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# THE QUANTITATIVE MEASUREMENT OF ROTATIONAL MOTION OF THE SUBFRAGMENT-1 REGION OF MYOSIN BY SATURATION TRANSFER EPR SPECTROSCOPY

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According to current models of muscle contraction (Huxley, H. E., Science 164: 1356–1366 [1969]), motion of flexible myosin crossbridges is essential to the contractile cycle. Using a spin-label analog of iodoacetamide bound to the subfragment #1 (S1) region of myosin, we have obtained rotational correlation times ($\tau_2$) for this region of the molecule with the ultimate goal of making quantitative measurements of the motion of the crossbridges under conditions comparable to those in living, contracting muscle. We used the newly developed technique of saturation transfer electron paramagnetic resonance spectroscopy (Hyde, J.S., and Thomas, D.D., Ann. N.Y. Acad. Sci. 222:680–692 [1973]), which is uniquely sensitive to rotational motion in the range of $10^{-7}$–$10^{-3}$ sec. Our results indicate that the spin label is rigidly bound to S1 ($\tau_2$ for isolated S1 is $2 \times 10^{-7}$ sec) and that the motion of the label reflects the motion of the S1 region of myosin. The value of $\tau_2$ for the S1 segment of myosin is less than twice that for isolated S1, while the molecular weights differ by a factor of 4, indicating flexibility of myosin in agreement with the conclusions of Mendelson et al. (Biochemistry 12:2250–2255 [1973]). Adding F-actin increases $\tau_2$ in either myosin or isolated S1 by a factor of nearly $10^3$, indicating rigid immobilization of S1 by actin. Formation of myosin filaments (at an ionic strength of 0.15 or less) increases $\tau_2$ by a factor of 10–30, depending on the ionic strength, indicating a decrease of the rotational mobility of S1 in these aggregates. The remaining motion is at least a factor of 10 faster than would be expected for the filament itself, suggesting motion of the S1 region independent of the filament backbone but slower than in a single molecule. F-actin has a strong immobilizing effect on labeled S1 in myosin filaments (in 0.137 M KCl), but the immobilization is less complete than that observed when F-actin is added to labeled myosin

 

monomers (in 0.5 M KCl). A spin-label analog of maleimide, attached to the SH-2 thiol groups of S1, is immobilized to a much lesser extent by F-actin than is the label on SH-1 groups. The maleimide label also was attached directly to F-actin and was sufficiently immobilized to suggest rigid binding to actin.

## INTRODUCTION

The two subfragment 1 (S1) regions of myosin are the parts of the myosin crossbridges that are attached to the thick filament backbone via an α-helical rod portion (S2) and form links with actin during the contractile cycle. It has been proposed that this interaction requires segmental flexibility within myosin, allowing S1 to rotate relative to the thick and thin filaments as part of the mechanism of force generation (1, 2). Evidence for segmental flexibility within isolated myosin monomers in solution (3) and dependence of crossbridge orientation on the functional state of glycerinated muscle fibers has been obtained from fluorescence studies (4). Previous electron paramagnetic resonance (EPR) studies carried out with conventional techniques have shown that an iodoacetamide spin label (5–8) attached to the so-called SH-1 thiol group, which is located in the S1 region, is strongly immobilized, the rotational correlation time ($\tau_2$) being $10^{-7}$ sec or longer.

Conventional EPR and fluorescence techniques have been limited to the measurement of correlation times shorter (faster) than $10^{-7}$ sec, but EPR techniques have recently been developed that have maximum sensitivity to rotational motion in the range $10^{-7} < \tau_2 < 10^{-3}$ sec (9–14), a time range inaccessible to most other physicochemical methods. The new EPR techniques are referred to as saturation transfer spectroscopy. Application of an intense microwave field to the spin-labeled sample perturbs the thermodynamic equilibrium by partially saturating the spin system. Rotational diffusion modulates the anisotropic magnetic interactions and causes spectral diffusion, thus transferring saturation from one spectral position to another. By employing various detection schemes (9–13), one obtains spectra that are quite sensitive to the rate of saturation transfer and hence to the rate of rotational motion. Optimal sensitivity is obtained when $\tau_2$ is comparable to the spin lattice relaxation time ($T_1$), which is about $10^{-5}$ sec for slowly tumbling nitroxides (15).

One of these saturation transfer techniques has proven applicable to the study of solutions of spin-labeled proteins: absorption EPR, detected 90° out-of-phase with respect to the unsaturated second harmonic EPR response to the modulation field, $H_m \cos \omega_m t$ (10, 11, 13). If $\tau_2 \sim 1/\omega_m \sim T_1 \sim 10^{-5}$ sec, and the spin system is partially saturated, the competition between spectral diffusion and field modulation, in governing the passage of spins through the resonance condition, gives rise to a significant signal component that lags behind the modulation by 90° and is sensitive in shape to rotational diffusion.

In the present study we have used the saturation transfer spectra of an iodoacetamide spin label attached to the S1 region of myosin to study its motion in intact myosin, in enzymatically cleaved fragments of myosin, in synthetic thick filaments, and in complexes with actin. Evidence is presented that the label is attached with sufficient rigidity so that the motion detected can be attributed to the motion of the S1 segment itself. Some experiments with maleimide spin-labeled S1 and maleimide spin-labeled F-actin have also been carried out with this technique.

## EXPERIMENTAL PROCEDURES

### Spin-Labeled Proteins

The preparation of myosin (16) and spin-labeling of sulfhydryl 1 (SH-1) groups with N-(1-oxyl-2, 2, 6, 6-tetramethyl-4-piperidinyl)-iodoacetamide (IASL) was carried out as described previously (8, 17). To minimize the amount of weakly immobilized label ($\tau_2 \leqslant 10^{-9}$ sec) bound to myosin, 1 mole of the label was used per mole of myosin. In labeling sulfhydryl 2 (SH-2) groups, the more reactive groups were blocked with N-ethylmaleimide and the SH-2 groups were then labeled with N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide (MSL) (17). Heavy meromyosin (HMM) and S1 labeled at the SH-1 groups were prepared by digesting IASL-myosin with trypsin (16). The labeled myosin was dialyzed exhaustively against a solution containing 0.5 M KCl and 0.05 M Tris HCl (pH 7.5). HMM and S1 were dialyzed against solutions containing 0.1 M KCl and 0.05 M Tris HC1 (pH 7.5). Myosin filaments were prepared by dialysis of IASL-myosin in 0.5 M KCl and 1 mM TES (pH 7.0) against 100 vol of a solution containing 0.137 M KCl and 0.02 M Tris (pH 8.3). More aggregated preparations were obtained by dialysis against a solution containing 0.03 M KCl and 1 mM Tris (pH 7.5), or 0.10 M KCl and 1 mM TES (pH 7.0).

Actin was prepared from acetone-dried muscle powder by extraction at 4°C, followed by two polymerization-depolymerization cycles (18). Polymerization was induced by adding 0.1 M KCl and 0.6 mM $MgCl_2$. The resulting F-actin was then dialyzed exhaustively against whatever buffer it was to be combined with (see experiments below). Some of the F-actin was spin labeled with MSL essentially as described previously (19).

Human oxyhemoglobin was labeled with MSL in 0.10 M potassium phosphate buffer, pH 7.0, and concentrated to 250 mg/ml by pressure dialysis. Glycerol (Eastman, "spectro" grade), buffer, and the concentrated hemoglobin solution were then combined to produce samples containing 25 mg/ml protein in glycerol concentrations from 0% to 90% (vol/vol). These samples are referred to as maleimide spin-labeled hemoglobin (MSL-Hb). Similarly, solutions of IASL-S-1 (20 mg/ml) in various glycerol concentrations were prepared. Viscosites were measured for the glycerol-water solvents at 20°C, 5°C, and −12°C, using Cannon-Fenske calibrated capillary viscometers

### EPR Experiments

In this work, a Varian E-9 X-band spectrometer was employed in the absorption mode. For conventional EPR, 100 kHz field modulation was used. The resulting spectra are designated $v_1$. A straightforward modification (10) permitted modulation of the magnetic field at 50 kHz with phase-sensitive detection at 100 kHz. In this configuration, normal second derivative (second harmonic) EPR spectra, which we designate $v_2$, are obtained when the reference phase is set "in-phase."

Saturation transfer spectra were recorded "90° out-of-phase" and are designated $v_2'$. The out of phase setting was obtained by adjusting the reference phase to obtain a minimum signal (typically 1% of the maximum second derivative signal) with the incident microwave power well below saturation (typically 1 mW). Saturation transfer spectra were recorded at 63 mW incident power, corresponding to a microwave field amplitude of 0.25 gauss, as determined by the method of perturbing metal spheres (20). The modula-

tion amplitude was 5.0 gauss. High quality $v_2'$ spectra were obtained in 2–4 min for Hb and in 30 min for S1.

Samples were contained in a standard Varian quartz flat cell, and the temperature of the entire E-231 cavity structure was controlled to within 1°C by circulation of cooled nitrogen gas through it.

The conventional $v_1$ EPR method of McCalley et al. (21) was used to determine correlation times of the order of $10^{-7}$ sec. Our $v_1$ experiments were performed with a modulation amplitude of 2.0 gauss and subsaturating microwave power (typically 10 mW). For each sample, at least 10 determinations were made of S, the separation, in gauss, between the outer extrema of the spectrum. Since the spin label was very strongly immobilized by addition of F-actin to IASL-myosin (see below), the value of S for acto-IASL-myosin was used as the rigid limit value $S_0$. A theoretical plot of $\Delta S = S_0 - S$ vs. $\tau_2$ (21) was then used to determine $\tau_2$, the correlation time for Brownian reorientation of the nitroxide principal axis.

### Reference Spectra

The saturation transfer method of determining long correlation times is based on comparison of $v_2'$ spectra with a set of reference spectra. One such set contained computer-simulated spectra calculated with modified Bloch equations (11). The only parameter varied was $\tau_2$, the correlation time for reorientation of the molecule-fixed principal axis of the hyperfine and g tensors of a nitroxide spin label undergoing isotropic Brownian rotational diffusion. To simplify calculation, these tensors were assumed to have axial symmetry. When possible (i.e. for $\tau_2 > 10^{-6}$ sec), $\tau_2$ for the reorientation of the principal axis of the spin label has been determined by matching the wings of a $v_2'$ spectrum with those of reference spectra; the wings, in contrast to the center of the spectrum, are essentially insensitive to small deviations from axial symmetry or to anisotropic rotation (22).

In addition to the calculated reference spectra, empirical reference spectra were obtained from spin-labeled hemoglobin and also from S1 itself. Dilution of Hb below 25 mg/ml and of S1 below 20 mg/ml resulted in no significant change in $v_1$ or $v_2'$ spectra. Therefore, the solutions are dilute enough that $\tau_2$ is determined by the solvent viscosity, and we have estimated $\tau_2$ values for Hb from the expression (23)

$$\tau_2 = \frac{4\pi\eta R^3}{3kT} \qquad (1)$$

Hb is assumed to be a sphere of effective radius R = 29 Å (10) undergoing isotropic Brownian rotational diffusion; $\eta$ is the measured solvent viscosity.

The spin-label concentration, determined by double integration of $v_1$ spectra, calibrated with standard solutions of MSL, was $4.9 \times 10^{-4}$ M for the Hb solutions and $1.0 \times 10^{-4}$ M for the S1 solutions.

Since S1 is believed to have approximately the shape and hydrodynamic properties of a prolate ellipsoid with an axial ratio of about 4 (1, 3), Eq. (1) cannot be used directly to estimate $\tau_2$ for the protein. The long axis of a rigid ellipsoid with an axial ratio of 4 changes its orientation with a $\tau_2$ that is 3.4 times longer than that of a sphere of the same volume (24). We therefore obtained estimates of the $\tau_2$ for reorientation of the long axis of isolated S1 by first calculating $\tau_2$ (Eq. [1]) for a spherical protein of the same molecular

weight (115,000, reference 25) and then multiplying it by 3.4.

We consider the Hb spectra more reliable and have given them more emphasis than the other reference spectra in the determination of $\tau_2$. The conclusions of this study are essentially the same regardless of which set of reference spectra is used.

## RESULTS

In order to establish a basis for interpretation of the saturation transfer spectra of spin-labeled myosin in complexes, we studied myosin and its fragments in solution.

### IASL-Myosin and Fragments

**$v_1$ experiments.** The left side of Table I shows the $\tau_2$ values obtained in $v_1$ experiments with myosin or its tryptic fragments, having an iodoacetamide label bound to the SH-1 thiol groups. The rotational correlation times were determined at a number of different protein concentrations and the values in the table were obtained by extrapolation to infinite dilution. Within the 10–20% statistical uncertainties, these values were unchanged by dilution beyond 20 mg/ml.

**Saturation transfer experiments.** S1 and Hb reference spectra are shown in Fig. 1 recorded under various conditions are arranged in such a way that adjacent S1 and Hb spectra correspond to similar $\tau_2$ values for the protein, estimated from molecular size and shape, as described above. Compared on this basis, the Hb and S1 line shapes agree fairly well with each other and with calculated line shapes (11), particularly in the wings of the spectra, with small discrepancies between calculated and experimental spectra for $\tau_2 \geqslant 10^{-4}$ sec. This agreement indicates that each label is undergoing rotational diffusion at a rate predicted for the host protein. This has also been found for MSL-Hb in other studies (10, 14, 21, 26).

Fig. 2 shows the $v_2'$ results for solutions of IASL-myosin and the two fragments. The only significant differences in line shapes (Fig. 2) are in the center of the spectrum. A parameter $C'/C$ (see Table I) has been used to analyze these spectra. The results are summarized in the center of Table I, and the averages of the results obtained by the $v_1$ and $v_2'$ methods are shown at the right of Table I. Changing the KCl concentration from 0.1 to 0.5 M did not affect the correlation times for S1 and HMM.

These values of $\tau_2$ reflect primarily the rate of reorientation of the principal axis of the spin label; knowledge of the orientation and motion of this axis with respect to S1 is important for extracting information about S1 rotation from the spin label spectrum. The $\tau_2$ found for the label on the isolated S1, $1.85 \times 10^{-7}$ sec, is approximately the value predicted for the reorientation of the long axis of a prolate ellipsoid with an axial ratio of 4–5 and the same volume as S1 (24); it is four times that predicted for a sphere of the same volume (Eq. [1]). The rotation of such an ellipsoid about its long axis would be about as fast as the rotation of the sphere (24). The long $\tau_2$ observed shows that the label is rigidly bound with its principal axis aligned approximately parallel to the long axis of S1, and that S1 has little internal flexibility. Assuming that the immobilization of the label is not changed by protein-protein interactions, we can use the spin label in the following experiments to probe the motion of S1 as a whole, using the $v_2'$ spectra to estimate $\tau_2$ for the reorientation of the long axis of S1.

**TABLE I. Determination of $\tau_2$ for IASL-Myosin and Fragments in Solution**

| | $V_1$ Experiments[1] | | | $V_2'$ Experiments[2] | | | Averages[3] | |
|---|---|---|---|---|---|---|---|---|
| | $\Delta S$ (G) | $\tau_2 \times 10^7$ | $\tau_2/\tau_2$ (myo) | $C'/C$ | $\tau_2 \times 10^7$ | $\tau_2/\tau_2$ (myo) | $\tau_2 \times 10^7$ | $\tau_2/\tau_2$ (myo) |
| Myosin | 0.68 ± 0.10 | 2.53 ± 0.35 | 1.0 | −0.67 ± 0.05 | 3.7 ± 0.6 | 1.0 | 3.0 | 1.0 |
| HMM | 0.795 ± 0.10 | 2.15 ± 0.30 | 0.85 ± 0.20 | −0.71 ± 0.04 | 3.2 ± 0.4 | 0.86 ± 0.25 | 2.6 | 0.85 |
| S1 | 1.01 ± 0.10 | 1.63 ± 0.20 | 0.64 ± 0.15 | −0.81 ± 0.04 | 2.2 ±0.4 | 0.59 ± 0.25 | 1.85 | 0.62 |

$\tau_2$ is the effective correlation time for rotation of the principal axis of the spin label, in sec. $\tau_2/\tau_2$ (myo) is the ratio of $\tau_2$ to that obtained for myosin by the same method. Data from four preparations are combined and extrapolated to infinite dilution. Uncertainties in $\Delta S$ and $C'/C$ reflect standard deviations.

[1] $\Delta S$ is the decrease in the separation of the outer hyperfine extrema, as described in text. $\tau_2$ was determined from a theoretical plot of $\Delta S$ vs. $\tau_2$ (21).

[2] C is the height of the central peak above the baseline, and $-C'$ is the depth of this peak below the baseline. $\tau_2$ was determined from a plot of $C'/C$ vs. $\tau_2$, obtained from MSL-Hb reference spectra.

[3] $\tau_2$ values (column 7) are averages of columns 2 and 5, weighted according to uncertainties. Ratio values (last column) are averages of columns 3 and 6.

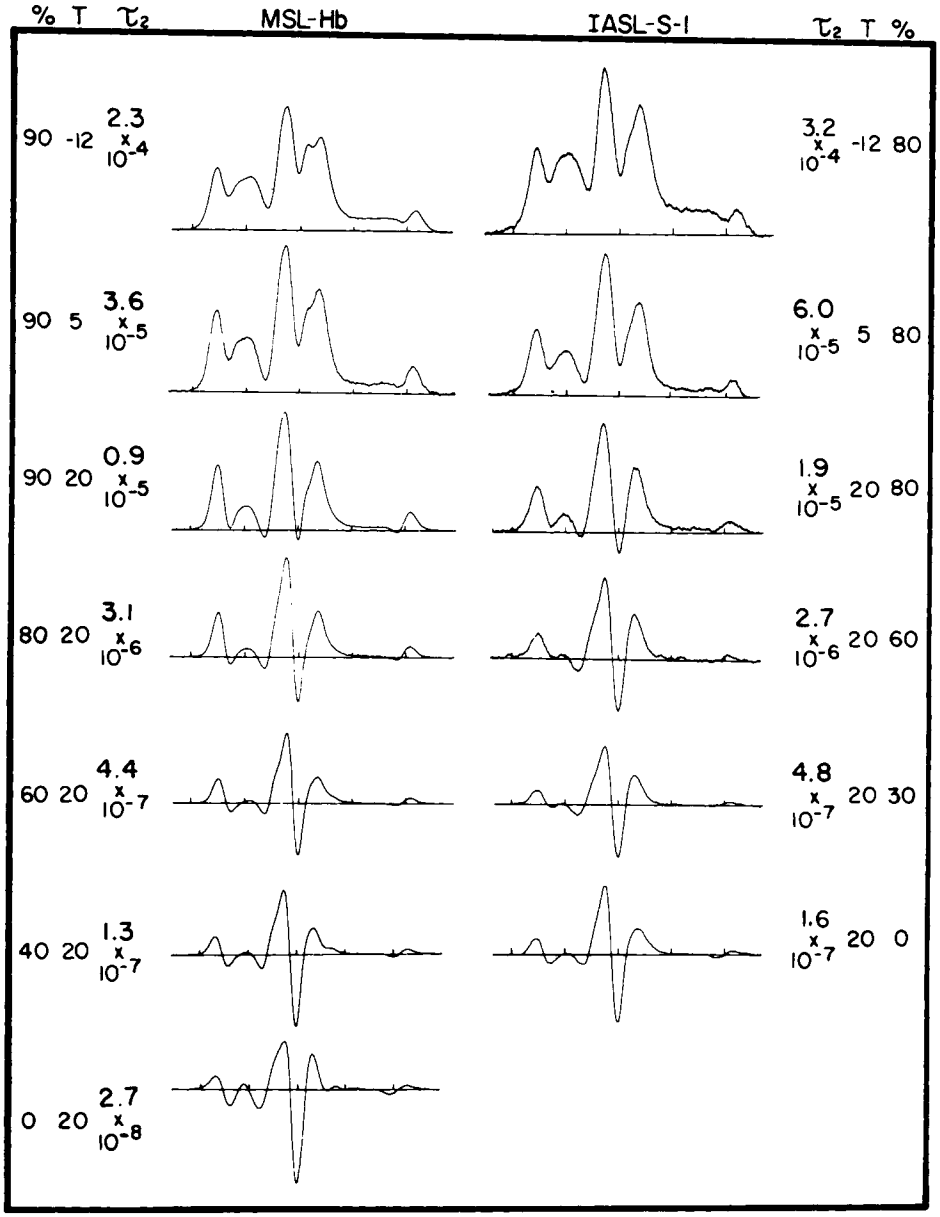


Fig. 1. Representative $v_2'$ reference spectra obtained from 25 mg/ml MSL-Hb (left) and 20 mg/ml IASL-S1 (right), in appropriate buffers (see text). Spectra were recorded and solvent viscosities were measured at glycerol concentrations (%) and temperatures (T, in °C) shown. Values of $\tau_2$ were estimated for the rotational diffusion of rigid bodies shaped like hemoglobin and S1 as described in the text, and spectra are arranged so that these $\tau_2$ values for adjacent Hb and S1 samples are similar. (We have compiled a collection of 40 MSL-Hb spectra available on request.)

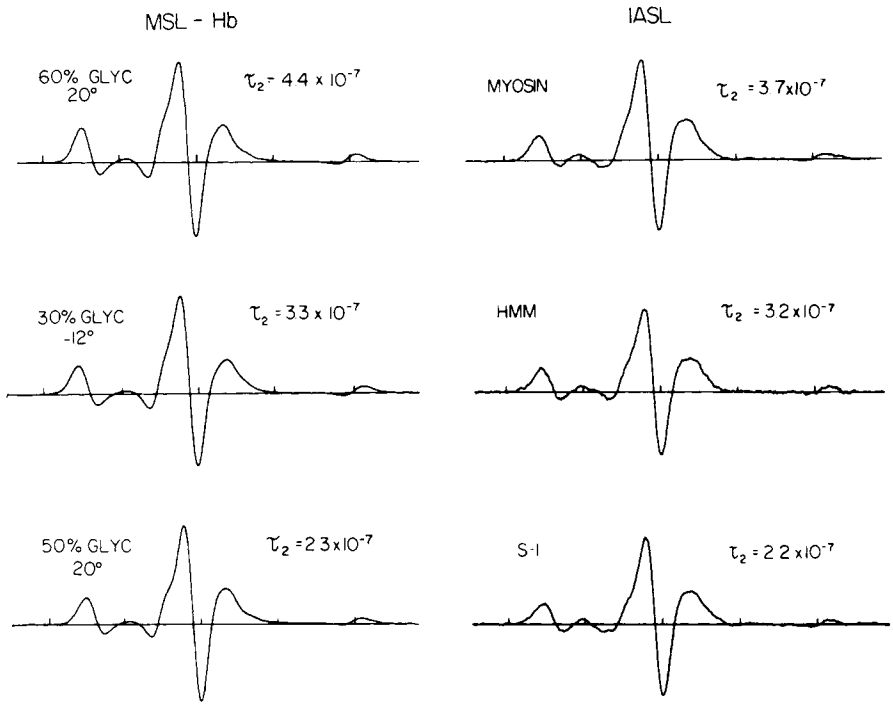


Fig. 2. Right (top to bottom): $v_2'$ spectra from solutions of IASL-myosin, HMM, and S1 (10 mg/ml) in appropriate buffers (see text), at 20°C, all from the same preparation of IASL-myosin. $\tau_2$ values are derived from comparison with Hb reference spectra, and are the averages from four myosin preparations (see Table I). Left: MSL-Hb reference spectra that are the closest line shape matches to the spectra on the right. $\tau_2$ values were calculated with Eq. (1). Spectra were recorded and solvent viscosities were measured at glycerol concentrations and temperatures shown. Ticks are at 20-G intervals.

### Supramolecular complexes

Saturation transfer is particularly useful in studying motion in supramolecular complexes (myosin filaments, actomyosin, acto-S1) where the correlation times lie between $10^{-7}$ and $10^{-3}$ sec. Formation of "synthetic" myosin filaments by dialysis against a solution containing 0.137 M KCl and 0.02 M Tris, pH 8.3, reduces the mobility of the S1 region by a factor of about 10 compared to myosin monomers (Fig. 3, bottom). Dialysis against solutions of lower ionic strength or pH (0.03 M KCl, 1 mM Tris, pH 7.5 or 0.1 M KCl, 1 mM Tes, pH 7.0) produces larger aggregates, which appear as a visible precipitate and in which the S1 mobility is 20–30 times less than in myosin monomers.

The combination of monomeric myosin with F-actin, in a ratio of two actin monomers per myosin monomer, reduces the mobility of the S1 region by a factor of nearly $10^3$ (Fig. 3, top), indicating that S1 binds quite rigidly to F-actin. The immobilization produced by actin is about 100 times that produced by formation of myosin filaments. Having an

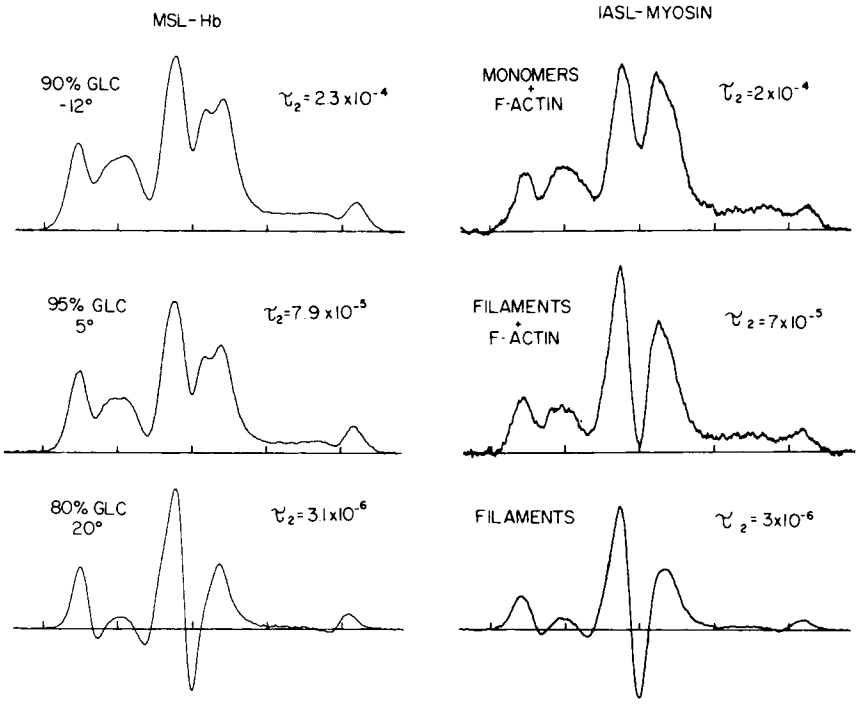


Fig. 3. Right (bottom to top): $v_2'$ spectra from IASL-myosin filaments (20 mg/ml in 137 M KCl, 02 M Tris, pH 8.3), acto-IASL-myosin filaments (1 ml of the same filaments solution plus 1 ml of a solution of F-actin, 5 mg/ml, in the same buffer) and acto-IASL-myosin (1 ml of the 20 mg/ml IASL myosin solution in 0.5 M KCl plus 1 ml of a 5 mg/ml solution of F-actin in the same buffer) all at 20°C; all from the same preparations of actin and IASL-myosin. $\tau_2$ values are from comparison with reference spectra, averaged for four myosin and actin preparations. The spin label concentrations were (top to bottom) $2 \times 10^{-5}$ M, $2 \times 10^{-5}$ M, and $4 \times 10^{-5}$ M, and each spectrum was obtained in a 30-min scan. Left: MSL-Hb reference spectra which are the closest line shape matches to the spectra on the right. $\tau_2$ values were calculated with Eq. (1). Spectra were recorded and viscosities were measured at glycerol concentrations and temperatures shown. Ticks are at 20-G intervals.

excess of actin over myosin heads (1.5:1) produced no significant effect on the $v_2'$ spectrum, suggesting that both heads of a single myosin molecule bind simultaneously to F-actin; when, however, there was an excess of myosin over actin (myosin heads–actin monomers, 2:1), a significant increase in the mean rotational mobility of S1 regions was observed. The spectrum of a mixture of myosin filaments and F-actin (Fig. 3, center) also indicates a mean mobility of S1 intermediate between that in myosin filaments and in actomyosin.

Fig. 4 demonstrates that F-actin immobilizes S1 isolated after tryptic cleavage, as well as the S1 segment of intact myosin. The $\tau_2$ for acto-IASL-S1 is about half that for acto-IASL-myosin. These results provide further evidence that the spin label is rigidly bound to the protein and indicate that S1 undergoes little or no independent motion when bound to F-actin. The actomyosin and acto-S1 samples contained about one actin monomer per S1. When G-actin was added to an IASL-S1 solution in a 1:1 molar ratio, both the $v_1$ and $v_2'$ spectra indicated only a slight immobilization.

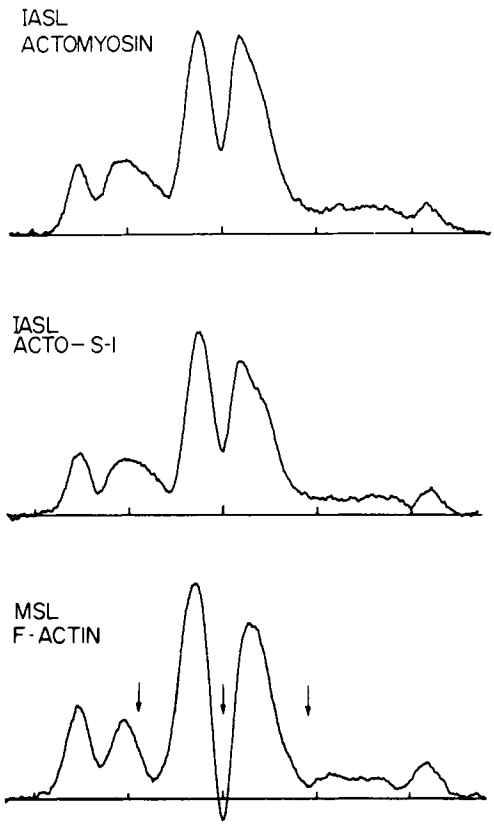


Fig. 4. $v_2'$ spectra for acto-IASL-myosin (1 ml of a 20 mg/ml IASL myosin solution plus 1 ml of a 5 mg/ml F-actin solution), acto-IASL-S1 (0.5 ml of a 20 mg/ml IASL-S1 solution plus 1 ml of a 5 mg/ml F-actin solution), and MSL-F-actin (5 mg/ml), in appropriate buffers (see text) at 20°C; all from the same preparations of actin and myosin. Estimated correlation times are (top to bottom) $2 \times 10^{-4}$ sec, and $5 \times 10^{-5}$ sec, by comparison with reference spectra. Arrows in the MSL-F-actin spectrum indicate where a small amount of loosely bound label affects the line shape. $\tau_2$ is therefore estimated mainly from the high-field portion of the spectrum. Ticks are at 20-G intervals.

The spectrum of maleimide spin-labeled F-actin (Fig. 4, bottom) reveals slow rotation of the label ($\tau_2 = 5 \times 10^{-5}$ sec), suggesting that this label is rigidly bound and that its motion therefore reflects the motion of the F-actin filament.

Figure 5 shows the $v_2'$ spectra obtained from myosin labeled at the SH-2 groups with MSL. The value of $\tau_2$ ($8 \times 10^{-8}$ sec) for MSL-myosin monomers in 0.5 M KCl (Fig. 5, bottom), is about four times shorter than that for IASL-myosin. Addition of F-actin to MSL-myosin increases $\tau_2$ by a factor of only about 70 (Fig. 5, top), resulting in a $\tau_2$ ($6 \times 10^{-6}$ sec) that is about 40 times shorter than in acto-IASL-myosin. The most likely explanation for these results is that MSL is less rigidly bound than IASL, so that immobilization of S1 by actin results in only partial immobilization of the label.

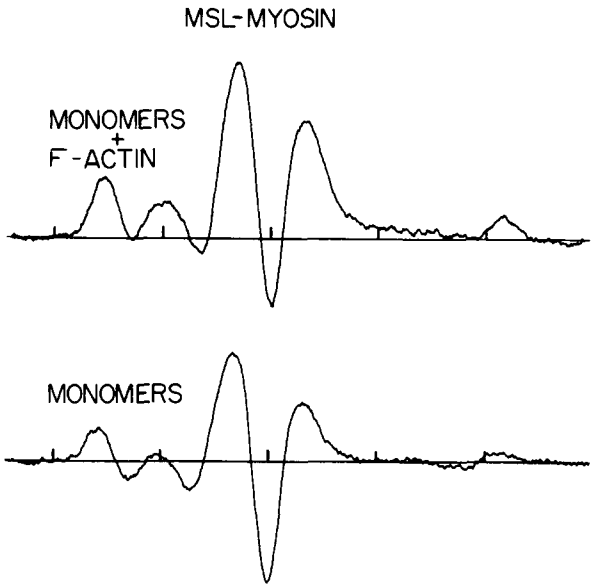


Fig. 5. $v_2'$ spectra for MSL-myosin monomers (20 mg/ml, bottom) and acto-MSL-myosin (1 ml of the same 20 mg/ml myosin solution plus 1 ml of a 5 mg/ml F-actin solution) both in 0.5 M KCl, 0.05 M Tris (pH 7.5). $\tau_2$ values from comparison with reference spectra are $8 \times 10^{-8}$ sec (monomers) and $6 \times 10^{-6}$ sec (actomyosin). Ticks are at 20-G intervals.

## DISCUSSION

The time scale accessible to the technique of saturation transfer EPR spectroscopy encompasses the likely range of crossbridge motion in intact, contracting muscle (2). This paper illustrates the applicability of the technique for studying the motion of the S1 region of myosin which is part of a crossbridge in intact muscle.

The iodoacetamide label bound to the SH-1 sulfhydryl groups appears to be rigidly attached. This conclusion is supported by the following facts: (a) $\tau_2$ for the label bound to S1 is three to four times longer than would be expected for a sphere of equivalent volume, but is in accord with the known axial ratio of S1; (b) the ratio of the $\tau_2$'s of IASL-S1 and MSL-Hb remains constant as the viscosity of the solvent is varied and $\tau_2$ changes by a factor of 1,000 (Fig. 1); and (c) the $\tau_2$ of actomyosin and acto-S1 is too long to reflect independent motion of the label.

Since the observed $\tau_2$ of S1 is three to four times longer than that of an equivalent sphere, it would appear that the principal axis is oriented more or less parallel to the major axis of S1 and that the observed values of $\tau_2$ are meaningful characteristic times for the reorientation of the major axis of S1.

Although the $v_1$ and $v_2'$ experiments (Table I) yield slightly different results, both methods show that (a) $\tau_2$ decreases by only about 40% when S1 is cleaved from the rest of myosin, even though the molecular weight decreases by more than a factor of 4, and (b) $\tau_2$ decreases by only about 15% when more than half of the "tail" is removed, producing

HMM. Mendelson et al. (3) interpreted similar results from the depolarization of the fluorescence of a dye bound to S1 as indicating flexibility within the HMM region of myosin, possibly at the postulated "hinge" (1) between S1 and S2. The fact that the motion of the S1 region in HMM and myosin is reduced by a factor of less than 2, compared with the isolated subfragment, suggests that the two "heads" move independently of each other. On the other hand we find no evidence for flexibility within the S1 region itself; as mentioned above, the rotation of isolated S1 is about four times slower than expected for a sphere of the same volume.

The rotational mobility of the S1 regions in myosin filaments is more relevant to the contraction mechanism than is the mobility of S1 in the myosin monomer. The latter decreases by a factor of 10 upon formation of synthetic myosin filaments (see Fig. 3), a decrease that is much less than would be caused by the change in molecular shape and volume alone: the fastest component of rotational motion of a $5 \times 10^7$-dalton protein ellipsoid with an axial ratio of 55 (27) would be at least 10 times slower than our observed result (24). The additional motion presumably reflects the rotation of the S1 regions independent of the filament as a whole. The $v_2'$ line shapes in the samples containing these filaments are more difficult to match with reference spectra than are the line shapes from other samples, suggesting that the motion of S1 in filaments may be more anisotropic or restricted to a narrower angular range. Because the motion of S1 in synthetic thick filaments is in a time range of high sensitivity for $v_2'$ experiments, this method promises to be useful in more detailed studies of thick filaments.

F-actin strongly immobilizes the S1 region, whether S1 is present in intact myosin monomers, in synthetic myosin filaments, or as the proteolytic fragment. Explanation of the results in terms of immobilization of only that portion of S1 containing the spin label, leaving the remainder of S1 free to move, is less plausible, since flexibility within S1 is inconsistent with the long $\tau_2$ of the isolated fragment. A direct interaction between actin and the spin label is also unlikely, since (a) the spin label can react with SH-1 groups whether F-actin is present or not, (b) the spin labels bound to SH-1 groups are equally accessible to dithiothreitol in the presence and absence of F-actin, and (c) the interaction between myosin and actin is not greatly altered by labeling the SH-1 groups (6).

The $v_2'$ spectrum of a mixture of actin and myosin filaments indicates somewhat faster motion than that found for complexes of actin with myosin monomers and with S1. This probably reflects binding of only a fraction of the myosin heads to actin. The interaction of a large polymer F-actin with an insoluble myosin filament would be expected to present steric problems in matching all the myosin heads with binding sites on actin, an interpretation supported by the fact that activation by actin of the ATPase activity of myosin is less effective with myosin filaments than with HMM or S1 (28). In this case $\tau_2$ should be interpreted not as a single correlation time but as an average of at least two populations of labels with greatly differing rotational mobilities.

The extremely long values of $\tau_2$ for acto-S1 and actomyosin indicate that the S1 region is rigidly attached to F-actin. The fact that $\tau_2$ for actomyosin containing two or more actin monomers per myosin molecule is even longer than that for acto-S1 indicates that both "heads" of a single myosin are immobilized.

Small changes in the shape of the $v_1$ spectrum (6, 29) and a decrease in signal amplitude at saturating levels of microwave power were observed on interaction of myosin

with F-actin, though the mechanism producing these effects was not clear. The present study using $v_2'$ spectra, which are much more sensitive to slow rotation, shows that these effects can be ascribed to immobilization of the S1 region of the myosin molecule by F-actin and not to internal changes in S1 induced by actin. The interaction with actin may well alter S1, as implied in the chemical scheme proposed for myosin-catalyzed hydrolysis of ATP (30) and by the effect of F-actin on the reactivity of the SH-2 thiol groups (31), but this is not reflected in the spin label bound to SH-1 groups. The $v_2'$ spectrum of a maleimide spin label attached to the SH-2 thiol groups does indeed show signs of an internal change in S1 produced by the interaction with actin. The maleimide label bound to SH-2 groups is not rigidly attached to S1, as indicated by the relatively rapid motion of this label in actomyosin. This label can be considered to undergo two types of motion: a rotation relative to the protein and the rotation of the protein itself. Assuming that

$$\frac{1}{\tau_{2obs}} = \frac{1}{\tau_{2i}} + \frac{1}{\tau_{2p}} \quad ,$$

where $\tau_{2obs}$ is the observed correlation time, $\tau_{2i}$ describes the rotation of the label relative to the protein, and $\tau_{2p}$ that of the protein itself, $\tau_{2i}$ of MSL bound to the SH-2 groups can be estimated. If one uses $\tau_2$ values obtained from proteins labeled with IASL at the SH-1 group for $\tau_{2p}$, then one obtains $\tau_{2i} = 1.0 \times 10^{-7}$ sec and $6 \times 10^{-6}$ sec for MSL-myosin and actomyosin, respectively. This corresponds to a 60-fold decrease in the rotational mobility of the label relative to S1. This treatment is only approximate and does not consider factors such as anisotropic and angularly restricted motion which may occur in these systems, but it seems unlikely that a 60-fold change could be attributed to these factors.

Motions of the label in actomyosin and in synthetic thick filaments occur in a time range of optimal sensitivity for the saturation transfer technique; this makes it possible to detect the large difference between the effects of filament formation and F-actin binding. Fluorescence depolarization measurements, having questionable applicability in this time range, yielded no significant difference between the two effects, and the estimated times were much shorter (3).

The motion of the maleimide spin label bound to F-actin ($\tau_2 = 5 \times 10^{-5}$ sec, Fig. 4) is much faster than the time scale accessible to the light scattering methods of Fujime et al. (32), who found evidence for F-actin flexing motions with correlation times longer than $10^{-3}$ sec. The spectra in Fig. 4 suggest that MSL, attached to actin, and IASL, attached to S1 and myosin, may both be reporting the motion of the F-actin structure, which is slowed by the binding of S1 and myosin. Although there is evidence that the principal axis of the maleimide label is approximately parallel to the filament axis (19), we cannot yet assign the observed $\tau_2$ to any particular mode of actin filament motion.

A possible application of the new EPR technique is to investigate the interaction of nucleotides with the myosin heads. Previous studies have shown that a "conformational change" takes place in myosin that is reflected in the increased mobility of the iodoacetamide spin label attached to SH-1 thiol groups. Recently, one of us (33) has shown that this conformational change probably is the same as that reflected in the ATP-induced

enhancement of tryptophan fluorescence (34). It should be mentioned parenthetically that the magnitude of changes in various "reporter" groups indicative of conformational changes can by no means be taken as a measure of a change in the energy content of the protein or of the role of a particular intermediate in the energy-transducing process.

Paradoxically, the fact that the iodoacetamide spin label is such a useful indicator of conformational changes upon interaction of ATP with myosin prevents at present its use for the study of changes in the motion of crossbridges or myosin heads upon interaction with nucleotides, because the label is no longer rigidly immobilized. We are in the process of extending these studies with the use of spin labels that remain rigidly immobilized in the presence of nucleotides.

Another possible area of interest is the interaction of calcium with myosin. Although in muscles of higher organisms the chief regulatory system that involves calcium is localized in thin filaments and contains tropomyosin and troponin (35), there is increasing evidence that some interaction of calcium and myosin may play an important role, if not in directly regulating the interaction of actin and myosin, at least in bringing about a change in the motion of crossbridges during and prior to attachment to actin (36, 37). This is particularly clear from X-ray studies on muscle stretched to the point where no overlap occurs between thick and thin filaments. A changed pattern of layer lines attributable to the crossbridge accompanies the onset of contraction. This suggests a direct effect of stimulation on the myosin filament presumably caused by $Ca^{2+}$. We have made preliminary measurements of the motion of the myosin heads of IASL-labeled filaments which indicate a small increase in mobility on addition of $Ca^{++}$. The significance of this change in relation to the X-ray diffraction studies is not yet clear.

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