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Spin-Label Studies of Glycerinated Muscle Fibers*

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ABSTRACT: Glycerinated rabbit psoas fibers were labeled with nitroxide spin labels having the following reactive groups: maleimide, isothiocyanate, and iodoacetamide. The electron paramagnetic resonance spectrum of the maleimide-spin-labeled fibers was anisotropic with respect to the fiber axis, and changes in the spectrum indicated that the rotational freedom of the labels was restricted when the fibers were adenosine triphosphate shortened. However, the spectrum did not change when the fibers were extended to lengths greater than l_0, where l_0 was the length which the fiber had when it was labeled. Neither iodoacetamide spin labels nor isothiocyanate spin labels showed any changes in spectrum when the fibers shortened. At any length, isometric addition of adenosine triphosphate or adenosine triphosphate + ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid did not change the spectrum of any of the spin labels. Proteins were extracted from maleimide-spin-labeled fibers and studied in solution. The spin labels on myosin and actin were not sensitive to myosin–actin–adenosine triphosphate interactions. The spin label on actin showed a restriction of the rotational freedom of the label when the actin polymerized. In addition, these studies indicate that myosin A extracted from glycerinated fibers had different properties from myosin A extracted from living fibers.

We conclude that the environment of maleimide spin labels changes on shortening of labeled fibers, yet these labels are not sensitive to actin–myosin–adenosine triphosphate interactions. Thus the labels indicate the existence of some as yet uncharacterized interactions which are dependent upon the length of the fiber.

The sliding filament model of muscular contraction has gained wide acceptance in recent years. However, the molecular events which actually produce the forces that cause one set of filaments to slide past another are still unknown. In this study we have used spin labels as probes to monitor changes that may occur in the conformation of the contractile proteins as the muscle fiber contracts, relaxes, etc.

A spin label is a stable organic free radical which can attach to the molecule of interest and report changes in conformation. We will not go into the details of spin-label spectra and techniques since there is an excellent recent review of this field (Hamilton and McConnell, 1968).

The electron paramagnetic resonance spectra are sensitive to the rotational freedom of the spin labels, and it is this property which allows the spin labels to be used as probes. When the spin is freely rotating in solution, the hyperfine interaction between the unpaired electron and the ^{14}N nucleus splits the spectrum into three sharp lines. When the spin label is completely immobilized the anisotropic spin orbit and hyperfine interactions further split the spectrum into three broad peaks. If the rotational freedom of the spin label is only partly restricted, the spectrum is complex and not amenable to simple theoretical analysis. In Figure 2, I_1 and I_5 are due to "strongly immobilized" spin labels while I_2 and I_4 are due to spin labels with a greater degree of rotational freedom, "weakly immobilized." I_3 is due to both strongly immobilized and weakly immobilized spin label. The two low-field peaks are well defined and clearly separated, and thus give the clearest indication of any change that occurs in the rotational freedom of the spin label. We define R as the ratio of the heights of these two peaks: $R = I_1/I_2$. The use of R to describe a spectrum neglects changes in line shape and position which could be caused by relaxation effects due to paramagnetic ions, etc. However, R expresses much of the information which a spectrum contains about the rotational freedom of the spin label, and we will use it as a "practical yard stick" to describe many of the spectra.

The spin labels are attached to the protein, and the shape of the protein neighborhood determines the degree to which the rotational freedom of the spin label is inhibited. Since the electron paramagnetic resonance spectrum is sensitive to the rotational freedom of the spin labels, any change of the conformation of the protein which affects the rotational freedom of the spin labels will be detected.

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All three of the spin labels used were attached to the proteins *via* covalent bonds. The maleimide spin labels react preferentially with "exposed SH groups" (Boeyens and McConnell, 1966), but may also attack amino groups (Griffith and McConnell 1966). The reaction of *N*-ethylmaleimide with both myosin and actin has been extensively studied (Sekine and Kielley, 1964; Martonosi, 1965; Bailen and Barany, 1967). The application of these studies to the reaction of maleimide spin label should be approached with caution, since the two reagents may not react in the same manner. The IAA[1] spin label is not fully characterized as a labeling reagent but seems to act as a general alkylating agent (Hamilton and McConnell, 1968). Isothiocyanate is known to react with alcoholic OH groups or with amino groups (Cram and Hammond, 1959; Varian Instrument Applications, 1968). Because of the abundance of reactive groups in a muscle fiber (myosin alone has approximately 40 reactive SH groups when in solution), the spin labels are distributed over a great many sites. The problem of detecting changes in the spectrum of the spin labels in one or more specific sites against the background due to a manyfold excess of extraneous spin labels can be overcome by achieving a high degree of accuracy of the electron paramagnetic resonance spectra.

Experimental Procedure

All electron paramagnetic resonance spectra were taken using the following spin labels which were purchased from Varian Co.: maleimide spin label, IAA spin label, and ITC spin label. Radioactive [^{14}C]maleimide spin label was synthesized according to the method of C. L. Hamilton and H. M. McConnell (unpublished data). The radioactive spin label was used only in the determination of the extent of labeling; 2.5 mM maleimide or IAA spin label was dissolved in 0.08 M KCl and 0.02 M Tris (pH 7.0); however, due to its low aqueous olubility, the ITC spin label was dissolved to saturation in a 50% glycerol–water mixture.

The electron paramagnetic resonance spectra were taken at 9500 MHz using a Varian E-3 spectrometer. All spectra of fibers were taken at a power setting of 100 mW and a modulation amplitude of 0.63 G. At these settings none of the lines was measurably broadened due to saturation or to modulation. The spectra of myosin and actin were taken with the same power settings but the modulation amplitudes were increased to 2.4 and 1.0 G, respectively. At these settings the sharper peaks were broadened approximately 5%; however, the greater modulation amplitude was necessary because of the low concentration of spin labels.

Spectra of solutions were taken using a glass "liquid flat cell" supplied by Varian Co. The inside dimensions of the cell measured 0.01 × 0.3 in. by several inches in length (only one cm of the length of the cell was actually in the microwave cavity). Spectra of fibers were taken using two types of cells.

[1] Abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; IAA, iodoacetamide; ITO, isothiocyanate; (amine spin label), *N*-2,2,6,6-tetramethylpiperidinenitroxide amine; (maleimide spin label), *N*-2,2,6,6-tetramethylpiperidinenitroxide maleimide; (IAA spin label), *N*-2,2,6,6-tetramethylpiperidinenitroxide iodoacetamide; (ITC spin label), *N*-2,2,6,6-tetramethylpiperdinenitroxide isothiocyanate; HMM, heavy meromyosin.

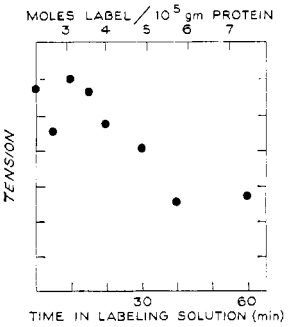

FIGURE 1: One bundle of fibers was sectioned into 1-cm lengths and each section was then labeled. The maximum tension developed by each section, measured in arbitrary units, is shown as a function of the amount of spin label with which it had reacted. Tension was generated by addition of 4 mM Mg-ATP plus 10^{-5} M $CaCl_2$ to the medium. The lower abscissa shows the length of time the fiber had spent in a 2.5 mM solution of maleimide-spin-label solution (25°). The upper abscissa shows the number of moles of spin label per 10^5 g of contractile proteins as determined by parallel radioactive experiments.

The first type, which was used to contrast spectra of fibers parallel and perpendicular to the magnetic field, consisted of a Kel F rod into which a rectangular cavity (0.01 × 0.3 × 0.3 in.) had been machined. In order to take spectra as a function of the length of the fiber a second type of cell was developed in which a fiber, secured on either end by silk threads, was suspended inside a capillary (1-mm i.d.). One thread was attached to a simple screw device which permitted the investigator to adjust the length of the thread. In this cell the long axis of the fiber was perpendicular to the magnetic field. The solution that bathed the fibers could be flowed through the capillary at a constant rate by the use of a mechanically driven syringe.

Rabbit psoas fibers were glycerinated from 1 to 4 weeks in a 50% glycerol–water mixture with 0.05 M KCl (pH 7.0) at −20°. After washing, to remove the glycerol, the fibers were incubated at room temperature in the spin-labeling solution, then washed repeatedly before spectra were taken. The solution in which the fibers were washed and the spectra were taken was 0.08 M KCl–0.02 M Tris (pH 7.0). ATP, EGTA, etc. were added to this medium.

Myosin A was extracted from labeled fibers with a modified Szent-Gyorgyi method (Tonomura *et al.*, 1966) in which the extracting solution consisted of 0.4 M KCl, 0.5 mM ATP, and 0.05 M histidine (pH 7.0). Actin was subsequently extracted from the fibers by the acetone powder method (Carsten and Mommaerts, 1963). Myosin B was extracted according to the method of Stowring *et al.* (1966).

Results

The reaction between spin label and glycerinated fibers decreased the maximum tension which the fibers could exert. Figure 1 shows the tension developed by fibers as a function of the amount of label which had reacted with the fiber. [^{14}C]-Maleimide spin label was used to determine how much label had reacted for a given length of time in the 2.5 mM labeling solution. The radioactivity study showed that approximately 2 moles of maleimide spin label/10^5 of contractile protein reacted quickly with the fiber. IAA spin label produced an effect on the tension of the fibers which was similar to that produced

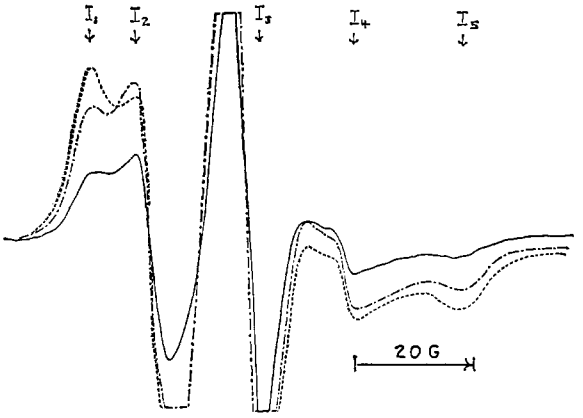

FIGURE 2: The electron paramagnetic resonance spectra of three glycerinated fibers which have reacted with different amounts of spin label: —, 2.7 moles of maleimide spin lable/10^5 g of protein; — - - - -, 3.5 moles of maleimide spin label/10^5 g of protein; — — —, 7.6 moles of maleimide spin label/10^5 g of protein. These spectra, as well as the other spectra of fibers which are shown, are taken in 0.08 M KCl–0.05 Tris (pH 7.0). All electron paramagnetic resonance spectra show the derivative of energy absorption plotted as a function of the field strength in gauss (G).

by maleimide spin labels, incubation in a 2.5 mM IAA spin label solution for 3 hr reducing the tension to one-half the original. A similar study of the effect of ITC spin label was not made; however, the amount of spin label used did not destroy the tension.

Figure 2 shows the electron paramagnetic resonance spectra of three glycerinated fibers which had reacted with increasing amounts of maleimide spin label. From Figure 2 we see that R increased from approximately 0.75 to 1.2 as more maleimide spin labels reacted with the fibers, indicating that the more slowly reacting groups had more strongly immobilized spin labels. The IAA spin labels were not as strongly immobilized as were the maleimide spin labels. The spectrum of IAA-spin-labeled fibers had an R of 0.2 after 3 hr in the labeling solution. The spectrum of ITC spin-labeled fibers had a R of approximately 1.0 after 1 hr in the labeling solution.

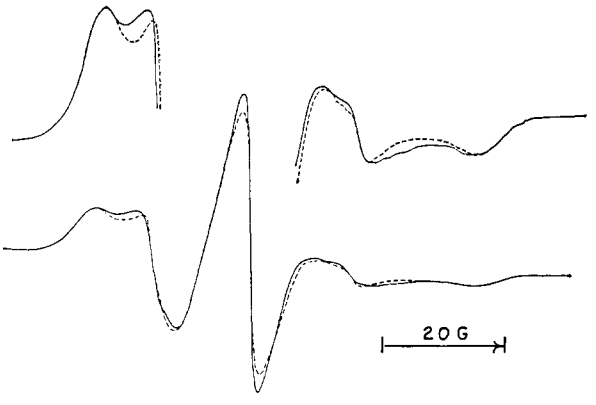

FIGURE 3: The electron paramagnetic resonance spectra of a glycerinated fiber at two different orientations to the external magnetic field: - - -, long axis of the fiber parallel to the magnetic field; —, long axis of the fiber perpendicular to the magnetic field. The fiber has reacted with 4.0 moles of maleimide spin label/10^5 g of protein. The inserts on top show the spectra at higher gain.

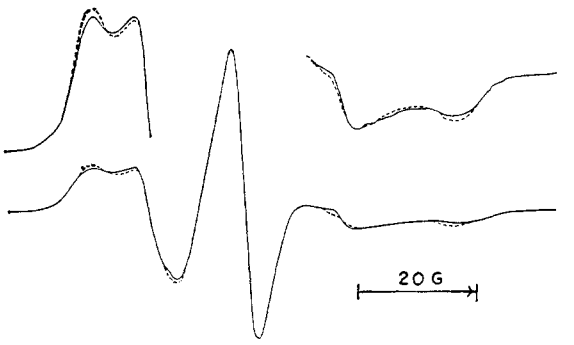

FIGURE 4: The electron paramagnetic resonance spectra of a glycerinated fiber (4.0 moles of maleimide spin label/10^5 g of protein) before (—) and after (- - - -) contraction with ATP. The long axis of the fiber is perpendicular to the magnetic field and the fibers were contracted to 60% of their original length.

The fibers could be aligned with their long axis either parallel or perpendicular to the magnetic field of the spectrometer. In the case of maleimide spin-labeled fibers the two alignments gave different electron paramagnetic resonance spectra (see Figure 3). (This effect was first seen by W. Hubbell and H. M. McConnell, personal communication). The dichroism persisted after the fibers had been contracted. No orientation effect was seen in the spectra of either IAA- or ITC-spin-labeled fibers.

When maleimide spin-labeled fibers were shortened with ATP, the ratio of the first peak to the second increased, corresponding to a restriction of the rotational freedom of some spin labels (see Figure 4). This ratio, R, is shown in Figure 5 as a function of the length of the fiber. In Figure 5, the fiber was labeled at some length l_0, 4 mM Mg-ATP was then added, and the fiber was allowed to shorten to a new length, at which it was held isometrically while a spectrum was taken. The fiber was then allowed to shorten still further and the process was

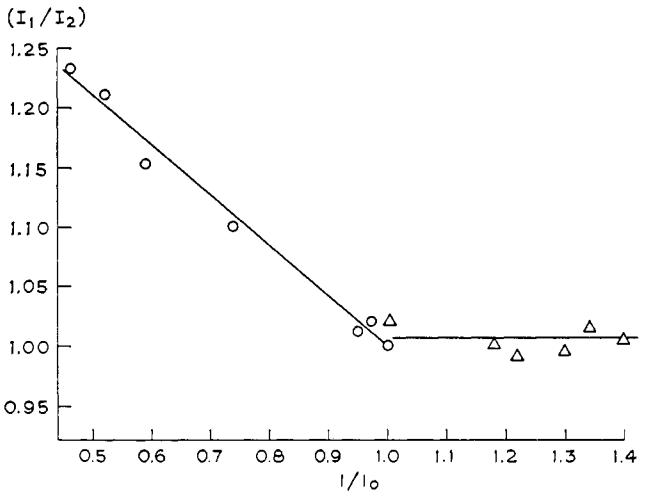

FIGURE 5: The ratio, R, $R = I_1/I_2$, as a function of the length of the fiber, l_0. l_0 is the length at which the fiber was labeled. The points for 1 less than l_0 (O) were taken with one fiber, while the points for l greater than l_0 (Δ) were taken for another fiber. The ratios for each fiber were then normalized such that the initial R, at $l = l_0$, was 1.0; 3.5 moles of spin label/10^5 g of protein was reacted.

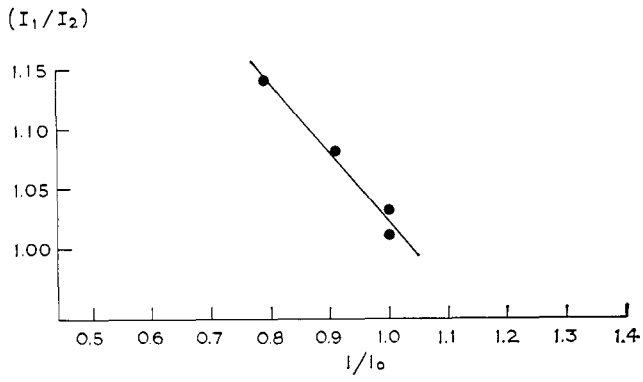

FIGURE 6: The ratio R, $R = I_1/I_2$, as a function of the length of the fiber. The fiber was incubated in 10 mM IAA for 30 min, then incubated in maleimide spin label until 3.5 moles of spin label/10^5 g of protein had reacted. l_0 is the length at which the fiber was labeled.

repeated. Figure 5 shows that R increased when the fiber contracted to a length shorter than l_0, but did not change when the fiber was extended beyond l_0. l_0 was varied over sarcomere lengths ranging from 1.9 to 3.0 μ. Fibers labeled at different lengths gave similar results. The amount of maleimide spin label which was reacted with the fibers was varied from 2.7 to 7.6 moles of spin label per 10^5 g of protein. The change of R on contraction occurred over the entire range. The data for Figure 5 were taken with the long axis of the fiber perpendicular to the magnetic field; however, the increase in R also occurred when fibers with their long axis parallel to the magnetic field were shortened.

A fiber which has been shortened to some length l (less than l_0) could be relaxed with 4 mM ATP plus 2×10^{-5} M EGTA and extended to the original length l_0. When this was done the ratio returned to its original value at l_0. Further extension of the fiber beyond l_0 had no further effect on the spectrum.

Fibers were incubated in 10 mM IAA for 30 min, washed, and then incubated in 2.5 mM maleimide spin label for 15 min. The initial ratio for these fibers was higher, and the slope of the graph of R *vs.* length was greater than for fibers not pretreated with IAA (see Figure 6).

At all lengths observed, addition of 4 mM ATP–$MgCl_2$ or 4 mM ATP–$MgCl_2$ plus 2×10^{-5} M EGTA to the bathing solution did not change the spectrum of maleimide-spin-labeled fibers. Addition of 25 mM $NaPP_i$ + 2 mM $MgCl_2$ or of 0.1 M polylysine also had no effect on the spectrum. The pyrophosphate ion, PP_i, could be expected to be a "plasticizer," *i.e.*, to disrupt actin–myosin interactions; polylysine was tried because recent experiments of K. Laki (personal communication) suggest the possible importance of transamidation in the muscle system. It is interesting to note that the fluorescence polarization ratio (A and M) which also shows a change on shortening and contraction is also insensitive to PP_i (and other plasticizers) but is sensitive to glycerol, when glycerol is applied to fibers (Aronson and Morales, 1969). At the labeling length, l_0, the addition of ATP or ATP plus EGTA did not have any effect on the spectrum of IAA-spin-labeled fibers or ITC spin-labeled fibers.

Fibers were soaked in a 10 mM solution of amine spin label, then blotted to remove excess solution and placed in the Kel F cell. The spectrum of the amine spin label in solution in the

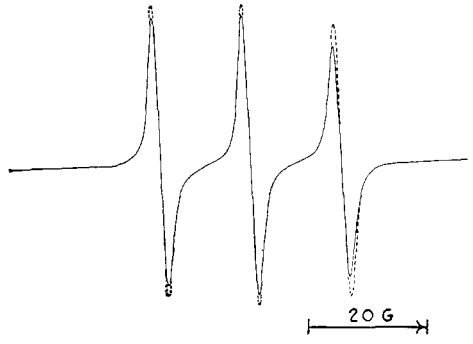

FIGURE 7: The spectrum of amine spin label in solution (dotted line) and in solution inside a glycerinated fiber (solid line).

fibers showed a small change relative to amine spin label in solution (see Figure 7). No further change occurred, however, when the fibers were contracted with ATP.

Spin-labeled proteins were extracted from maleimide spin-labeled glycerinated fibers. The spectra of myosin A in 0.6 M KCl is shown in Figure 8. The spectrum in Figure 8 was taken immediately following extraction. After 4 days at 0° the ratio of the first two peaks, R, had diminished from 1.15 to 0.7, and after 6 days had changed to 0.5. At room temperature R changed from 0.7 to 0.6 in about 0.5 hr. The rate of change was greatly accelerated at 37°.

Myosin was extracted from living rabbit fibers and labeled *in vitro* with moles of maleimide spin label/mole of myosin (see Figure 9). The myosin was labeled for 25 hr, 0° in 0.6 M KCl plus 0.05 M Tris (pH 8.0). The unreacted label was removed by dialysis at the end of the labeling and the amount of reacted label was not determined. This spectrum had a value of R of 0.72, which showed no time dependence up to 12 hr at room temperature. Myosin A was then extracted from unlabeled glycerinated fibers and labeled *in vitro* as above with 5 moles of spin label/mole of myosin. The spectrum of this myosin was quite different from that of myosin extracted from living fibers (see Figure 9).

Addition of ATP (1 mM) plus $MgCl_2$ (0.1 mM) in 0.6 or 0.06 M KCl did not change the spectrum of myosin A. Precipitating the protein by dilution to 0.06 M KCl produced an increase in R (see Table I). Addition of unlabeled actin to myosin A, 3 moles of actin/mole of myosin, in either 0.6 or 0.06 M KCl did not change the spectrum.

Myosin B, extracted from labeled fibers, had a spectrum

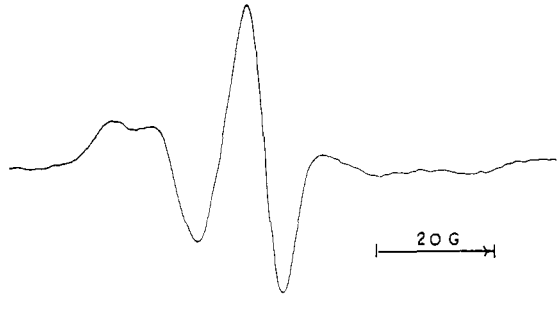

FIGURE 8: The electron paramagnetic resonance spectrum of myosin A, 0.6 M KCl (pH 7.0), extracted from glycerinated spin-labeled fibers. This spectrum was taken immediately after extraction.

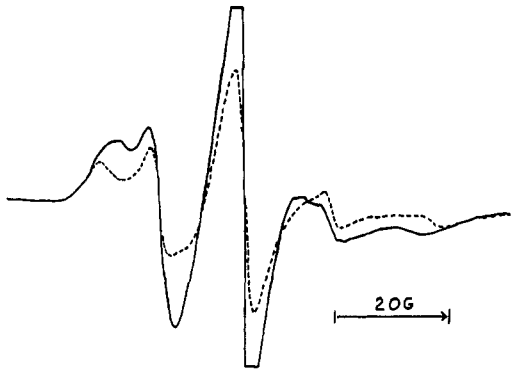

FIGURE 9: The electron paramagnetic resonance spectrum of myosin A extracted from living fibers and labeled in solution with 5 moles of maleimide spin label/mole of myosin (solid line). The dotted line shows the electron paramagnetic resonance spectrum of myosin A labeled as above but extracted from glycerinated fibers. Both spectra are taken in 0.6 M KCl and 0.05 M Tris (pH 7.0).

similar to that of myosin A, also extracted from labeled fibers (see Table I). Although the time dependence of the spectra of myosin B was not closely checked, we observed no time dependence of the order of magnitude of that observed for myosin A. Addition of ATP (1 mM) plus $MgCl_2$ (0.1 mM) to myosin B in either 0.6 or 0.06 M KCl did not change the spectrum. As with myosin A, myosin B showed a slight increase in R when the protein was precipitated at 0.06 M KCl. In Table I, the actin and myosin were extracted from the same fibers; however, the myosin B was extracted from a separate group of fibers in which the degree of labeling may have been different.

The spectrum of F-actin, extracted from spin-labeled fibers, had a ratio, R, of 1.1. Addition of unlabeled myosin A or unlabeled HMM (3 moles of actin/mole of myosin or HMM) to the actin did not change the spectrum. However, if this actomyosin is precipitated in 0.06 M KCl and then spun down, the precipitate has a ratio of 1.4 (see Figure 10) while the supernatant contains a small residue of signal with a ratio of approximately 0.1. The spectrum of the precipitated actomyosin

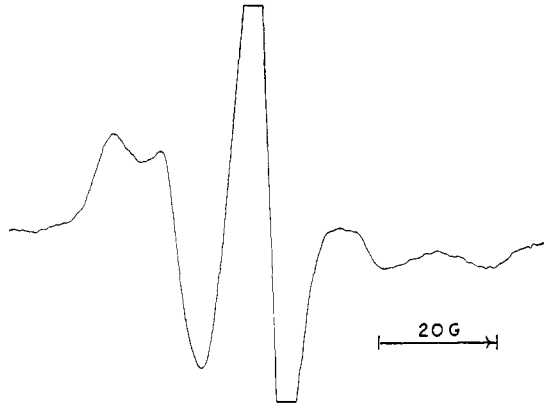

FIGURE 10: The electron paramagnetic resonance spectrum of F-actin, extracted from spin-labeled fibers. The actin is combined with myosin A (3 moles of actin/mole of myosin A) in 0.6 M KCl–0.05 Tris (pH 7.0).

TABLE I: The Ratio, R, of the Spectra of Proteins Extracted from Maleimide-Spin-Labeled Glycerinated Fibers.[a]

	0.6 M KCl	0.6 M KCl + ATP	0.06 M KCl	0.06 M KCl + ATP
Spin-labeled myosin B	1.09	1.08	1.35	1.32
Spin-labeled myosin A	1.15	1.15*	1.4*	1.4*
Spin-labeled myosin A plus actin	1.15*	1.15*	1.4*	1.4*
Spin-labeled F-actin plus myosin A	1.45	1.40	1.37	1.40
Spin-labeled G-actin	0.7 (in 0.2 mM ATP no KCl)			

[a] Owing to the change in the spectrum of myosin A which occurred with time, the values of R marked by (*) are extrapolated to correspond to R for myosin A in 0.6 M KCl. The actual measured increase in R for myosin A (4 days after extraction) upon dilution from 0.6 M KCl to 0.06 M KCl was from 0.73 to 0.91. All values ±0.05.

does not change when the protein is redissolved in 0.6 M KCl, nor is it sensitive to addition of ATP at either high or low ionic strength (see Table I).

F-actin was centrifuged and redissolved in 0.2 mM ATP to give G-actin. The spectrum of G-actin had a ratio of 0.7. The spectrum returned to that of F-actin, R = 1.1, upon addition of 50 mM KCl.

Discussion

The only change which was seen in the study of spin-labeled glycerinated fibers was the restriction of the rotational freedom of maleimide spin label which occurred when the fibers were shortened. This effect was a linear function of the length of the fiber relative to the length at which the fiber had been labeled. The change in the spectrum was seen only with the maleimide spin label and not with either IAA spin label or ITC spin label. IAA spin label reacts with many of the same groups with which maleimide spin label also reacts, such as SH groups. Thus the change seen with maleimide spin label was specific to some sites occupied by maleimide spin label and was not a general property of spin labels attached to fibers.

The internal macroviscosity of the fibers was checked by the use of noncovalently bound amine spin label. The results show that there was no change in the rotational relaxation time of the amine spin label when the fibers contracted. Thus, from the point of view of a small molecule in solution there was no change in the internal viscosity of the fibers. When groups were masked with IAA prior to reaction with maleimide spin label the effect on R was increased. This result can be interpreted as an indication that either the "sensitive" maleimide spin labels were on groups which did not react with IAA or that they were

on slowly reacting groups and that the IAA had masked the quickly reacting groups. Both explanations require that the increase in R be caused by maleimide spin labels on some specific site.

We conclude that the change in spectrum which occurred when the fibers contracted was due to changes in one or more specific groups of maleimide spin-labeled sites on the contractile proteins. Since IAA-spin-labeled fibers did not generate a spectral change on contraction, we assume that either IAA spin label did not react at the sites which gave the changes in maleimide-spin-labeled fibers, or that the IAA spin label did react at these sites but was not sensitive to the changes in protein environment.

In the sliding filament model of muscle contraction the cross bridge goes through a cyclic process in which it attaches to the thin filaments, in some way produces a thrust, then is detached and reattached to another site to repeat the cycle. After the translation from one actin site to another, however, the environment of the individual cross bridge is exactly the same as before. At those sarcomere lengths, below that at which all cross bridges are attached to actin sites, any further contraction of the fiber will not change the environment of the individual cross bridge. Since the spectrum continued to change linearly at sarcomere lengths well below the length at which all cross bridges were interacting with actin sites, we can assume that the groups responsible for the change in the spectrum upon contraction were not on the cross bridge.

We must look for a mechanism which not only explains the changes in the spectrum upon contraction, but which also explains the lack of change in the spectrum when the fiber was extended beyond the length at which it was labeled. It should be noted that the length at which the spectrum ceased to change as the fiber was extended occurred at the length at which the fiber was labeled and was independent of the sarcomere length. This last phenomenon could result from labeling a protein group on the thin filaments which was reactive in the I bands but which was protected in the A bands. If, in addition, the rotational freedom of a spin label attached to this protein group was restricted when the thin filaments entered the A bands, the spectral change can be explained. It is possible that a steric hindrance, which protected this protein group in the A bands, was also responsible for the restriction of the rotational freedom of any spin label which then entered from the I bands as the fiber contracted.

An alternative hypothesis is that the spin labels responsible for the changes in the spectrum were on the thick filaments, and that the reactive site was protected by the thin filaments as they entered the A bands. This hypothesis is essentially the mirror image of the previous one. As we have said before the spectral changes cannot be explained by groups on the cross bridges, so that the above hypothesis would have to invoke some interaction between the thin filaments and the thick filaments which did not involve the cross bridges. In the present formulation of the sliding filament model the thin and thick filaments are separated by about 60 Å and interact only *via* the cross bridges. Fibers with initial sarcomere lengths of 2.3–2.5 μ were shortened to 60% of their initial length to sarcomere lengths of 1.4–1.5 μ. Over this range the restriction of the spin label was a linear function of the length of the fiber. However, over this same range the thin filaments left the region of cross bridges, entered the pseudo H bands, and then began to overlap each other (Huxley, 1968). Thus we must assume that the postulated interaction which changed the spectra of spin labels on the thick filaments must not only be independent of whether the thin filaments were in the A or pseudo H bands, but must also be independent of whether the thin filaments were overlapping each other. This assumption, coupled with the fact that the interaction is known to be specific to particular groups, makes this second hypothesis less attractive than the first. However, we cannot completely eliminate it as a possibility.

Returning to the first hypothesis, *i.e.*, that the spin labels responsible for the spectral changes were on the thin filaments, it is reasonable to assume that the restriction of the spin labels was caused by an interaction with the cross bridges. HMM is known to interact with actin, and is also known to protect an SH group on actin (Bailen and Barany, 1967), a group which would react with maleimide spin label. However, the hypothesis that HMM was affecting the actin quickly runs into trouble. The spectra of maleimide-spin-labeled fibers showed no change upon addition of ATP, ATP plus EGTA, or $NaPP_i$. If the restriction of the spin labels was caused by an interaction of thin filaments with the HMM cross bridges, we would expect that substances which break that interaction would influence the spectra. Either ATP plus EGTA or $NaPP_i$ is thought to inhibit the formation of HMM actin links.

The hypothesis that HMM affects the spin label on actin runs into even greater trouble in the *in vitro* experiments. The spin label on extracted actin was not sensitive to interactions with myosin or HMM and in addition the spin label on myosin was not sensitive to interactions with actin. We are thus forced to conclude that the ATP-sensitive interaction between myosin or HMM and actin did not play a role in the restriction of the spin labels which occurred on shortening.

The maleimide spin label was immobilized upon precipitation of both myosin B and myosin A. The changes in spectra were similar in both cases and we assume that the changes seen in the precipitation of myosin B were due to the spin labels on myosin A. It is logical to expect that some spin labels may have been immobilized by the close aggregation of the myosin molecules which occurred on precipitation.

The studies on myosin A were confused by the time dependence of the spectrum. The R of the spectrum of myosin A, extracted from labeled glycerinated fibers, decreased with time. A decrease of R, *i.e.*, greater rotational freedom of the spin labels, could indicate progressive denaturation. When myosin A was extracted from both living and unlabeled glycerinated fibers and labeled *in vitro* the two resulting spectra were different. The spectra of spin labels on myosin A extracted from living fibers was not a function of time. From these studies it appears that some properties of the myosin molecule have been modified during the glycerination of the fibers. A time-dependent maleimide-spin-labeled spectrum may not indicate a time-dependent protein conformational change, since this labeling agent may undergo a time-dependent hydrolysis (Boeyens and McConnell, 1966). However, we believe that is is unlikely that a time-dependent hydrolysis is occurring in myosin A extracted from labeled fibers, since none was observed for the myosin A labeled *in vitro*.

When myosin A was added to spin-labeled F-actin in either 0.6 or 0.06 M KCl there was no change in the electron paramagnetic resonance spectrum, showing that the spin labels on actin were not sensitive to interactions of actin with myosin. The insensitivity of the spin labels to actin–myosin interac-

tions may be due in part to the fact that spin labels on actin tend to inhibit superprecipitation or ATPase of actomyosin (Stone and Botts, 1969). Regardless of the mechanism which renders the actin spin labels insensitive to actin–myosin interactions the above results still eliminate actin–myosin interactions from playing a role in the explanation of the results obtained from the fibers. When the actomyosin was precipitated in 0.06 M KCl and sedimented, the R of the pellet was 1.5 as opposed to a R of 1.1 for F-actin. We believe that this effect was caused by contamination of the F-actin with some protein, possibly denatured actin, which does not form a bond with myosin A. In support of this hypothesis the supernatant showed a "weakly immobilized" spectrum which would account for the difference between the spectra of F-actin before and after precipitation with myosin. Stone and Botts (1969) labeled both G- and F-actin *in vitro* with 2 moles of maleimide spin label/mole of actin. The resulting spectra depend upon the state of the actin when it was labeled. The spectra of our actin, extracted from labeled fibers, closely resembled the spectra of actin which had been labeled *in vitro* as F-actin. These results may not indicate that the spin label attacks different groups in the F and G forms, since the resonance of maleimide-spin-labeled oxyhemoglobin depends upon whether the hemoglobin was in the oxy or deoxy form when it was labeled, although the label is doubtless attached at cysteine $\beta 93$ in either case (Boeyens and McConnell, 1966).

The only effect which the *in vitro* studies produced which might explain the changes that occurred when fibers were shortened was the increase in R upon polymerization of actin. The data require that the actin have a G-like spectrum in the I bands and a F-like spectrum in the A bands. It is difficult to imagine the actin in the I bands to actually be G-actin; however, in the ordered array of the muscle fiber some interaction may take place which involves the spin label in a way similar to the F-to-G transition. Recent studies have shown that spin labels are sensitive to actin-relaxing protein interactions (Y. Tonomura, S. Watanabe, and M. Morales, to be published), and it is possible that these interactions may be responsible for the increase of R on contraction of the fibers (the relaxation mechanism of the fibers was at least partially intact; however, the presence or absence of Ca^{2+} did not influence the spectrum of the spin labels). The spin-label studies alone cannot decide what interactions are occurring to explain the fiber data, and further work is needed to explore these intriguing hypotheses.

Summary

In the sliding filament theory, as presently formulated, the only interaction between the thin and thick filaments occurs *via* the cross bridges, and the thin filaments play only a passive role, that of providing a handle onto which the cross bridges can hook. Our results show that the above picture is complicated by some additional interactions. The environment of maleimide spin labels changed when the fibers to which they are attached are shortened, yet these labels were not sensitive to actin–myosin–ATP interactions. We must either postulate an additional interaction between the thin filaments and myosin, influencing the spin labels on myosin, or we must postulate some change in the conformation of the thin filaments as they enter the A bands. *In vitro* studies suggest that this transformation may involve groups similar to those involved in the G-to-F transition of actin.

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