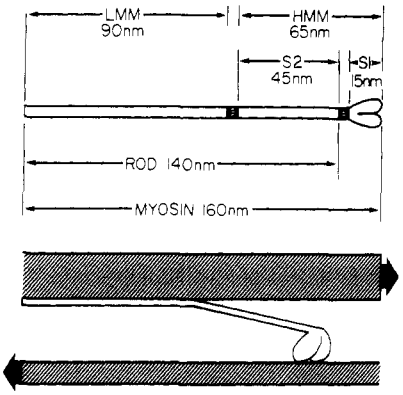


FIGURE 1: Substructure of the myosin molecule and a schematic diagram of the relationship between myosin substructure and the interaction of the thick and thin filaments. The dimensions are from Tonomura (1973), and the cross-hatched regions indicate the areas of proteolytic sensitivity. It is not known how much of the rod lies within the thick filament. We have shown S2 reaching into the interfilament space, but this is conjecture.

with rapid chilling to 0 °C. The digestion solution was then centrifuged at 63 000*g* for 1 h. The precipitate, containing rods, undigested myosin, and perhaps other fragments, was resuspended in 50 mL of 0.6 M KCl, 50 mM Tris, pH 8.0. Three volumes of ethanol was then added dropwise, with stirring, and the resulting suspension was stirred at room temperature for 2 h. After centrifugation at 22 000*g* for 30 min, the proteins were resuspended in 20 mL of 0.6 M KCl, 50 mM Tris, pH 8.0, and dialyzed against three changes of this same solvent to remove all ethanol. The rods were redissolved by this procedure. The other proteins were removed from the solution by centrifugation at 63 000*g* for 1 h. The resulting rods were homogeneous and uncontaminated with other proteins, judging from sodium dodecyl sulfate gel electrophoresis. Intrinsic viscosities of the rods ranged from 2.7 to 2.9 dL/g, indicating the absence of contamination by LMM or S2 (Lowey et al., 1969; Harrington and Burke, 1972). Protein concentrations were determined by the method of Lowry (1951).

Since ANS does not covalently bond to proteins, ANS labeling was achieved by mixing ANS and rods in a molar ratio of 1:1. ANS does not fluoresce when free in aqueous solution, so only the bound dye contributes to the observed anisotropy decay curves (Stryer, 1965). For labeling with either DNS-Cl or 1,5-IAEDANS, rods were incubated overnight with a dye:protein molar ratio of 0.8. Incubation was carried out at 4 °C, in the dark. The labeled protein was then dialyzed against three changes of 30 volumes of 0.6 M KCl, 50 mM Tris, pH 8.0, to remove any dye not covalently bonded to the protein. Samples were diluted in this same solvent. Harrington and Burke (1972) believe that even at high salt concentrations myosin rods associate, and that a rapidly reversible equilibrium exists, probably between monomers and dimers. As a precaution, all samples for fluorescence depolarization measurements were prepared at a concentration of 0.04 mg/mL or less. At this concentration the monomer:dimer ratio is greater than 50:1 (Harrington and Burke, 1972), and any dimers present will make a negligible contribution to the decay of the fluorescence polarization anisotropy.

Fluorescence Depolarization. When a solution of fluorescent macromolecules is excited with a pulse of polarized light, the resulting fluorescence is polarized. The fluorescence anisotropy

$$A(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \qquad (1)$$

is determined by measuring $I_{\parallel}(t)$ and $I_{\perp}(t)$, the components of fluorescence polarized parallel to, and perpendicularly to, the direction of polarization of the exciting light. The time course of the decay of the anisotropy depends on the rotational Brownian diffusion of the macromolecule. If the macromolecule is rigid and has the shape of an ellipsoid of revolution, $A(t)$ is the sum of three exponentials

$$A(t) = \sum_{i=1}^{3} c_i e^{-t/\phi_i} \qquad (2)$$

The correct relationship between the molecular parameters and the parameters of eq 2 was derived independently by three different groups (Belford et al., 1972; Chuang and Eisenthal, 1972; Ehrenberg and Rigler, 1972) and has been verified by Monte Carlo simulations (Harvey and Cheung, 1972). The characteristic decay times, ϕ_i, depend on the rotational diffusion coefficients, which, in turn, depend on the size and axial ratio of the ellipsoid and on the temperature and solvent viscosity (Perrin, 1934). The coefficients, c_i, are functions of the orientations of the absorption and emission dipoles in the macromolecule. If the molecule has significant internal flexibility, the anisotropy will decay more rapidly than in the case of an identically shaped rigid molecule with the same dye orientation, because the bending motion increases the rate at which the alignment of emission dipoles is randomized.

Fluorescence lifetime and anisotropy decay curves were measured with a single photon fluorometer (Yguerabide, 1972). The instrument was built in our laboratory and uses two photomultipliers, allowing the concurrent measurement of $I_{\parallel}(t)$ and $I_{\perp}(t)$. The two phototubes were calibrated with respect to one another just before each experiment, as follows: With the sample in place, the analyzers were turned parallel to the polarization of the exciting light, so they both measured $I_{\parallel}$. A balance factor, f, was then determined by accumulating several million counts and calculating the ratio of the number of photons observed in the two phototubes

$$f = I_1/I_2$$

where I_1 and I_2 are the intensities measured in the tubes which would later measure $I_{\perp}$ and $I_{\parallel}$, respectively. Variable apertures were then adjusted to get f as close to 1.0 as possible, and the process was repeated until f fell in the range 0.995–1.005. The resulting balance factor was used to correct the data by multiplying measured values of $I_{\parallel}$ by f before calculating the anisotropies using eq 1. With several million counts accumulated to determine f, a detailed analysis of error propagation shows that the error in the anisotropy due to errors in f is small, for the error in $fI_{\parallel}$ is nearly all due to the Poisson-distributed error in $I_{\parallel}$ (see the note at the end of this paper regarding supplementary material).

The exciting pulse came from a spark bursting in air. Excitation wavelength was selected by a grating monochromator set to 336 nm, or by either of two Corning band pass filters (7-37: transmission maximum near 360 nm; or 7-60: transmission maximum near 355 nm). In the latter two cases, excitation is due to a combination of the nitrogen lines at 336, 350, and 370 nm. For some samples, both lifetimes and anisotropies were measured first with the monochromator and then with excitation filters, and the observed decays were found to be independent of the method of selecting the excitation wavelength. Emission was observed through a Corning 3-144 filter followed by a Corning 3-72, giving a cutoff wavelength near 450 nm. The 3-144 is a low-fluorescence filter. When it was omitted, light scattered by the rods caused appreciable secondary fluorescence from the 3-72 filter, biasing the mea-

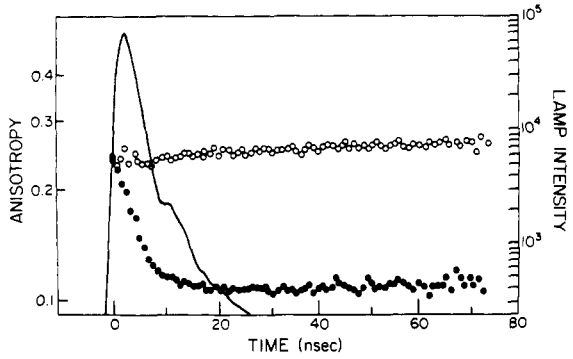


FIGURE 2: Anisotropy decay curves for myosin rods labeled with DNS-Cl (open circles) and with 1,5-IAEDANS (closed circles). A semilog plot of the exciting light pulse is also shown (solid line and scale at right). The fitted rotational correlation time for the DNS-rods curve is −780 ns. T = 7.5 °C, pH 8.

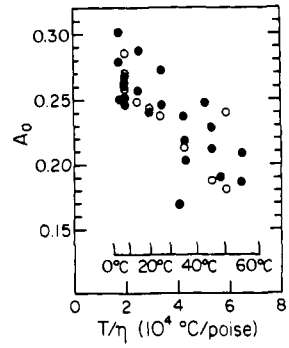


FIGURE 3: Dependence of the limiting anisotropy, A_0, on temperature. T/η has been chosen for the abscissa so this figure could be compared with Figure 7; no functional relationship is implied here. Open circles indicate ANS-rods, closed circles, DNS-rods.

sured decays. Employment of the 3-144 eliminated this secondary fluorescence.

Lifetime data were deconvoluted with a computer program based on the method of moments (Isenberg et al., 1973). Deconvolution of the anisotropy decay curves did not affect measured rotational correlation times, since these were much longer than the characteristic times of the lamp pulse.

Because of their length (140 nm), myosin rods scatter appreciable amounts of light, particularly the short wavelength exciting light. Although most of this is removed by the emission filters, the filters do have a finite, nonzero transmittance, and some excitation photons which are scattered by the sample do get counted by the fluorometer. To correct for this, blanks containing unlabeled rods were run at conditions identical with those of each sample, and the counts from these blanks were subtracted, channel by channel, from the sample decay curves. The background counts comprised 5–15% of the total counts at temperatures below 10 °C and between 30 and 70% of the total counts for temperatures above 50 °C. It should be pointed out that our correction procedure does correct for direct scattering of excitation photons into the photomultipliers. It does not, however, compensate for the fact that the lamp pulse is slightly depolarized before absorption because some photons are scattered before being absorbed. Nor does it compensate for depolarization of the fluorescence due to scattering of the emitted photons. The size of these scattering effects is discussed below.

Results and Discussion

Figure 2 shows typical decay curves for the fluorescence anisotropy of myosin rods labeled with DNS-Cl and with 1,5-IAEDANS. The 1,5-IAEDANS is apparently not rigidly bound to the rod, and that decay curve will be discussed later. Rods labeled with ANS give curves which are similar to those of DNS-rods under the same conditions.

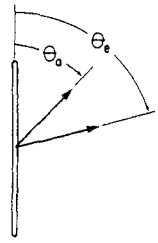
The DNS-rods curve is striking in that it is, as far as we know, the first experimentally observed case of a rising anisotropy curve, i.e., a negative rotational correlation time. We need to carefully examine the experimental conditions to be certain that these observations are not artifacts.

First, we note that we have made a total of nine different rod preparations, and rising curves have been observed in all of them. Also, we have made anisotropy measurements on other large fluorescent macromolecules and have not seen this effect either with myosin labeled with 1,5-IAEDANS, where we repeated the measurements of Mendelson et al. (1972, 1973), nor with F-actin labeled with 1,N^6-ethenoadenosine diphosphate (Harvey et al., 1977), nor with F-actin labeled with other fluorophores (Cheung et al., unpublished results). In all of these cases, either positive correlation times or flat anisotropy curves, indicating very long rotational correlation times, were obtained. At pH 8, we have measured the rotational correlation times for 15 different samples of ANS-rods and DNS-rods in the temperature range 4–8 °C. The average slope for those experiments was 0.286 μs^{-1} with a standard error of the mean of 0.139 μs^{-1}. Thus, the positive slope (negative correlation time) is statistically significant ($t = 2.06$, $P < 0.05$, 14 df). A detailed error analysis is available (see note at the end of this paper regarding supplementary material).

Although the ANS-rods and DNS-rods decay curves are affected by the background subtraction procedure, the uncorrected data show slopes that are about the same as the corrected data, so the effects of the correction process are small. As mentioned above (Materials and Methods), our background subtraction procedure does not compensate for any scattering depolarization of either the exciting light or the fluorescence. Both of these effects will produce lower values of A_0, the limiting anisotropy, and this does occur at higher temperatures, where samples become more turbid (Figure 3). The scattering effects are time independent; the photon is either scattered or it is not, but the probability of scattering does not depend on the time after excitation. Thus, a constant fraction of the emitted light is scattered throughout a given experiment. The anisotropy is reduced by a time-independent scaling factor, and the measured decay time should not be biased in either direction. We have used our correction procedure on visibly turbid samples of F-actin and acto-heavy meromyosin, and have obtained the same result as did Martonosi and Teale (1965) in their steady state measurements on DNS-F-actin: polarizations are affected by scattering, but measured rotational relaxation times are not.

The procedure for balancing the two photomultipliers (Materials and Methods) cannot account for the observed negative correlation times, because these experiments were made over a period of several months. Since the instrument was used for other experiments during that time, the balancing procedure had to be repeated before each series of measurements on the rods. As a consequence, the effects due to the errors in the balance factor are random. A detailed error analysis also shows that they are small, so errors in the balance factor are not responsible for the rising anisotropy curves.

Our samples probably contain mixtures of rods of different lengths, but this cannot be the cause of the observed negative correlation times. Anisotropies are additive, so, in the case of several molecular shapes, the observed anisotropy is the weighted average anisotropy (Rigler and Ehrenberg, 1973).



CURVE NO.	θ_a	θ_e
1	-15°	15°
2	0°	30°
3	20°	50°
4	40°	70°
5	60°	90°
6	75°	105°
7	Random	Orientation

FIGURE 4: Geometry of the model used for the labeled rod in the calculations outlined in the text. θ_a and θ_e indicate the angles between the rod axis and the absorption and emission dipoles, respectively. An angle of 30° between the two dipoles has been used throughout. At the right are given the values of θ_a and θ_e for the curves in Figure 5.

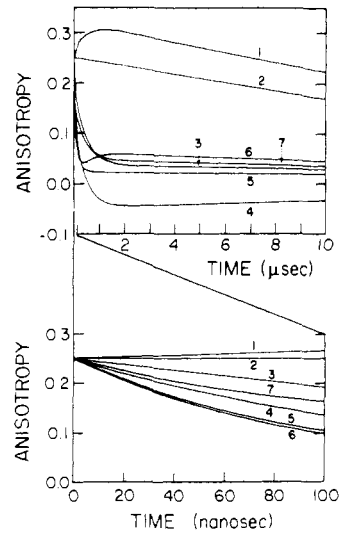


FIGURE 5: Theoretical anisotropy decay curves for the rod model discussed in the text. The different curves correspond to the various dye orientations specified in Figure 4. The upper set of curves shows the anisotropy decay over a period of 10 μs, a time scale appropriate for the end-over-end rotation of the rod. The lower set of curves shows only the first 100 ns of the same decay, a time scale appropriate to the dye lifetime and to the rotation of the rod about its long axis.

If rods of a given length were characterized by a positive correlation time (monotonic decay of anisotropy), then the mixture of rods of various lengths would also exhibit a monotonically declining anisotropy. Consequently, our rising curve (Figure 2) is not the result of the heterogeneity of rod lengths.

Thus, the observed negative correlation times are real, not artifacts. What is their cause?

In his original treatment of the effects of Brownian motion on fluorescence polarization, Perrin (1936) pointed out that Brownian rotations could, under special circumstances, produce an initial increase in polarization. We have calculated anisotropy decay curves for rigid myosin rods from eq 2 and Perrin's equations (1934), using the model shown in Figure 4. Approximating the rod by a prolate ellipsoid introduces errors in correlation times which are a few percent, at most (Broersma, 1960). We assume a molecular weight of 2.3×10^5 (Lowey et al., 1969), a hydration of 0.3 g of H_2O/g of protein, a dry protein density of 1.37 g/cm^3, and a rod length of 140 nm, giving an axial ratio of 60. An angle of 30° between absorption and emission dipoles has been chosen, to agree with the observed A_0 of 0.25. The rod axis and the two dipoles have been chosen to be coplanar, because initially rising curves are only produced if the two dipoles each make a small angle with the rod axis and lie on opposite sides of it. Figure 5 shows the decay curves for different orientations of the fluorophore.

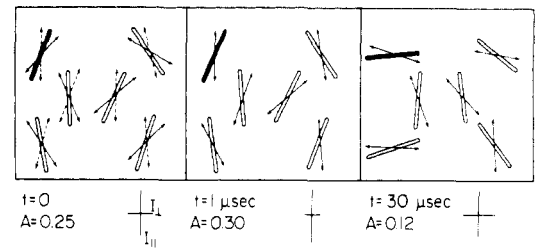


FIGURE 6: Schematic diagram of the physical basis for the rising anisotropy decay curves. Absorption dipoles are shown only at $t = 0$ (dashed arrows). As discussed in the text, the rotation *about* the rod axis produces an increased alignment of the emission dipoles (solid arrows) at $t = 1$ μs, while the slower rotation *of* the rod axis slowly depolarizes the fluorescence, giving a lower anisotropy at $t = 30$ μs. One particular rod has been darkened; its motion is typical and illustrates this process. Translational diffusion does not affect the anisotropy, so it has been ignored.

Curve 1, like our data, has an initially positive slope. (It should be noted that the curve shapes do not depend on the position of the dye binding site on the rod. The angles between the transition dipoles and the rod axis determine the coefficients, c_i, in eq 2, so these angles are the only parameters of dye orientation which affect $A(t)$. Trypsin digestion of DNS-rods suggests that DNS-Cl binds nonspecifically, for both the LMM and S2 fractions were found to contain DNS-labeled protein. Since ANS binds noncovalently, it probably binds to several sites on the rod, too.)

The physical basis for the original increase in anisotropy can be understood by reference to Figure 6. We consider the exciting light pulse to be vertically polarized. It selects, at time zero, those molecules whose absorption dipoles (dashed arrows) are most nearly parallel to the direction of polarization. Since the rotational diffusion of a rod or ellipsoid about its long axis is much more rapid than is the end-over-end diffusion, the Brownian motion will initially cause a rotation of the emission dipoles (solid arrows) around the rod axes, which stay essentially stationary. This motion works to swap the locations of the emission and absorption dipoles, causing an increased alignment of the emission dipoles along the vertical axis. The average projection of the emission dipoles grows along the direction of $I_\parallel$ and decreases along $I_\perp$, so the anisotropy grows with time. Only after long times, when the end-over-end rotation has moved the rod axes from their initial positions, will the orientations of the emission dipoles randomize enough to cause a drop in anisotropy. For myosin rods, the time constant for rotation about the rod axis is a few hundred ns, while the end-over-end rotation has a time constant of about 30 μs. The fluorescence lifetimes of the labels we have used are less than 20 ns, so it is only possible to observe the anisotropy through its rising phase. The eventual decline in anisotropy due to the end-over-end rotation occurs too slowly to be observed with these fluorophores. If appreciable rod bending were present, the bending motion should produce positive correlation times which are observable with these dyes, for such bending motions have been seen in myosin (Mendelson et al., 1972, 1973) and in immunoglobulin G (Yguerabide et al., 1970).

The observed negative correlation time is, then, consistent with that which would be produced by a rod which is rigid (or nearly so), with the dye orientation such that the transition dipoles are on opposite sides of the long axis of the molecule (curve 1 of Figure 5).

We have studied the effect of acid pH on the rotational correlation time of rods labeled with DNS-Cl at a temperature of 7.5 °C. At pH 8, the mean correlation time is −3500 ns (N = 15). At pH 6, the rod bends very little, if at all, for the cor-

relation time is still negative, $\phi = -730$ ns. At more acid pH, however, the correlation times are positive, with $\phi = 184$ ns at pH 4 and $\phi = 70$ ns at pH 2. The low pH decays are not due to denaturation or to a gross change in molecular shape (from a rod to a more compact form), because the helical content of the rod and the intrinsic viscosity of LMM are both unchanged as the pH is lowered to 2 (Burke et al., 1973). Nor is it likely that the positive correlation times are produced by local dye motion. If the rod stayed rigid and the dye had some motion about its bond to the rod, the anisotropy decay curve would have a pronounced two exponential character, as does the decay curve for 1,5-IAEDANS-rods (Figure 2). That curve falls rapidly at first, with a correlation time of only a few nanoseconds for the dye motion, and then it is nearly flat. The DNS-rods curves at pH 4 and pH 2 exhibit almost no curvature, however, and the decay times are too long to be due to dye motion. Thus, the data are consistent with a model where the dye remains rigidly bound to a rod that bends fairly freely at low pH. This result is in agreement with the conclusion of Burke et al. (1973) and correlates with thermal melting studies in which it was concluded that the absorption of protons accompanies the localized denaturation of the region between S2 and LMM (Goodno and Swenson, 1975).

The effect of temperature on rotational correlation times is shown in Figure 7. Accurate measurement of correlation times becomes more difficult as the temperature is raised, because increasing sample turbidity produces two effects. First, increased light scattering raises the background count rate, lowering the signal:noise ratio. Second, the scattering lowers the measured anisotropies (Figure 3). At lower values of the anisotropy, there is more statistical scatter in the data, so curve fitting is more subject to error (Yguerabide, 1972). Since the correlation time is the negative reciprocal of the slope of the anisotropy plot, and since these slopes are near zero, small errors in slope result in large errors in ϕ. These problems are reflected in the large scatter in the data in Figure 7 at temperatures above 30 °C. Above 50 °C, decay curves are highly variable, and we have been unable to get consistently repeatable results; we have observed single decays with both positive and negative correlation times, and we have obtained curves with multiexponential behavior.

While sample turbidity does cause considerable scatter in the data between 30 and 50 °C, we believe that rod bending, if present, would manifest itself through appreciable depolarization and observable *positive* correlation times. In this regard, we note that the signal:noise ratio is also degraded in experiments at acid pH, because of acid quenching of the fluorescence. In spite of high background rates (50% at pH 2), the rod bending was easily observed. None of the correlation times shown in Figure 7 even approaches the value at pH 2 ($\phi = 70$ ns; $1/\phi = 14\ \mu s^{-1}$).

Burke et al. (1973) observed that as the temperature was raised from 5 to 35 °C, rods showed a significant drop in intrinsic viscosity, while the intrinsic viscosity of LMM did not change. From these facts and their observation that both rods and LMM maintain nearly 100% helical structure over that temperature range, they concluded that the rod bends about the trypsin-sensitive region between LMM and S2. The rod bending at temperatures below 35 °C which is proposed by Burke et al. (1973) does not appear in our measurements.

There is a possible explanation for the apparent discrepancy between our results at temperatures below 35 °C and those of Burke et al. (1973). Viscosity measurements are made on flowing solutions. Even though the shear stresses are low, the forces on the macromolecule could be large enough to produce bending of the macromolecule under conditions where Brownian motion alone would not do so. In other words, if the molecule has a semirigid region, it may be deformed under the stresses of viscosity measurements but remain free of deformation in the undisturbed solvent of the fluorometer.

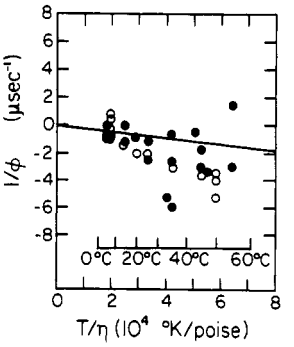


FIGURE 7: Effect of temperature on the rotational correlation time. A reciprocal plot has been used, because the experimentally measured parameter is the slope of the anisotropy decay curve, $-1/\Phi$. The solid line shows the harmonic mean correlation time for the calculation outlined in the text. Open circles indicate ANS-rods, closed circles, DNS-rods.

A significant loss of α-helical structure is known to occur at 45 °C, accompanied by a sharp drop in intrinsic viscosity of both LMM and myosin rods (Burke et al., 1973), so the supposed flexibility of the rod might be expected to manifest itself in positive rotational correlation times as the temperature is raised above 40 °C. Our depolarization measurements do not detect the anticipated flexibility (Figure 7). As discussed above, the repeatability and reliability of our measurements are degraded at elevated temperatures, because of turbidity and lower signal:noise ratios. Nonetheless, it is difficult to reconcile our results above 40 °C with the optical rotation and viscosity results of Burke et al. (1973). Consequently, in the discussion which follows, we confine our attention to temperatures below 40 °C.

We conclude that, at acid pH, the rod has considerable flexibility, probably due to a nearly free hinge in the region between LMM and S2. At neutral pH and temperatures below 40 °C, the rod can bend if it is subjected to external stresses. This bending is apparently opposed by appreciable stiffness in the hinge region, for it does not manifest itself in our fluorescence depolarization measurements. Physiologically, the ability to switch from a free-bending to a springlike hinge could give the myosin rod an important role in controlling the position of S1. The release of protons from nearby actomyosin ATPase activity would make the hinge flexible, allowing HMM to swing over to the thin filament; at a later time, the return to neutral pH would stiffen the rod, helping to swing HMM away from the thin filament between cycles (Pepe, 1967; Huxley, 1969).

One aspect of the temperature dependence of the rotational correlation time merits attention. The decay times can be calculated from the equations of Perrin (1934) and eq 2, as described above. It is easily shown that, for a fixed molecular geometry, the correlation time is inversely proportional to T/η, where T is the temperature and η the solvent viscosity. In order to get the largest possible negative slope to the plot of $1/\phi$ vs. T/η, we have chosen a value of $\theta_a = -15°$ and $\theta_e = 15°$ (see Figure 4), so the absorption and emission dipoles are on opposite sides of the rod axis. The results of one of these calculations, for a temperature of 20 °C, are given in Table I.

What value of ϕ do we expect to observe? We can expand the exponential functions of eq 2 in power series, dropping terms in t^2 and higher. This will give an approximate expression for $A(t)$ for early times, $t < \phi_i$. It will be valid for times up to

TABLE I: Parameters for Theoretical Anisotropy Decay Curves for Labeled Rigid Ellipsoids of Axial Ratio 60 (T = 20 °C).

$c_1 = 0.0013$	$\Phi_1 = 97$ ns
$c_2 = -0.0750$	$\Phi_2 = 385$ ns
$c_3 = 0.3237$	$\Phi_3 = 2.7 \times 10^4$ ns

$$A_0 = \Sigma c_i = 0.25$$

$$\Phi^* = \Sigma c_i / \Sigma \left(\frac{c_i}{\Phi_i}\right) = -1480 \text{ ns}$$

100 ns, even though $\phi_1 = 97$ ns, because of the small value of c_1. As an approximation, then

$$A(t) = \Sigma[c_i(1 - t/\phi_i)]$$

$$A(t) = (\Sigma c_i)(1 - t/\phi^*) \quad (3)$$

where the index of summation ranges from 1 to 3. ϕ^* is the harmonic mean correlation time, given by

$$\frac{\Sigma c_i}{\phi^*} = \Sigma \left(\frac{c_i}{\phi_i}\right)$$

Since ϕ^* has a large magnitude (Table I), for the time scale of interest eq 3 represents the series expansion of

$$A(t) = A_0 e^{-t/\phi^*} \quad (4)$$

where $A_0 = \Sigma c_i$. Equation 4 shows that the expected single exponential decay time for the rods anisotropy curves will be ϕ^*, the harmonic mean correlation time. The temperature dependence of ϕ^* is shown by the solid line of Figure 7.

Even with the most favorable dye orientation, and in spite of the scatter in the data in Figure 7, there is a clear difference between our data and the expected decay times for rigid ellipsoids. If we choose any other dye orientation, the slope of the line in Figure 7 is less steep, so the difference is not due to our choice of dye orientation.

The only other parameters which we have considerable latitude to adjust are the amount of bound water we attribute to the rod and the axial ratio. A wide range of hydration values is reported for different proteins, from about 0.1 to 1.0 g of H_2O/g of protein. We have chosen an intermediate value, 0.3 g/g. This is a reasonable value, based on the results of a variety of techniques (Kuntz et al., 1969). Even if we used a hydration value of 0.1 g/g, there would be only a 20% change in the slope of the theoretical line in Figure 7. Such a low hydration value would still leave a clear discrepancy between our data and the theoretical values.

The effect of changing the axial ratio is even smaller. ϕ_1 and ϕ_2 are insensitive to axial ratio, changing by less than 6% over the range of axial ratios from 20 to 200. ϕ_3 is very sensitive to axial ratio, but its impact on ϕ^* is relatively small. Calculated values of ϕ^* at 20 °C range from −2200 ns for an axial ratio of 20 to −1400 ns for an axial ratio of 200.

Thus, the parameters of the rigid ellipsoid model cannot be adjusted enough to make the theoretical line of Figure 7 coincide with our data. What we are evidently observing is a rotational diffusion about the rod axis at a rate higher than that predicted for a rigid ellipsoid.

We have argued that, if the rod were very flexible, the bending motion would depolarize the fluorescence and give positive rotational correlation times. Consequently, our observed negative correlation times indicate that, if the rod bends at all, such bending must be very limited. The rod is either rigid or nearly so. If the rod were a perfectly rigid ellipsoid, the data points in Figure 7 would be scattered along the theoretical line. Let us suppose, however, that the rod had *limited* flexibility, with the bending opposed by a restoring force, as described above. In that case, existing theory would not allow us to predict the time course of the decay of the anisotropy. It is possible that such limited flexibility would produce the dependence of ϕ^* on temperature shown by the data in Figure 7.

There is another possible explanation for the fact that the rotational diffusion of the rod is faster than that predicted for a rigid ellipsoid. It has been shown (Brenner, 1967) that the estimation of diffusion coefficients for macromolecules is subject to serious error whenever the macromolecular geometry is such that the translational and rotational diffusions are coupled. This coupling occurs in cases where the molecule possesses a screwlike assymetry. This could be the case for the myosin rod: since it is a coiled coil, its surface may be helical, like a screw. If this were so, the Brownian motion of translation along the rod axis would cause torques about the axis, and these torques would cause rotations. The rotational diffusion coefficient would be larger than it would be in the absence of this rotational-translational coupling. Although it is not possible to determine exactly how large these effects would be, our data are consistent with such a coupling mechanism.

Acknowledgments

We are indebted to Dr. R. A. Mendelson for suggesting this problem and for numerous discussions. Essential technical assistance was supplied by Ms. Susan Hamel and by the staff of the UAB Multilaboratory Computer Center.

Supplementary Material Available

Appendix I, a detailed error analysis, establishing the statistical significance of the negative rotational correlation times, and Appendix II, which examines the propagation of error of the balance factor, showing that it does not affect the results (7 pages). Ordering information is given on any current masthead page.

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Reactivity of Essential Thiols of Myosin. Chemical Probes of the Activated State†

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ABSTRACT: ^{14}C-Labeled fluorodinitrobenzene and *N*-ethylmaleimide have been used as chemical probes of the conformational states of myosin induced by the binding of MgADP and MgATP. The results indicate that in the high-energy conformation, M**MgADP·P_i, the essential thiols are protected from modification but their diminished reactivity does not result from depletion of the reagent by reaction at nonessential thiols. The binding of MgADP to myosin exposes the essential thiols as reflected by an increased rate of their modification. The influence of the divalent cations Mg^{2+} and Ca^{2+} on the conformation of the M** species has also been investigated. By monitoring the incorporation of fluorodinitrobenzene, the conformations of the M** state in the presence of these cations can be clearly discerned.

A large body of evidence is at hand which demonstrates that the ability of myosin to hydrolyze MgATP is closely linked to the reactivity of two sulfhydryls (designated SH_1 and SH_2) in each of the two globular, subfragment I regions of the molecule (Sekine and Kielley, 1964; Yamaguchi and Sekine, 1966). Recent work from this laboratory has shown that these sulfhydryls exist in close spatial proximity (12–14 Å); they can be bridged by the bifunctional reagent *p*-phenylenedimaleimide (pPDM) (Reisler et al., 1974b). It has also been observed that the near-ultraviolet (UV) circular dichroic (CD) spectrum of myosin covalently bridged between SH_1 and SH_2 by pPDM resembles closely that of myosin in the presence of MgATP, that is in the M**MgADP·P_i conformation (Burke et al., 1976). The M**MgADP·P_i state has been identified with the long-lived, rate-limiting species in the sequence of steps proposed for the binding and hydrolysis of MgATP to myosin (Bagshaw et al., 1974):

$$\mathrm{M} + \mathrm{MgATP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{M{\cdot}MgATP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{M^*MgATP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{M^{**}MgADP{\cdot}P_i} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{M^*MgADP{\cdot}P_i} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \mathrm{M^*{\cdot}MgADP} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} \mathrm{M{\cdot}MgADP} \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} \mathrm{M} + \mathrm{MgADP}$$

The involvement of SH_1 and SH_2 in the interaction with the substrate is also suggested by the observation that in the M**MgADP · P_i conformation these thiols are not readily reacted with sulfhydryl reagents, whereas in the M*MgADP conformation they are both exposed to such reagents (Schaub et al., 1975). The decreased reactivity of the essential sulfhydryls in the M** state was attributed by us to a protective effect imposed by the bound nucleotide (Reisler et al., 1974a). In fact, the quantitative measurement of the protective effect was used as a chemical assay of the M** state and was shown to monitor the shift in the relative population of this state in response to changes in temperature or ionic strength of the solvent (Harrington et al., 1975).

Recently, Schaub and Watterson and their collaborators (Schaub et al., 1975; Watterson et al., 1975) have suggested from their studies of incorporation of [^{14}C]MalNEt[1] that the decreased reactivity of the SH_1 and SH_2 thiols in the M**MgADP · P_i state results from a depletion of the reagent through reaction with a class of nonessential thiols exposed by the binding of the nucleotide. This conclusion is not consistent with our interpretation and with our postulate on a direct interaction between the sulfhydryls and the substrate in the M**MgADP·P_i conformation. Since the properties of the M** complex and the state of the essential sulfhydryls in this complex are central to our understanding of the mechanism of ATP hydrolysis we have reexamined the effect of the nu-

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[1] Abbreviations used are: MalNEt, *N*-ethylmaleimide; N_2phF, 1-fluoro-2,4-dinitrobenzene (FDNB is used in the figures); EDTA, ethylenediaminetetraacetic acid.