ORIENTATION OF SPIN-LABELED NUCLEOTIDES BOUND TO MYOSIN IN GLYCERINATED MUSCLE FIBERS

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ABSTRACT Electron paramagnetic resonance (EPR) spectroscopy of paramagnetic derivatives of ATP has been used to probe the angular distribution of myosin in glycerinated muscle fibers. Three nucleotide spin labels have been prepared with the nitroxide free radical moiety attached, via an ester linkage to either: (*a*) the 2′ or 3′ positions of the ribose unit of ATP (SL-ATP), (*b*) the 2′ position of 3′ deoxy ATP (2′SL-dATP), or (*c*) the 3′ position of 2′ deoxy ATP (3′SL-dATP). In muscle fibers, these nucleotides are quickly hydrolyzed to their diphosphate forms. All three diphosphate analogues bind to the nucleotide site of myosin with similar affinities: rabbit psoas fibers, 7×10^3/M; insect flight muscle, 5×10^3/M; and rabbit soleus muscle, 2×10^4/M. Analysis of the spectra showed that the principal z-axis of the nitroxide attached to bound nucleotides was oriented with respect to the filament axis. The principal axes of 3′SL-dADP and 2′SL-dADP appeared to be preferentially aligned at mean angles of 67° ± 4° and 55° ± 5°, respectively. The distribution of probes about these angles can be described by Gaussians with widths of 16° ± 4° and 13° ± 5°, respectively. The spectrum of bound SL-ADP was a liner combination of the spectra of the two deoxy analogues. These orientations were the same in the three muscle types examined, indicating a high degree of homology in the nucleotide binding site. Applying static strains as high as 0.2 N/mm^2 to muscle fibers caused no change in the orientation of myosin-bound, spin-labeled nucleotides. When muscle fibers were stretched to decrease actin and myosin filament overlap, bound SL-ADP produced EPR spectra indicative of probes with a highly disordered angular distribution. Sodium vanadate and SL-ATP caused fiber stiffness to decrease, and the EPR spectrum of the bound analogue indicated an increase in the fraction of disoriented probes with a concomitant decrease in the fraction of oriented probes. These findings indicate that when myosin is bound to actin its nucleotide site is highly oriented relative to the fiber axis, and when this interaction is removed the orientation of the nucleotide site becomes highly disordered.

INTRODUCTION

Muscle contraction involves a cyclic interaction between two proteins, myosin and actin. Myosin heads extend from the myosin filament and bridge the interfilament space to interact with the actin filament. These cross-bridges generate force during a power-stroke, resulting in the "sliding" of the actin filament past the myosin filament (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). At the end of the power-stroke, ATP binds to myosin, causing the cross-bridge to dissociate from actin. ATP is hydrolyzed by myosin and then myosin again binds to actin, repeating the contraction cycle (Lymm and Taylor, 1971). It has been postulated that the force-producing event of the power-stroke involves cross-bridge rotation (Reedy et al., 1965; Huxley and Simmons, 1971; Morales et al., 1982). To test this hypothesis, cross-bridge orientations have been monitored using electron microscopy, x-ray diffraction, and spectroscopic probes attached to the myosin head. The latter studies have concentrated on the spectra of fluorescent and paramagnetic probes attached to a reactive sulfhydryl (SH-1) and on fluorescent nucleotides that bind to the ATPase site on the myosin head. We report here that paramagnetic analogues of adenine nucleotides bind to the cross-bridge in muscle fibers and that they can be used to measure the orientation of this portion of myosin.

Rigor is induced by the removal of ATP from the muscle so that the dissociation step in the cycle of the cross-bridge is inhibited. Rigor muscle cannot work and is very stiff. However, rigor muscles can sustain isometric forces that are as large as those developed during contraction. The rigor state has been suggested to represent the end of the cross-bridge power-stroke. A number of studies have led to the conclusion that in rigor muscle most myosin heads are attached to actin in an ordered configuration. Electron micrographs of rigor insect flight muscle displayed cross-bridges that made an acute angle with respect to the filament axis (Reedy et al., 1965; Reedy, 1968). Measurements of the polarization of fluorescence from dyes attached to the SH-1 site (Nihei et al., 1974; Tregear and Mendelson, 1975; Borejdo and Putnam, 1977) and from fluorescent nucleotides bound to the nucleotide site of

myosin (Yanagida, 1981) indicated that the cross-bridges in rigor muscle fibers are ordered, but these studies could not define the exact angular distribution. Electron paramagnetic resonance (EPR) spectra can provide considerable angular resolution, and spectra of probes attached to the reactive sulfhydryl showed that the probes had a narrow angular distribution that could be approximated by a Gaussian distribution with a full width at half maximum of ~12° (Thomas and Cooke, 1980; Barnett et al., 1986). X-Ray diffraction data of rigor muscle have also indicated high degrees of order in myosin cross-bridges (Reedy et al., 1965; Huxley and Brown, 1967; Haselgrove, 1980; Holmes et al., 1980). Because of different monomer repeat distances in myosin and actin filaments, some disorder in the protein structures must be present for a majority of the myosin heads to attach to actin. Cross-bridge disorder has been detected by both electron microscopy and x-ray diffraction (Taylor et al., 1984; Haselgrove and Reedy, 1978; Poulson and Lowey, 1983; Heuser and Cooke, 1983).

When muscle fibers are exposed to physiological concentrations of ATP in the absence of calcium, they become relaxed. Relaxed muscle has a very low stiffness and is easily extensible. EPR spectroscopy of a spin label attached to SH-1 in relaxed muscle fibers indicated a high degree of disorder in the cross-bridge (Thomas and Cooke, 1980; Arata and Shimizu, 1981). Saturation transfer EPR (ST-EPR) of the maleimide spin label attached to SH-1 of myosin showed that the spin label is rigidly attached to myosin and that the cross-bridges were undergoing Brownian rotations in a time scale of 1–10 μs (Thomas et al., 1980). Studies of fluorescent probes also indicated much more disorder in relaxed muscle than in rigor muscle fibers (Borejdo and Putnam, 1977; Burghardt et al., 1983; Yanagida, 1981). However, x-ray diffraction and electron microscopy of relaxed muscle have usually been interpreted as indicating more cross-bridge order than detected by EPR and fluorescence measurements. Analysis of the diffraction data leads to a model in which the myosin heads are arranged in a helical array about the thick filament (Haselgrove, 1980). Addition of nucleotides and vanadate produces a state that has mechanical properties and x-ray diffraction patterns that resemble those of relaxed muscle (Goodno, 1979; Goodno and Taylor, 1982; Goody et al., 1980).

The results cited above show that the apparent angular distribution of cross-bridges depends upon the method used to measure the distribution. In particular, paramagnetic probes placed at SH-1 have measured less disorder in rigor muscle and more disorder in relaxed muscle than deduced from some other methods. Spectra obtained from probes at additional sites can help resolve these controversies and can give additional insight into the mechanism of contraction. The nucleotide site on the myosin head is an important region to probe because of its role in energy transduction. In an effort to probe this site by EPR spectroscopy we have used paramagnetic analogues of adenine nucleotides. We show that these spin labels bind to the nucleotide site of myosin and that they can be used to determine the orientation of this site in rigor and in muscle fibers relaxed by the addition of vanadate plus nucleotides. These studies complement and extend previous work that used fluorescent nucleotides because considerably more information can be obtained on the angular distribution of the spin labels. The entire angular distribution of the probes can be determined with a resolution of a few degrees and the contributions due to bound and unbound nucleotides can be separated.

EXPERIMENTAL METHODS

Striated muscle fibers were derived from rabbit psoas and soleus muscles, and insect flight muscles were obtained from Lethocerus. Small strips of psoas muscle fibers (1–2-mm diam) were dissected and tied to wooden sticks. Soleus muscle fibers were cut into rectangular strips and pinned to styrofoam. Muscle fibers were incubated at 0°C in 50% glycerol/50% solution of 0.24 M potassium acetate, 10 mM magnesium acetate, 10 mM EGTA, 40 mM TES, pH 7. After 24 h, the solution was changed, and the muscle fibers were stored at −20°C. Insect flight muscles were provided by Dr. M. Reedy. The day before use, the fibers were incubated in rigor solution (0.12 M potassium acetate, 5 mM magnesium acetate, 1 mM EGTA, and 20 mM TES, pH 7) and 0.02% Triton X-100 at 0°C overnight and then washed with rigor solution before use. The EPR spectrum of SL-ATP–treated muscle fibers, not exposed to Triton, displayed a significant number of randomly distributed spin labels that were much reduced in Triton-treated fibers. These probes probably represent SL-ADP bound to membrane components such as the sarcoplasmic reticulum, which is known to have ATPase activity. Sodium orthovanadate was purchased from Fisher Scientific Co., Pittsburgh, PA. Stock solutions of 60 mM vanadate were prepared by weight and boiled at pH 10 to destroy polymeric species (Goodno, 1979).

Fibers prepared as described above had sarcomere lengths of 2.2–2.4 μM. To obtain long sarcomere lengths, the fibers were first stretched mechanically in a relaxing solution (rigor solution plus 5 mM ATP) at 0°C, then washed with rigor solution and incubated in the experimental solution. Sarcomere lengths were determined from their diffraction patterns. Measurements of fiber stiffness were made as described in Crowder and Cooke (1984). Fiber stiffness was high in rigor fibers, with a Youngs modulus of 20 ± 4 N/mm^2 and was changed by <10% by addition of either ADP or SL-ADP. Fiber stiffness fell to about one-third of its rigor value after incubation with SL-ATP and vanadate. Physiological measurements were made on single muscle fibers in conjunction with EPR experiments by using aliquots of the EPR solutions.

EPR measurements were made with an ER/200D EPR spectrometer from IBM Instruments, Inc., Danbury, CT. X-band, first-derivative, absorption EPR spectra were obtained with the following instrument settings: microwave power, 25 mW, modulation, 0.2 mTesla at 100 kHz. Muscle fiber EPR spectra were obtained in one of two experimental configurations. In one protocol, muscle fibers (50–100) were placed in a capillary (0.7-mm inside diam) that extended through side wall holes (3-mm diam) of a TM_{110} cavity such that the fibers were aligned parallel with the static magnetic field (H_0). The fibers were secured at their ends with surgical silk, and the capillary was attached to a flow system that allowed a rapid perfusion of the fibers. However, EPR spectra were obtained in the absence of flow so that all nucleotides had been hydrolyzed to their diphosphate forms by the fiber ATPase activity. The EPR spectra of fibers in the capillary contained a large contribution from free spin-label due to solution external to the fibers. To minimize the signal from free spin-labels, a rigor solution was used to briefly flush the capillary. This procedure produced the best EPR spectra of myosin-bound SL-ADP by minimizing the EPR signal due to free spin-label. Alterna-

tively, EPR measurements were performed with a TE_{102} cavity and a specially designed flat cell made of rexolyte. Small bundles of muscle fibers (7-mm long) were blotted, enclosed, and oriented parallel or perpendicular to the magnetic field. All studies of nucleotide affinities were performed using the flat cell. All spectra were obtained at 25° ± 2°C. Application of stress to muscle fibers was applied as described by Cooke (1981).

EPR computer simulations were performed on a VAX 11/80 using an EPR program written by Dr. David Thomas. This program includes two angular distributions, θ, the axial angle between the principal z-axis of the nitroxide and the direction of the magnetic field (fiber axis), and ϕ', the angle corresponding to rotation of the probe about its z-axis. Changes in ϕ' produce small shifts in the center of gravity of the three lines with almost no change in the splittings between the lines. Because different angular distributions for ϕ' led to similar simulated spectra, this distribution is not well determined by the experimental spectra, and ϕ was taken to be random. Thus, the spectra were fit with two parameters that described the angular distribution of θ: the mean angle of a Gaussian distribution and its width. The g tensor was assumed to be rhombic (g_x = 2.0086, g_y = 2.0066, g_z = 2.0032), and the hyperfine tensor axial (Ax = 4.7 gauss, Ay = 4.7 gauss, Az = 31 gauss) (Griffith et al., 1965). Details of the calculations are discussed by Thomas and Cooke (1980) and by Barnett et al. (1986).

Spin-label Synthesis

Spin-labeled ATP (2'-3'-0-[1-oxyl-2,2,5,5-tetramethyl-3-carbonyl pyrrolidine] adenosine 5'-triphosphate) was synthesized by a modified method of Guillory and Jeng (1977) and Streckenbach et al. (1980) (see Fig. 1). Dry *N*, *N'*-carboxydiimidazole was mixed with an equal concentration of 2,2,5,5-tetramethylpyrrolidine-3-carboxy-1-oxyl (800 μM) in anhydrous dimethylformamide (DMF) at room temperature for 30 min. Aqueous ATP (800 μM) was added to the reaction mixture and mixed for 45 min.

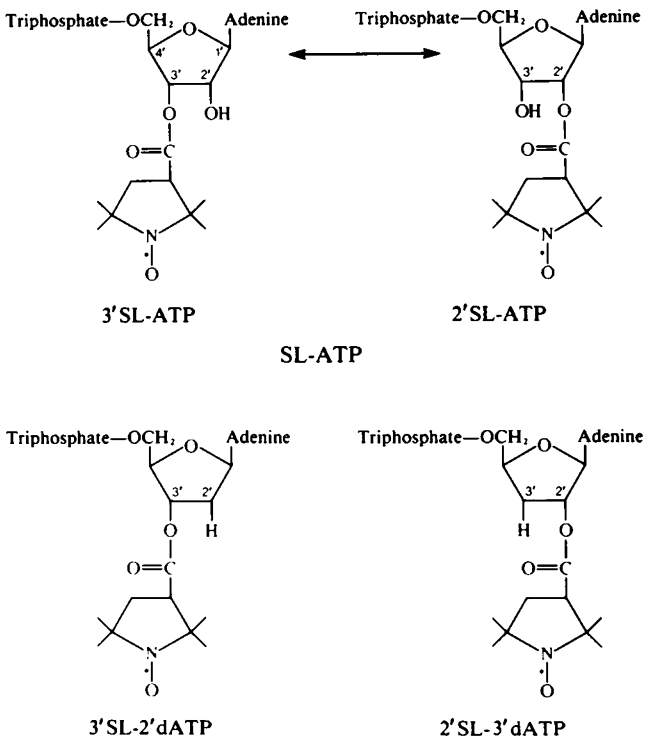

FIGURE 1 Structures of spin-labeled nucleotide analogues. SL-ATP, shown above, exists in two forms with the label attached to either the 2' or 3' position. Shown below are the two deoxy ATP spin labels: 3'SL-dATP and 2'SL-dATP.

Acetone was added (at 10 × vol) to precipitate ATP and SL-ATP. The precipitate was then washed several times with acetone to remove residual reactants. 2'SL-dATP and 3'SL-dATP were synthesized in the same manner except 3'dATP and 2'dATP, respectively, replaced ATP as the starting material.

SL-ATP was purified either by chromatography on Affi-gel 601 (Bio-Rad Laboratories, Richmond, CA) or by reversed phase separation in a high pressure system. An Affi-gel 601 column was equilibrated with 0.1 M LiCl, 1.0 M ammonium acetate at pH 8.5, and the precipitate from the synthesis was dissolved in 10 ml of the same solution and loaded on the column. ATP binds to this column while SL-ATP is eluted. SL-ATP was separated from LiCl with a Sephadex G-10 column, lyophilized, and stored at −20°C. Alternatively, SL-ATP, 2'SL-dATP, and 3'SL-dATP were purified using an LC/9533 IBM Instruments, Inc. liquid chromatograph equipped with an LC/9523 variable wavelength UV detector (Toranji et al., 1985). Fig. 2 shows the reversed phase high performance liquid chromatography (HPLC) separation profile when the SL-ATP reaction mixture is loaded on a silica-based methyl column. On the basis of absorption of 260 nm, three elution peaks were detected of which two had EPR signals. EPR analysis was performed by taking 50-μl aliquots of eluent every 30 s and measuring the EPR spectrum. The first peak has no EPR signal and has a retention time similar to free nucleotides (ATP, ADP, AMP, 2'dATP, 3'dATP). The second elution peak has a large EPR signal and has a retention time similar to carboxylic acid spin-label. The third peak has a retention time similar to that obtained when pure SL-ATP is loaded on the column. Manganese (II) binds to either SL-ATP or SL-ADP causing the EPR signal, due to the nitroxide to broaden and decrease in intensity (Toranji et al., 1985). Manganese (II) had a dramatic effect on the EPR signal intensity of the third peak but only a small effect on the second peak. When eluent of peak three is loaded on polyethyleneimine-impregnated cellulose, it migrates as SL-ATP, indicating that little if any SL-ADP is present. These findings indicate: peak three is due to product, SL-ATP; peak two is due to unreacted spin label; and peak one is due to unreacted nucleotide and other material of the reaction mixture. Similar results were obtained with the SL-dATP analogues. Product was concentrated by vacuum drying

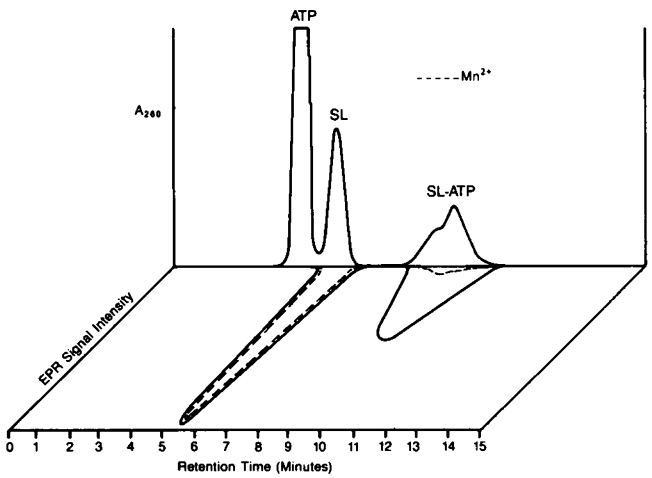

FIGURE 2 Three-dimensional elution profile of reversed phase HPLC run of SL-ATP reaction mixture loaded on a methyl column. At retention time = 0 (injection of reaction mixture) the mobile phase was 500 μm Tris at pH 7. At time = 3 min, a gradual gradient of methanol was applied so that at time = 3.5 min the final mobile phase was 10% methanol/90% 500 μm Tris. Mobile phase rate was 1 ml/min. EPR analysis was performed by taking 50-μl aliquots of a 0.5-ml eluent and measuring the EPR spectrum in the absence and presence of 500 μm $MnCl_2$. Assignments of peaks are based upon retention times of pure components and EPR analysis (see text).

and stored at −30°C until use. Before using SL-ATP (2'SL-dATP and 3'SL-dATP), the manganese (II) quench experiment was performed to assure that no decomposition of spin-labeled ATP had occurred. Periodic chromotography on polyethyleneimine showed that no hydrolysis had occurred.

3-Carboxy-1-oxyl-2,2,5,5-tetramethyl pyrrolidine was purchased from Molecular Probes, Inc., Junction City, OR. ATP, ADP, and 1,1'-carbonyldiimidazole were purchased from Aldrich Chemical Co., Milwaukee, WI. AMP, 2'-deoxyadenosine 5'-triphosphate (2'dATP), and 3'-deoxyadenosine 5'-triphosphate (3'dATP) were obtained from Sigma Chemical Co., St. Louis, MO. HPLC grade methanol and doubly distilled water was used for preparation of the samples and buffers. Manganese (II) chloride tetrahydrate was used for the EPR analysis. Preliminary studies were performed with SL-ATP given to us by Drs. R. Grosse and B. Streckenbach.

RESULTS

Binding of Diphosphate Analogues to Muscle Fibers

Glycerinated muscle fibers were incubated in a rigor solution with 50 μM SL-ATP. After several minutes, allowing sufficient time for hydrolysis of SL-ATP to occur, the fibers were transferred to an EPR flat cell and excess solution was removed. The EPR cell was aligned so the muscle fibers were oriented parallel with the static magnetic field. The resulting EPR spectrum for SL-ADP is shown in Fig. 3. This spectrum is complex and represents the superposition of strongly immobilized (bound) SL-ADP in equilibrium with freely tumbling unbound SL-ADP in the interfilament space of the muscle fiber. The spectra of unbound SL-ADP measured in the absence of a fiber consists of three intense narrow EPR signals, seen in Fig. 3, which are due to the hyperfine interaction between the free radical and the nitrogen nucleus of the nitroxide group. These signals can be used to determine the concentration of unbound SL-ADP in the muscle fibers. The spectra of bound SL-ADP consists of broader peaks, two of which are easily discernible, labeled P_1 and P_2 in Fig. 3. In addition, the central peak is broader and there is negative intensity to the right of the narrow high field line. These relatively weak signals represent SL-ADP bound to myosin, and they are dependent upon the orientation of the muscle fiber relative to the static magnetic field. To show that SL-ADP is bound at an ATPase site, we added ADP to these fibers. The resultant EPR spectrum, shown by a dotted line in Fig. 3, has no P_1 and P_2 signals. ADP competes with SL-ADP, and the spectral components from bound analogues decrease while those from free SL-ADP increase in intensity. This is strong evidence that SL-ADP binds at a site that is specific for nucleotides.

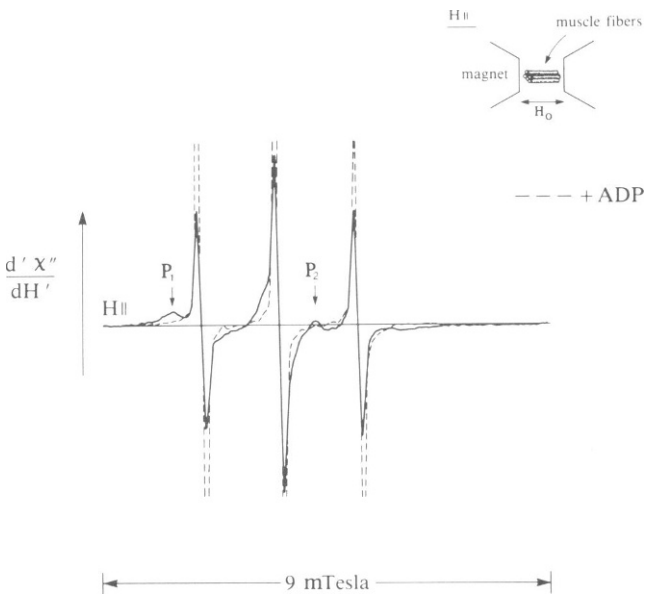

FIGURE 3 First derivative X-band absorption EPR spectrum of 50 μM SL-ADP in rabbit soleus muscle fibers. Several bundles of ~25 muscle fibers were blotted and placed in a flat cell that was aligned parallel ($H\parallel$, see *inset*) to the static magnetic field (H_0). P_1 and P_2 refer to signals that decrease upon the addition of 1 mM ADP (*dotted line*) and are due to spin label bound to the nucleotide site. EPR conditions: TE_{102} cavity microwave power, 25 mW; modulation, 0.2 mTesla at 100 kHz. Data are an average of 25 scans.

Simulations of the EPR spectrum obtained when the muscle fibers are aligned parallel to the magnetic field indicate that a distribution of bound SL-ADP about a single orientation cannot explain the EPR spectrum. It appears that at least two orientations of bound SL-ADP are required to simulate the spectrum. We show below that the two orientations arise from the two isomers of SL-ADP and not from two orientations of the nucleotide site. SL-ATP has two isomers with the nitroxide spin-label attached to either the 2' or the 3' position of ATP by an ester linkage with rapid interconversion of the isomers. The relative proportions of the two isomers are not known but significant fractions of each are expected. Because the interconversion is slow compared with the temporal resolution of the EPR measurement, effectively two different spin labels exist within the SL-ATP mixture. To clarify experimental results obtained with SL-ATP, we synthesized two nucleotide spin labels from 2' and 3' deoxy ATP. These analogues exist in one form with the nitroxide reacting with a single hydroxyl of the ribose group of deoxy ATP. A spin label attached to the 3' position of 2' deoxy ATP is called 3'SL-dATP, while the other form, 2'SL-dATP, has the spin label attached to the 2' position of 3' deoxy ATP (see Fig. 1).

Fig. 4 shows a comparison between the EPR spectra obtained with SL-ADP (4 *A*), 3'SL-dADP (4 *B*), and 2'SL-dADP (4 *C*) when the magnetic field was parallel to the muscle fiber axis. The spectra of the deoxy analogues are more easily analyzed. As described below, each can be shown to consist of two components, three broad peaks that arise from a population of analogues bound to and oriented by the nucleotide site on myosin and three narrow lines that arise from unbound probes. The spectrum obtained with 3'SL-dADP (Fig. 4 *B*) had features in common with the SL-ADP spectrum. The P_2 signal was present and the

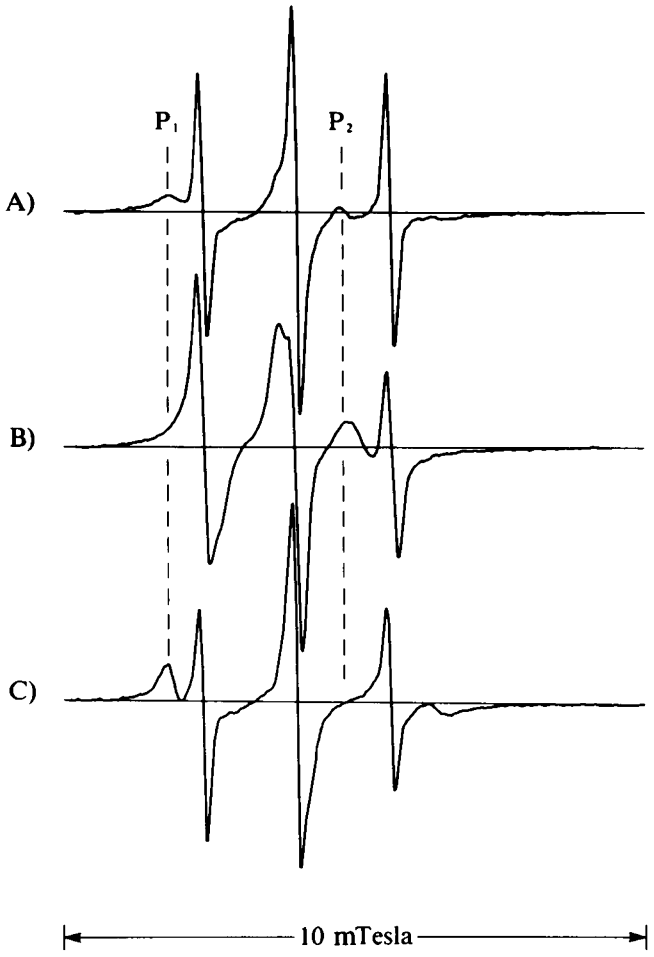

FIGURE 4 Comparison of the three nucleotide spin labels (50 μm) incubated in soleus muscle fibers (*A*) SL-ADP, (*B*) 3'SL-dADP, and (*C*) 2'SL-dADP. The more complex spectrum of SL-ADP (*A*) can be seen to be composed of a superposition of the spectral components detected with 3'SL-dADP and 2'SL-dADP. Each spectrum was obtained with a different set of muscle fibers, and different spectra may contain different amounts of free label that are external to the fibers. Spectra were obtained as described in Fig. 3.

P_1 signal was absent. In addition, two other broad peaks associated with bound 3'SL-dADP were visible: One appeared as a shoulder on the low magnetic field signal, while the other gave rise to double peaks in the center of the spectrum. In the case of 2'SL-dADP, the P_1 peak was present while the P_2 peak was absent (see Fig. 4 *C*). Again, the other two broad peaks, due to myosin-bound 2'SL-dADP, were visible as a broadening of the center line and a clear dip at high magnetic fields. These findings establish that the bound EPR components of SL-ADP in muscle fibers are a mixture of two populations due to two isomers of SL-ADP (see Fig. 1).

Although the spectra of the two deoxynucleotides are simpler than those of SL-ATP, we have pursued the latter for several reasons. SL-ATP has one ribose hydroxyl available for hydrogen bonding to the protein, while the deoxynucleotides have none. Although the present studies find that all three analogues bind with about equal affinity to the nucleotide site of myosin, SL-ATP may prove to be a superior analogue in future studies, e.g., in active fibers. Because SL-ATP effectively provides two labels, its spectra also provide more information than do either of the deoxynucleotides. Finally, SL-ATP is more cheaply synthesized.

The angular distribution of the bound analogues was determined by comparison with simulated spectra. Spectra were simulated by solving the spin Hamiltonian and calculating the intensities expected for various angular distributions (see Methods). The spectrum of 3'SL-dADP can be decomposed into a component due to free analogue and a bound component that has an angular distribution of θ that can be approximated by a Gaussian distribution centered at 67° ± 4° with a full width at half maximum of 13° ± 5° (shown in Fig. 5, right). The spectrum of bound 2'SL-dADP can be fit by assuming a Gaussian distribution in θ centered at 55° ± 5° with a width of 16° ± 4° (Fig. 5, *left*). The mean angle of the distribution is determined largely from the positions of the three peaks, while the spread in the angles is determined by the relative peak heights (Barnett et al., 1986). The errors in the above parameters were estimated by determining the errors in peak heights and in the peak positions from a series of spectra (four to six) and obtaining the corresponding errors in the angles from the spectral analysis of Barnett et al. (1986). These simulations show that the bound analogues have a rather narrow distribution of orientations on the myosin head, and thus that the nucleotide site is well oriented by the interaction with actin. Because the peaks for 3'SL-dADP are slightly sharper than those for 2'SL-dADP, the peak heights are approximately twice as high for a given quantity of spins. Spectra were simulated for equal numbers of spins for the two isomers, and the positive peak heights were normalized to the center peak of 2'SL-dADP. The low field, center field, and high field peak heights were found to be 0.72, 1.0, and 0.22 for 2'SL-dADP and 1.11, 1.98, and 0.48 for 3'SL-dADP.

Additional information on the orientation of bound analogues is provided by a comparison of EPR spectra obtained with the magnetic field applied at different orientations to the muscle fiber. This was most clearly seen with 3'SL-dADP when the EPR spectra are obtained with the muscle fiber aligned parallel and perpendicular to the magnetic field (see Fig. 6). The EPR spectrum of unbound spin label was independent of magnetic field direction; however, the bound component was dramatically dependent upon orientation of the muscle fiber in the magnetic field. When the fiber is perpendicular to the field the probe angles relative to the field are distributed between 90° and ~90°–67°, giving rise to the broad peaks at high and low magnetic fields. Although a program to provide a rigorous simulation of this spectrum is not available, the observed peaks occur at approximately the correct positions

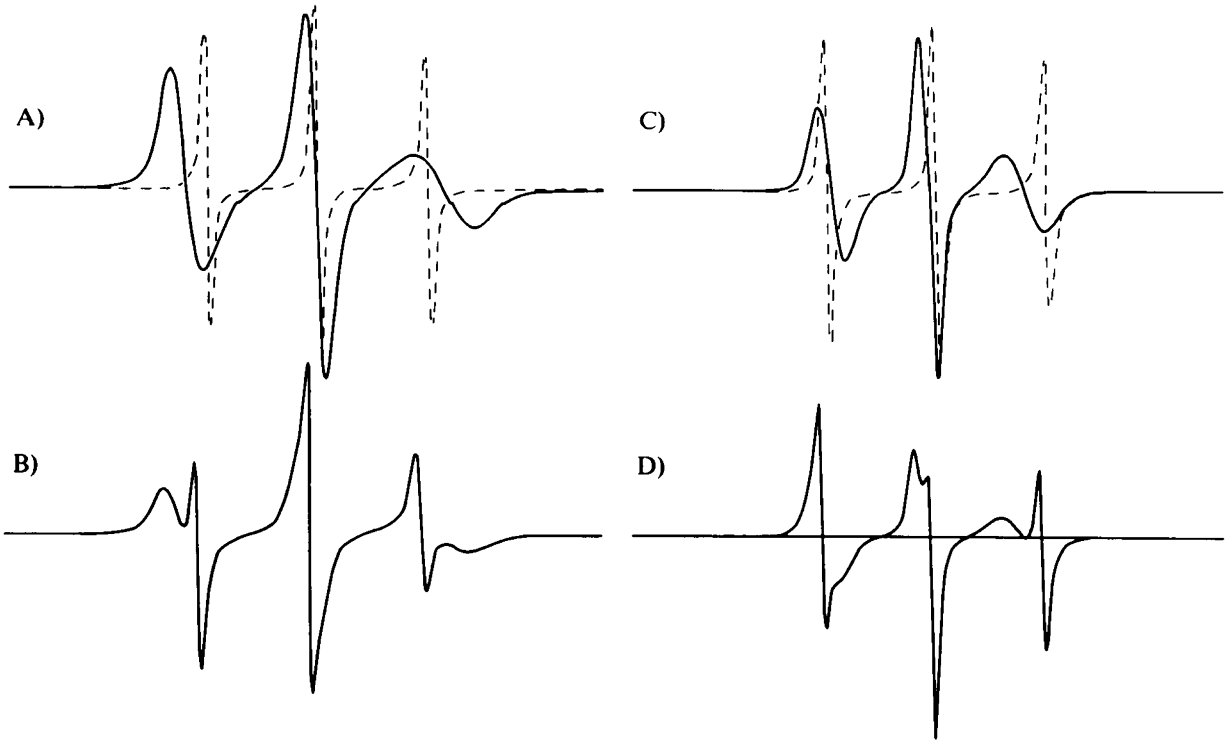

FIGURE 5 Simulations of EPR spectra. The simulations on the top left show that the spectrum of 2'SL-dADP bound to fibers can be closely approximated by two components, one due to free analogue (*dotted line*) and one due to bound analogue (*solid line*). The spectrum of bound analogue was approximated by a Gaussian distribution in θ centered at 55° ± 4° with a full width at half maximum of 16° ± 4°. The simulation shown on the bottom left is a composite of these components, and is a close approximation to the spectrum shown in Fig. 4 *C*. The simulations on the right similarly show that the spectrum of 3'SL-dADP bound to fibers (Fig. 4 *B*) is closely approximated by a component due to free analogue and a bound component described by a Gaussian distribution in θ centered at 67° ± 4° with a full width at half maximum of 13° ± 5°.

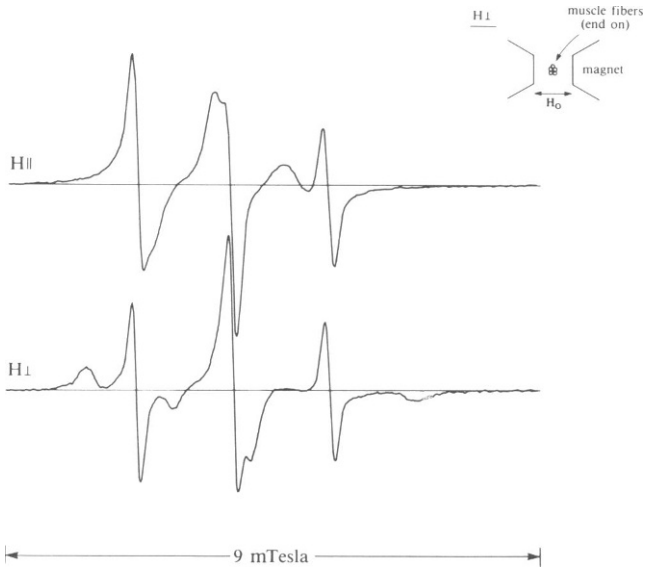

FIGURE 6 Comparison of the EPR spectra of 3'SL-dADP (50 μM) in soleus muscle fibers aligned parallel ($H\parallel$) and perpendicular ($H\perp$, see *inset*) to the static magnetic field (H_0) in a flat cell. These spectra clearly demonstrate the high degree of anisotropy of the bound nucleotide. The three narrow signals, which are independent of the orientation of muscle fibers, represent mobile probes that are not bound to myosin. Spectra were obtained as described in Fig. 3.

expected for probes that are aligned at 67° to the fiber axis.

In summary the EPR spectrum of SL-ADP in muscle fibers gives rise to three spectral components: (*a*) three sharp isotropic peaks due to unbound nucleotides; (*b*) three broader peaks with narrow splittings that arise from the 3' isomer of SL-ADP bound to the nucleotide site of myosin; (*c*) and three broader peaks with greater splitting that arise from the 2' isomer of SL-ADP bound to the nucleotide site of myosin. The bound 2' and 3' isomers each give rise to three broad peaks, but only one from each isomer is clearly visible due to the overlap of these peaks with those from unbound label.

The relative concentrations of free and bound nucleotides in muscle fibers can be measured from their respective EPR spectral components and used to determine the affinity of the analogue for the nucleotide site of myosin. A straight forward method would be to subtract the EPR spectrum of unbound spin-label from the spectra obtained from muscle fibers and to doubly integrate the resulting spectrum, which would be due to bound, immobilized SL-ADP. However, this method is very difficult because the spectrum of the free SL-ADP is altered by the fiber, possibly because it is encountering a range of microviscosities giving rise to a distribution of rotation correla-

tion times. This made an accurate subtraction difficult. Thus, to determine the affinity of the label for the myosin ATPase site we measured the relative population of unbound and bound components. A number of experiments in which ADP competed with SL-ADP showed that a unit height of the low field peak, P_1, represented 10 ± 2 times as many spins as did a unit height of the low field peak from the free spin label. As shown in Fig. 3, the addition of ADP causes a dramatic increase in the signal intensity of the unbound component of SL-ADP, while the bound EPR signal decreases.

Binding constants of the analogues were easily calculated by using the signal intensities of the EPR signals to determine the numbers of bound and free probes. The number of myosin sites has been determined previously (myosin heads = 220 μM; Marston [1973]). The intensities due to the two isomers of SL-ADP were approximately equal so that the affinities of the two analogues for myosin do not differ by more than a factor of 2. Binding constants were measured using SL-ADP, using the height of P_1 to determine the concentration of the bound 2′ isomer, and assuming that both isomers bound with similar affinities. The fractions of free probe, bound probe, and free myosin were determined at several concentrations of free probe (25–100 μM) and used to calculate the binding constants. There was no systematic variation of the binding constant with concentration, and the various values were averaged. Insect flight muscle and psoas muscle have similar affinities, 5×10^3/M and 7×10^3/M, respectively, however, soleus muscle fibers bind spin-labeled nucleotide more tightly, with an association constant of 2×10^4/M. The uncertainty in the absolute value of these constants is approximately ±50%, which arises from several aspects of the method used. The largest uncertainty comes from variation in the volume of solution surrounding the fibers. However, the relative binding affinities are more precise (±20%). The higher binding constant for soleus is in agreement with the notion that muscle types with slower ATPases have a higher affinity for nucleotide (White, H., personal communication).

Fig. 7 shows the EPR spectra obtained when 50 μM SL-ADP was added to soleus, psoas, or insect flight muscle fibers. Comparison of these spectra shows that the angular distribution of bound SL-ADP is almost the same in all three, indicating a high degree of homology in both the structure of the nucleotide binding site and in the structure that orients this site on the actin filament. A small difference betwen vertebrate and insect muscle, seen to the left of the central peak, is probably due to a slightly wider distribution of the labels in insect fibers.

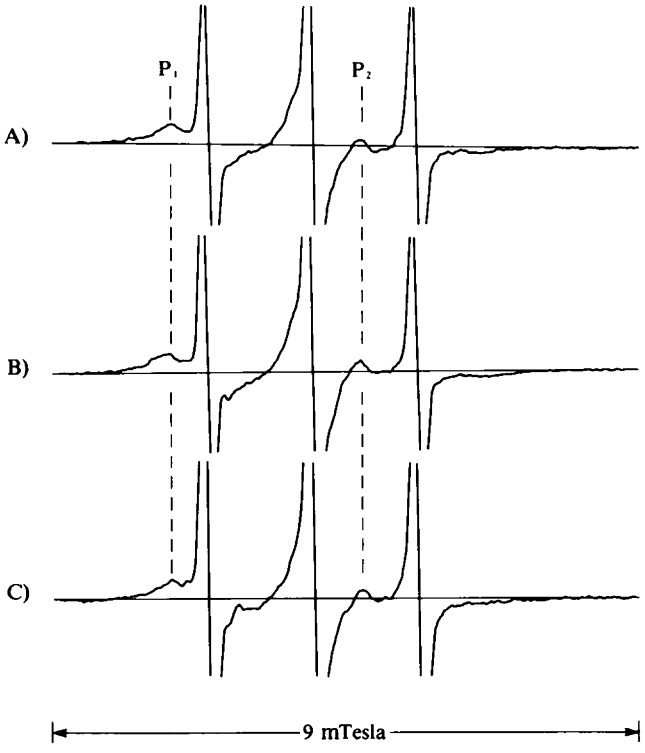

FIGURE 7 Comparison of the EPR spectra of 50 μM SL-ADP in the three muscle types: (*A*) rabbit soleus, (*B*) rabbit psoas, and (*C*) Lethocerus insect flight muscle. The shapes of the spectra are almost identical in the three muscle types. In particular the positions of P_1 and P_2 signals occur at the same position in all muscle types. Due to the lower affinity of the probe for myosin in psoas and insect flight muscles, the amplitude of the three narrow peaks from unbound label is greatest in spectrum *C*, and spectrum *B* shows more unbound label than spectrum *A*. Lethocerus spectrum was obtained with a flat cell while the mammalian muscles were mounted in a capillary and spectra obtained in a modified TM_{110} cavity.

Effect of Stress

Active force is thought to be generated by a change in cross-bridge conformation, leading to the possibility that a passive force could in turn alter cross-bridge conformation. We investigated the influence of external tension on the cross-bridge by monitoring the EPR spectrum of bound analogues as a function of stress applied to the muscle fiber. Muscle fibers were mounted in a capillary that extended through the microwave cavity such that the long axes of both capillary and muscle fibers are parallel with the magnetic field. One end of the bundle of fibers (~75 fibers) was secured with surgical thread and fixed. The other end was tied with surgical thread that was passed over a pulley and attached to a weight. The muscle fiber was bathed in rigor solution with 50 μM SL-ADP (or one of the deoxy analogues). The EPR spectrum was monitored as stress was applied to the fiber. The EPR spectrum was recorded with no stress on the fiber and again while stress was being applied. With minimal stress, a slight sharpening of lines was detected with no change in the splitting between the low and high field peaks of bound SL-ADP. We believe this to be due to better alignment of the fibers with respect to the magnetic field (Cooke, 1981). Tension was increased in small increments until the fibers were torn apart. No additional changes were detected in the EPR spectrum until the fibers broke. A comparison of

the experimental spectra with a series of simulated spectra leads to the conclusion that a change in the principal axis of either isomer by >5° would have generated a detectable change in the signal. We conclude that a passive tension, which was as much as that generated by active fibers (0.2 N/mm^2), does not alter the angular distribution of spin-labeled nucleotides attached to the nucleotide site on myosin.

Fibers at Longer Sarcomere Lengths

When fibers are stretched beyond filament overlap, the interaction between actin and myosin is eliminated. Fig. 8 compares rest length fibers (8 *A*) to stretched fibers (8 *B*). EPR spectra obtained with a high degree of non-overlap between myosin and actin filaments (sarcomere length, 3.5 μm) showed EPR signals characteristic of immobile probes with a highly disordered angular distribution and a decrease in the amount of the oriented components seen at full overlap. At a sarcomere length of 3.5 μm, ~35% of the cross-bridges remain in contact with the thin filaments. This explains the presence of the oriented probes in Fig. 8 *B*, which are seen more prominently at P_2 than at P_1, since P_1 is buried between two other peaks. Fig. 8 *D* shows the EPR simulation of randomly oriented immobilized probes. Intermediate sarcomere lengths produced EPR spectra with larger numbers of ordered and disordered components. This is strong evidence that the immobilized EPR signals represent SL-ADP bound to cross-bridges and that in the absence of a bond with actin the orientation of the probes becomes disoriented. A similar disordered distribution in stretched fibers was also observed for probes on SH-1 (Thomas and Cooke, 1980; Barnett and Thomas, 1984).

Vanadate-induced Relaxation

At low concentrations of nucleotides fibers do not relax, due to the presence of rigor bonds which block the inhibitory effects of troponin and tropomyosin. Because spectra must be obtained at low concentrations of analogue to minimize the signal from unbound probes, it was difficult to obtain spectra of fibers relaxed by SL-ATP. An alternative to SL-ATP–induced relaxation is the use of SL-ADP and vanadate. After nucleotide hydrolysis, vanadate forms a long lived complex with myosin-ADP, which inhibits further ATPase activity (Goodno, 1979; Goodno and Taylor, 1982). When vanadate and ATP are added to insect flight muscle, contraction is inhibited and the muscle stiffness and x-ray diffraction pattern mimic the relaxed state (Goody et al., 1980). Fig. 8 *C* shows the EPR spectrum obtained when insect flight muscle fibers are incubated with 50 μm SL-ATP and 5 mM vanadate. The ordered component detected when SL-ADP is added to rigor fibers was decreased with a concomitant increase in disoriented spin probes. Similar spectra were obtained when rabbit psoas or soleus fibers were incubated with

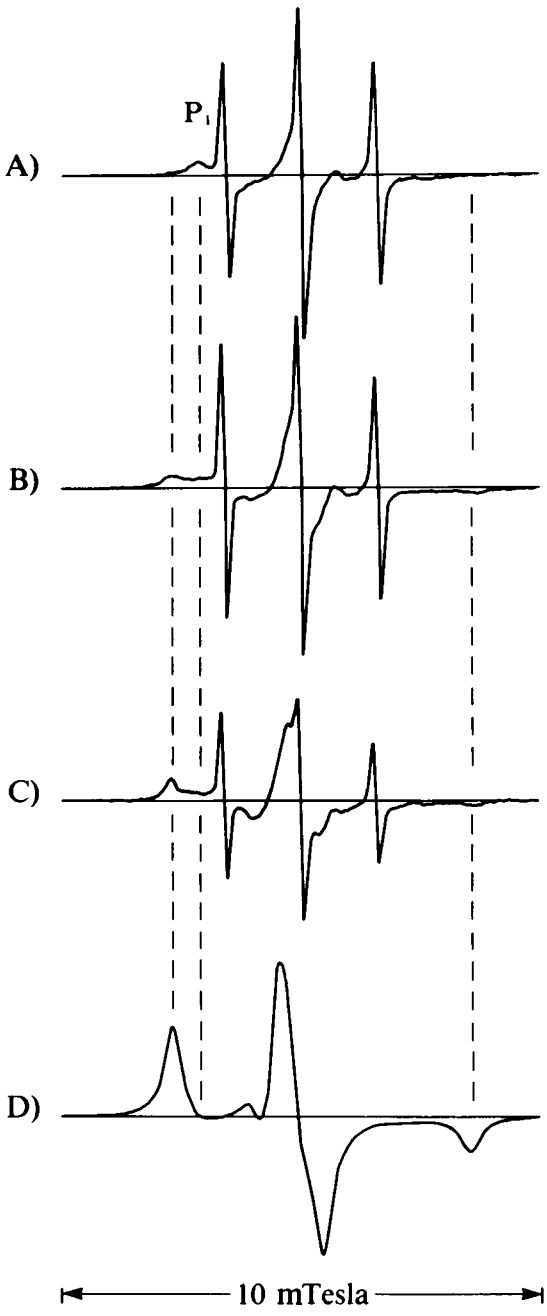

FIGURE 8 EPR spectra of SL-ADP in (*A*) rest length soleus fibers, (*B*) stretched soleus fibers (different set of fibers, sarcomere length 3.5 μm), (*C*) insect flight muscle treated with orthovanadate (5 mM), and (*D*) computer simulation of randomly oriented and immobilized spin probes. Spectra *A* and *B* were obtained from fibers mounted in a capillary using the modified TM_{110} cavity, while spectrum *C* was obtained as described in Fig. 3.

SL-ATP and vanadate. The spectral components seen in spectrum 8 *C* closely resemble the spectrum obtained from randomly distributed probes, spectrum 8 *D*, superimposed on the spectrum of unbound probes. Spectrum 8 *C* suggests that cross-bridges detach from actin, in the presence of SL-ADP and vanadate, undergo large angular motions

that are slow on the EPR time scale, i.e., with a rotational correlation time longer than 10^{-7} s.

The addition of vanadate and 3'SL-dATP did not appear to induce relaxation of muscle fibers. The EPR spectrum was identical to that of fibers bathed in 3'SL-dADP. However, vanadate and 2'dATP did induce a randomization of bound probes. Thus, it appears that when the spin label is attached at the 3' position of ribose, it may interfere with the binding of vanadate. We detected no ordered components with SL-ATP and vanadate, suggesting that the vanadate complex with the 2' isomer of SL-ADP may occupy all of the myosin sites. Although we did not examine the rate of vanadate relaxation in detail, an initial population of spin labels did randomize, while a rather long incubation time (12 h) was required to completely relax the cross-bridges. This may represent a tautomerism-induced delay in the formation of the 2'SL-dADP vanadate complex at the active site of myosin.

DISCUSSION

Although the power-stroke of the muscle cross-bridge is thought to involve large changes in protein conformation, the exact nature of these events remains unknown. It has been proposed that the power-stroke is driven by an alteration in the orientation of some portion of the cross-bridge while it is attached to actin, and such alterations are easily monitored by probes (fluorescent and paramagnetic) whose orientation can be determined from their spectra. Previous studies have concentrated on fluorescent and paramagnetic probes attached to SH-1, and on fluorescent nucleotides. Here we present evidence that a paramagnetic nucleotide can also provide information on cross-bridge orientation. The spectra of paramagnetic probes can provide considerably more information on the distribution of probe orientation than can be obtained using fluorescent probes, so these studies extend and amplify the results obtained with fluorescent nucleotides (Yanagida, 1981, 1983, 1985).

Several experimental results show that the spin-labeled nucleotides are binding largely to the nucleotide site of myosin. Virtually all bound nucleotides are removed by addition of ADP, indicating that the analogue is bound to sites that also bind ADP. The affinity of SL-ADP for these sites is approximately the same as that of ADP for actomyosin or for fibers (discussed below). The most compelling argument, however, is that the orientation of the bound nucleotides depends upon factors that would be expected to influence cross-bridge orientation. The diphosphate analogues bound to fibers are highly ordered and either stretching the fibers or formation of the complex with vanadate caused a loss in both the interaction of cross-bridges with actin and in the ordered component of the bound probes.

The analogues bind to myosin with an affinity that is very similar to that of ADP. An association constant of 7×10^3/M was found for SL-ADP in psoas muscle fibers, which agrees with the association constant of ADP with the acto-S1 complex (Green and Eisenberg, 1980; Highsmith, 1976) but is a little less than that measured for the binding of ADP to rabbit psoas fibers (Marston, 1973). The association constant for SL-ADP in insect flight muscle was very similar to that of psoas muscle fibers (5×10^3/M). However soleus muscle fibers had a higher association constant, of 2×10^4/M. The stronger affinity may be explained by the results of Marston and Taylor (1980), who found that the ADP dissociation rate constant for slow red muscle acto S1 was much smaller than for fast white muscle acto S1.

The spectra shown in Fig. 3–6 lead to the conclusion that diphosphate analogue are highly oriented when bound to the myosin site. Two different populations are found with approximately equal numbers of probes oriented with their principal axis at 67° and 55° relative to the fiber axis. The distribution of probe orientations about these mean angles can be adequately described by a Gaussian with a full width at half height of 13° and 16°, respectively. The spectra of 2'SL-dADP and 3'SL-dADP confirm that the two different angles detected with SL-ADP arise from the two isomers of the analogue and not from different cross-bridge orientations. Thus, the nucleotide site of myosin is highly oriented with respect to the fiber axis in the presence of diphosphate analogues. This conclusion agrees with that obtained previously with fluorescent nucleotides, although the report of two different cross-bridge orientations found in the presence of large concentrations of ϵ-ADP is not confirmed at similar concentrations of SL-ADP (Yanagida, 1981). Virtually identical angular distributions of spin-labeled nucleotides are found in the three muscle types studied, showing that the structure of this site and its alignment by the actomyosin bond are highly conserved despite the large evolutionary distance between vertebrate skeletal and insect flight muscles. Because both isomers of SL-ADP and two fluorescent nucleotides, ϵ-ADP and 2-aza-ϵADP, all appear oriented in muscle fibers, the nucleotide site of myosin is highly ordered in all three axes in this state. This conclusion is identical to that obtained from probes bound to SH-1. The portion of myosin that is oriented by its interaction with actin is rather large. Fluorescence energy transfer measurements have shown that the nucleotide binding site on myosin is 20–30 Å from SH-1, and both are at least 50 Å away from various sites on actin (Takashi, 1979; Trayer and Trayer, 1983; dos Remedios and Cooke, 1984).

Because myosin and actin filaments are formed by helical arrays of subunits with different repeats, some disorder must exist in the array of cross-bridges that are attached to actin. Disorder in rigor muscle fibers has been implied from the presence of diffuse scattering x-ray diffraction patterns and has been visualized in electron micrographs (Poulson and Lowey, 1983; Heuser and Cooke, 1983; Reedy et al., 1965; Taylor et al., 1984). The

fact that little disorder is seen in probes that are placed at either SH-1 or the nucleotide site shows that the flexibility required to form the actomyosin bond is not found at the interface between the two proteins or in the region between the actomyosin bond and the probe sites. This flexibility may exist in myosin somewhere between the probe sites and the core of the thick filament. There is some evidence that the portion of the myosin head that is close to the thick filament maybe disordered in rigor fibers (Taylor et al., 1984). Thus a synthesis of the evidence showing highly ordered probes and the evidence for some disorder in cross-bridge orientations suggests that some flexibility may exist within the myosin head, with one portion of the myosin head rigidly fixed to actin while a second portion of myosin, more distal from actin, can assume a variety of attitudes.

The application of stress to fibers in the presence of SL-ADP causes no change in the orientation of the probe. Similar results have been found for fluorescent nucleotides, fiber tryptophans, and probes at SH-1 (Yanagida, 1983; Cooke, 1981; dos Remedios et al., 1972). Measurements of the transient mechanical response of active fibers after step changes in muscle length have shown that some compliance exists within the cross-bridge (Huxley and Simmons, 1971; Ford et al., 1985). An apparently similar compliance is also found in rigor fibers (Tawada and Kimura, 1984). The results obtained with probes suggest that this compliance is found between the probes and the core of the thick filament. It should be noted, however, that a stress-induced rotation of the cross-bridge about the fiber axis would not be detected because rotations of probes about the axis of the magnetic field do not produce spectral changes. The possibility of such rotations is suggested by evidence that the helix of actin monomers can rotate about the thin filament axis and that rotations may occur in rigor fibers (Egelman et al., 1982; Taylor et al., 1984).

Although there has been general agreement that all myosin heads are bound to actin in rigor vertebrate muscle, there has been considerable speculation that some fraction of the myosin heads is not attached to actin in rigor insect flight muscle (Offer et al., 1981). Because nucleotides bind to free myosin more tightly than to actomyosin in vertebrate muscle it is likely that even a small fraction of free myosin in insect flight muscle would give rise to a disordered fraction of probes particularly at the lower probe concentrations (Highsmith, 1976; Greene and Eisenberg, 1980). No such fraction was observed, and we place an upper limit of 20% on the fraction of myosin not bound to actin in the presence of SL-ADP.

Fibers did not relax well in the low levels of nucleotides at which most of our experiments were carried out. To obtain spectra of relaxed fibers we incubated fibers with SL-ATP and vanadate. It has been shown that ADP and vanadate form a tight complex at the nucleotide site of myosin and that this complex has many properties of an ADP·P_i complex thought to be an intermediate in the hydrolysis of ATP (Goodno, 1979; Goodno and Taylor, 1982; Goody et al., 1980). The complex of ADP and vanadate on the myosin head effectively relaxes fibers as determined from x-ray diffraction patterns, suggesting that the myosin–ADP–vanadate complex in fibers is either not bound to actin or is bound very weakly. The results found here show that the SL-ADP–vanadate complex causes almost random disorder in the probes. This disorder is similar to that seen in stretched fibers in the presence of SL-ADP and suggests that the cross-bridges are highly disordered when not bound tightly to actin. We cannot rule out the possibility that the disorder of the probes may be due, at least in part, to a disorder of the probe relative to the myosin head. However, a similar disorder was observed by probes on SH-1 and by fluorescent nucleotides in relaxed fibers (Thomas and Cooke, 1980; Yanagida, 1981). Similar observations with different probes strongly suggest that the orientation of the entire head is highly disordered.

A number of previous studies of spin labels attached to the reactive sulfhydryl on the myosin head have established that this site is well oriented in rigor fibers, that this orientation is not affected by stress, and that the site is highly disordered in relaxed fibers (Thomas and Cooke, 1980; Thomas et al., 1983; Cooke, 1981). The present study has shown that spin labels at the nucleotide site on the myosin head give results that are similar to those obtained in the previous studies, and they lead to identical conclusions concerning the orientation of the myosin head. These conclusions are also in agreement with those obtained from measurements of the orientation of fluorescent nucleotides bound to muscle fibers (Yanagida, 1981, 1983, 1985). This study provides the framework for an investigation of the orientation of the myosin nucleotide site in contracting muscle.

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