Mobility and Orientation of Spin Probes Attached to Nucleotides Incorporated into Actin†

Nariman Naber* and Roger Cooke

Department of Biochemistry and Biophysics and the Cardiovascular Research Institute, University of California, San Francisco, San Francisco, California 94143-0524

Received August 23, 1993; Revised Manuscript Received January 26, 1994®

ABSTRACT: Each actin molecule contains a nucleotide, tightly bound in a deep cleft that divides the molecule. To probe conformational changes within this region of the molecule, we have incorporated two spin label analogues of ATP into actin. In both analogs the spin label was attached to the 6 position on the adenine ring, either directly (6nSLATP) or via a longer thioacetamido linker (6sSLATP). Electron paramagnetic resonance spectra of randomly oriented actin filaments showed that both the probes possessed considerable rotational mobility relative to the protein surface. The 6nSLADP has two degrees of rotational mobility that can be approximately modeled by rapid diffusion within cones with half angles of 30 ± 1° and 42 ± 1.5°. The 6sSLADP displayed one degree of rotational mobility approximated by rapid motion within a cone with a half-angle of 38 ± 1°. The rotational mobility of the probes is determined by the protein structure surrounding them, and changes in this structure should alter the mobility. The mobility of the probes was unchanged by addition of 20 mM P_i, which forms an ADP–P_i complex. However, binding of myosin heads (S1) shifted the population of 6nSLADP toward the more highly restricted cone, while binding of DNase-I shifted it toward the less restricted cone. We conclude that this region of actin is unchanged by binding of phosphate, while the binding of S1 or DNase-I produces only a modest shift in conformation. When actin filaments were oriented by flow into capillaries, the spectra were strongly dependent on the orientation of the capillary relative to the magnetic field of the spectrometer, showing that although the probes are mobile, the average angles of all the probes are similar, calculated as 70° for the 6nSLADP and 67° for 6sSLADP. These results show that the nucleotide region is highly aligned in the oriented gels.

Actin is involved in the generation of force in all eukaryotic cells, and it is a major component of the cytoskeleton which helps determine cell shape. Each actin monomer (G-actin)[1] binds one molecule of ATP which undergoes hydrolysis upon polymerization [see Carlier (1989) for review]. The ADP remains tightly bound to the polymer (F-actin). The nucleotide plays a role in regulating polymerization, leading to the dynamic equilibrium between actin monomers and the actin filaments of the cytoskeleton, F-actin–ADP is more easily depolymerized than F-actin–ATP.

Recently, the atomic structure of the actin monomer complexed with the enzyme DNAse-I was determined both in the ATP and the ADP form by X-ray crystallography at effective resolutions of 2.8 and 3 Å (Kabsch et al., 1990). An atomic model for the actin filament was proposed based on the structure of the subunit and the X-ray diffraction data from gels of oriented actin filaments (Holmes et al., 1990). The molecule consists of four subdomains. In the model of the filament, subdomains 3 and 4 form the core of the filament while subdomains 1 and 2 extend out away from the filament. Subdomain 1 contains the major site involved in the binding of the myosin head (Holmes et al., 1990; Sutoh, 1982). The nucleotide is bound in a deep cleft which divides subdomains 3 and 4 from 1 and 2. The nucleotide interacts with residues on either side of the cleft, and it may play a pivotal role in determining the conformation of the cleft.

Actin participates in a number of interactions that could involve conformational changes. The actin monomer could be altered upon polymerization, a possibility enhanced by the fact that the bound nucleotide is hydrolyzed during this process. The bond between the actin monomer and DNase-I is a very strong one, suggesting the possibility that formation of this complex could also alter the conformation of the actin monomer (Lazarides & Lindberg, 1974). Because the crystal structure was derived from the actin–DNase-I complex, it is of particular importance to understand whether this structure is altered from that found in the F-actin filament. Of course the most important conformational changes are those that accompany the interaction of myosin. Recently, a more active role for actin in the generation of force has been proposed by Schutt and Lindberg (1992).

The presence of putative conformational changes can be easily studied using spectroscopic probes, and the conformation of actin has been monitored using both fluorescent and paramagnetic probes [for review, see Cooke (1986) and Thomas, (1987)]. Spin probes attached to Cys-374 have shown that actin has a rotational correlation time of about 100 μs which increased several fold upon interaction with myosin (Thomas et al., 1979; Ostap & Thomas, 1991). Probes at several sites are sensitive to polymerization or to the binding of myosin, suggesting that at least small changes in actin conformation occur during these events (Ando & Hiroshi, 1979; Hegyi et al., 1974; Bender et al., 1976; Burtnick, 1984; Lehrer & Elzinga, 1972; Miki & Whal, 1984; Miki et al., 1987). Fluorescent nucleotides incorporated into the actin

† Supported by a grant from the NIH (AR 30868).

* Author to whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* March 1, 1994.

[1] Abbreviations: 6sSLATP, 6-mercapto[*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)]acetamido-9-β-D-ribofuranosylpurine 5′-triphosphate; 6nSLATP, 6-amino[*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)]-9-β-D-ribofuranosylpurine 5′-triphosphate; tempoamine, 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl; S1, myosin subfragment-1; G-actin, monomeric actin; F-actin, polymeric actin; EPR, electron paramagnetic resonance; Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; τ_c, rotational correlation time.

0006-2960/94/0433-3855$04.50/0

site have also shown some changes upon interaction with myosin (Yanagida & Oosawa, 1978, 1980). Many of the sites discussed above, including Cys-374, Cys-10, and Gln-41 are located in subdomains 1 and 2, and only the base of the nucleotide provides the opportunity to probe changes occuring in subdomains 3 and 4 as well as motion within the cleft.

As part of our interest in studying the contractile proteins in muscle fibers, we have used EPR spectroscopy to study the rotational mobility and orientation of spin-labeled analogs of ATP that bind to actin at the nucleotide site. These probes are located in an ideal position to monitor conformational changes occurring in the center of the actin monomer and in particular, to sense opening or closing of the deep cleft which runs through the center of the molecule. We find that the structure of the nucleotide region undergoes only minor alterations when actin binds S1, P_i, or DNase-I. In addition, when gels of actin filaments are oriented by flow into capillaries this region of actin is highly ordered, a result which resolves an ambiguity concerning the degree of order observed for spin probes attached to Cys-374 (Ostap et al., 1992; Naber et al. 1994).

MATERIALS AND METHODS

Synthesis of Spin Label ATP Analogs. 6-Amino[*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)]-9-β-D-ribofuranosylpurine 5′-triphosphate was synthesized according to the method suggested by Gaffney (1976a) with several modifications.

6-Amino[N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)]9-β-D-ribofuranosylpurine 5′-Monophosphate. The sodium salt of 6-chloropurine monophosphate (250 μmol) was converted to the free acid on a Dowex-50W-X column. The free acid was eluted from the column with water. The solution was evaporated to dryness, and the residue was dissolved in water and adjusted to pH 8.5 with tempo amine. Tempo-amine (500 μmol) and 1.5 mL of *tert*-butanol were added to the residue. The mixture was refluxed for 3 h and left overnight at room temperature. The reaction was followed by thin layer chromatography run on silica gel plates (Baker flex IB2-F) purchased from VWR company, using *n*-propanol/ammonia/water (6/3/1). The resulting mixture was diluted to 100 mL with 5 mM triethyl ammonium bicarbonate buffer, pH 8.2, and applied to a Q-Sepharose column (30 × 2 cm) equilibrated with 5 mM triethylammonium bicarbonate buffer, pH 8.2. The product was eluted with a 600-mL gradient of 5–200 mM triethyl ammonium bicarbonate buffer, pH 8.2. Fractions that had an EPR signal and absorbance at 267 were pooled and evaporated to dryness three times with water. The final yield was 155 μmol.

6-Amino[N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)]-9-β-D-ribofuranosylpurine 5′-triphosphate (6nSLATP). The triethyl ammonium salt of the monophosphate (100 μmol) was passed through a Dowex-50W-X in the pyridine form. The column was washed with water until no significant absorbance was noticed at 267 nm. Anhydrous tributylamine (100 μmol) in 1 mL of anhydrous pyridine was added, and the mixture was evaporated to dryness three times. The residue was then dissolved in 1 mL of dimethylformamide, 500 μmol of carbonyldiimidazloe was added, and the mixture was left overnight. Anhydrous methanol (0.015 mL) was added, and the mixture was left for 2 h at room temperature. Anhydrous tributylammonium pyrophosphate (500 μmol) was then added, and the mixture was left overnight. The reaction was followed by thin layer chromatography using the solvent system described above. The precipitated salt was filtered, and the solution was diluted to 100 mL with ice water and adjusted to pH 7.0. It was then extracted with ether and chromatographed on a Q-Sepharose column using 600 mL of 50–500 mM triethylammonium bicarbonate buffer at pH 8.2. Fractions that had an EPR signal and absorbance at 267 nm were pooled and evaporated to dryness, and then washed and evaporated to dryness three times with methanol.

The presence of spin-label triphosphate was checked by observing the change in signal intensity produced by addition of 1 mM manganese chloride to a solution of 100 μM analogue in 100 mM Tes, pH 7.0. The paramagnetic manganese binds to the triphosphate and quenches the signal of the attached label. Manganese at this concentration has little effect on labels that are not attached to tri- or diphosphates.

6-Mercapto[N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)]acetamido-9-β-D-ribofuranosylpurine 5′-Triphosphate (6sSLATP). This spin label ATP analog was synthesized according to the method of Cooke and Duke (1971). An iodoacetamido spin label was reacted with 6-mercapto-ATP provided as a gift by Dr. Al Murphy.

Incorporating Analogs into Actin. F-Actin was prepared from the acetone powder by the method of Spudich and Watt (1971). A solution of F-actin (4–10 mg/mL) was centrifuged for 1 h at 100000*g*. The pellet was washed several times with 2 mM $MgCl_2$ and 25 mM Tes, pH 7.0, and then resuspended to 3–4 mg/mL in the same buffer. The spin label ATP analog was added to a concentration of 100 μM. The mixture was sonicated several times at 4 °C for 15 s each and left on ice overnight. KCl was then added to a final concentration of 50 mM to polymerize the actin. Except where noted, all subsequent manipulations of F-actin and spectral acquisition were performed in 50 mM KCl, 2 mM $MgCl_2$, and 25 mM Tes, pH 7.0.

Orienting Spin-Labeled F-Actin in Capillaries. F-Actin was oriented in capillaries following the method of Popp et al. (1987). A solution containing 30 mg of labeled F-actin was concentrated in a 10-mL Amicon cell to 1–1.5 mL. The supernatant was removed, and the gel was scraped to the center of the filter and then introduced into 0.5-mm capillaries. The effect of inorganic phosphate on probe orientation was observed after diffusion of phosphate into the capillaries. One-centimeter capillaries opened at both ends, containing F-actin (approximately 70 mg/mL) labeled with SLADP, were soaked in a solution of 20 mM Tes, 2 mM $MgCl_2$, and 50 mM KCl containing 20 mM inorganic phosphate, pH 7.0. The EPR spectra of these samples were taken after 2 h, 5 h, and overnight. The ability of small molecules to diffuse through the gel during this time was determined by measuring the signal of a small fraction of free spin-labeled nucleotide. This nucleotide had diffused out of the capillary after 5 h.

A randomly oriented sample of labeled F-actin was obtained by centrifuging the concentrated F-actin solution on top of the gel at 50K for 1 h. The pellet was smeared on the EPR flat cell, and the EPR spectrum was taken with the EPR cell oriented parallel or perpendicular to the magnetic field.

Effect of DNase-I Binding. To a sample of 5 mg/mL F-actin labeled with 6nSLATP was added 1 molar equivalent of DNase-I in 20 mM Tes and 2 mM $MgCl_2$, pH 7.0. The mixture was left for 1 h and then incorporated into capillaries. A decrease in the viscosity of the solution indicated that DNase-I had depolymerized the F-actin and little actin (<1%) was sedimented under conditions that would have sedimented any remaining F-actin. The EPR spectra were taken before and after the addition of DNase-I.

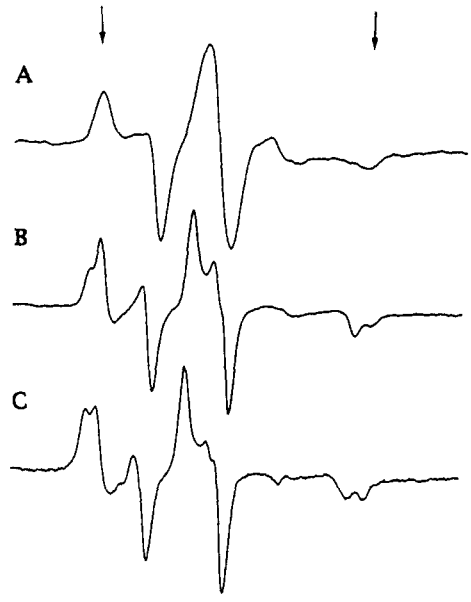

FIGURE 1: EPR spectrum of spin-labeled nucleotide analogs incorporated into F-actin. The actin filaments are randomly oriented with respect to the magnetic field. (A) F-Actin labeled with 6sSLADP; (B) F-actin labeled with 6nSLADP; (C) F-actin labeled with 6nSLADP and complexed with myosin heads. The high and low field peaks, whose position provides information on probe mobility, are identified by vertical arrows. The derivative of absorption is plotted as a function of the magnetic field. The width of all spectra shown is 10.0 mT.

EPR Spectroscopy. EPR measurements were made with an ER/200D EPR spectrometer from Bruker Instruments, Billerica, MA. X-band, first-derivative absorption EPR spectra were obtained with the following instrument settings: microwave power, 25 mW; modulation, 0.1–0.2 mT at 100 kHz; with a total sweep width of 10 mT.

RESULTS

Incorporation of ATP Analogs into Actin. We first tested several spin-labeled ATP analogues for binding to actin. We found that the association was weak when the spin label was attached to the 2′ or 3′ position of the ribose ring. The best incorporation into F-actin was obtained with spin labels attached to the 6 position on the adenine ring. These analogs could be incorporated easily into 15–20% of the actin monomers using protocols described under Materials and Methods. We have used two of these analogs, one with the spin label attached directly to the nitrogen atom at the 6 position; in the second one the label is attached via an acetamido link to a sulfur atom that replaces the nitrogen. Following incorporation of the analogs into actin, as described under Materials and Methods, the actin was sedimented and resuspended to remove free analog. To determine the relative concentrations of analogs attached to G- and to F-actin, an aliquot of this sample, 3–4 mg/mL actin, was sedimented a second time. More than 95% of the analog was found in the pellet, showing that little analog was bound to G-actin under the conditions used for spectral aquisition.

Rotational Mobility of the Probes. EPR spectra are capable of providing information on both the orientation and the rotational mobility of spin probes. When the orientations of the sample are random, the shape of the spectrum is sensitive to rotational motion with correlation times (τ_c) in the range from 10^{-12} to 10^{-6} s. In the absence of motion, i.e., $\tau_c > 10^{-6}$ s, the splitting between high field and low field peaks (defined in Figure 1) is typically about 7.0 mT (Barnett et al., 1986; Gaffney, 1976b). As τ_c becomes shorter and/or the amplitude of the rotational motion becomes greater, this splitting decreases.

The spectra of both 6sSLADP and 6nSLADP on randomly oriented F-actin filaments is shown in Figure 1. The splitting observed for 6sSLADP is 5.83 ± 0.03 mT, which is considerably less than that expected for a probe that is rigidly immobilized on the surface of actin. For 6nSLADP both high and low field peaks are composed of two components, with splittings of 5.64 ± 0.07 and 6.25 ± 0.05 mT (Figure 1B,C), indicating that the probe is mobile on the protein and that there are two populations with different degrees of freedom. The two populations could arise from different amplitudes of rotational motion, from different correlation times, or from different polarities, leading to different values of the hyperfine tensor. In any case, the spectra suggest two populations of probes. As discussed below, the probes were also oriented relative to the actin filament, showing that the motion of the probes is not isotropic but is restricted to a relatively small solid angle by the protein surface.

Making the assumption that the probes are undergoing rapid rotational diffusion within a cone, the width of the cone can be estimated following the calculations of Griffith and Jost (1976), who have determined the spectra expected for diffusion within cones of varying angular spread. The measured value of the splitting, defined in Figure 1, is compared to the value obtained in the absence of motion to calculate a value for an order parameter that is in turn related to the amplitude of the rotational motion. We assume that in the absence of any motion the rigid limit for the splitting between high and low field peaks, defined in Figure 1, is 7.0 mT. This is a value characteristic of this six membered nitroxide ring in an aqueous environment [for examples, see Figure 1 of Barnett et al. (1986)]. The splitting between high and low field peaks observed in Figure 1A is 5.83 ± 0.03 mT which is equal to 83% of the rigid limit. This value can be compared to calculated spectra from Figure 16 or from Table II of Griffith and Jost (1976). This comparison leads to a value for the order parameter of 0.7, corresponding to motion within a cone of half angle 38 ± 1°. A similar calculation for the amino analog suggests that the half-angles of the two degrees of mobility are 30 ± 1° and 42 ± 1.5°. The degree of mobility of the probe was independent of the actin concentration in the capillary and the method used for concentrating actin.

Effect of DNAse-I. DNase-I is a protein that binds tightly to G-actin, producing the complex whose structure was determined by X-ray crystallography (Kabsch et al., 1990). It is thus of great interest to determine how the binding of DNase-I alters the structure of actin. We added an equimolar amount of DNAse-I into a solution of F-actin (5 mg/mL) labeled with 6nSLADP and incorporated the mixture into a capillary. The EPR spectra showed that the two components of the spectrum had the same splitting between low and high field peaks, but that the bond with DNAse-I had shifted the population into the more mobile component (see Table 1).

Effect of Inorganic Phosphate (P_i). G-Actin binds one molecule of ATP which is hydrolyzed to ADP and P_i upon polymerization. Isolation of the nucleotide spin labels and analysis by thin layer chromatography, showed that the analogs were also in the disphosphate state when incorporated into actin. However, P_i can rebind to the nucleotide site on the F-actin with a dissociation constant of 1.5 mM (Carlier & Pantaloni, 1988). The EPR spectra of the spin label taken before and after the addition of 20 mM P_i indicated no change in the rotational mobility of the probe (Table 1). Assuming

Table 1: Spectral Parameters for 6nSLADP Bound to Samples of Randomly Oriented Actin[a]

	$2T_{\parallel}'(1)$	$2T_{\parallel}'(2)$	I_1/I_2
F-actin	5.64 ± 0.07	6.25 ± 0.05	1.5 ± 0.2
actin-S1	5.54 ± 0.07	6.24 ± 0.06	1.0 ± 0.2
actin + 20 mM P_i	5.60 ± 0.05	6.31 ± 0.07	1.5 ± 0.3
actin–DNase-I	5.60 ± 0.05	6.31 ± 0.05	2.2 ± 0.4

[a] $2T_{\parallel}'(1)$ is the splitting (mT) between the two innermost doublets of the high and low field peaks identified by the vertical arrows in Figure 1, and $2T_{\parallel}'(2)$ is the splitting between the two outermost doublets. I_1/I_2 is the ratio of the height of the innermost doublet to that of the outermost doublet for the low field doublet. The errors represent the standard error of the mean for at least four measurements. The concentration of the actin was 50–100 μM in all samples, and the samples were in 50 mM KCl, 2 mM $MgCl_2$, and 25 mM Tes, pH 7.0, along with the additions noted. For the samples containing S1 and DNase-I, the ratio of these proteins to actin was 1:1.

that the binding constant of P_i for actin is not altered by the presence of the spin label, virtually all actin monomers should have a bound P_i under these conditions.

Binding of Myosin Heads. The interaction between myosin and actin generates the force of muscle contraction, requiring large conformational changes in protein structures [for review, see Cooke (1986)]. Although the major changes are thought to occur in myosin, the possibility that changes within actin also contribute to force generation has not been ruled out. The effect of the binding of S1 on the structure of actin in the vicinity of the nucleotide can be monitored by measuring probe mobility. Although the addition of a stoichiometric amount of S1 made no change in the splitting observed between high and low field peaks for either analog, there was a distinct change in the ratio of the two peak heights observed for the 6nSLADP analog (see Figure 1C and Table 1). Thus the population of spin probes in the less mobile fraction of 6nSLADP increased, suggesting that at least a small change in the protein conformation had occurred in this region. However, the lack of change in the rotational mobility of either spectral component suggests that no new conformations of the protein had been produced. The binding of S1 to actin labeled with 6sSLADP produced a splitting of 5.89 ± 0.08 mT, which was not significantly different from that in the absence of S1.

Probe Orientation. F-Actin forms a highly viscous gel in which long range electrostatic interactions occur between neighboring filaments. When F-actin gels are introduced into capillaries the actin filaments become oriented along the axis of the capillary by the shear forces induced by flow. Once oriented, the interactions between filaments maintain the orientation for periods of weeks. When labeled F-actin was oriented in this fashion, the EPR spectrum depended dramatically upon the orientation of the capillary relative to the magnetic field. Spectra for both analogs are shown in Figures 2 and 3. Although the probes possess rotational mobility relative to the protein surface, the average angles for probes on different actin monomers are similarly aligned relative to the filament axis. To a first approximation, the signal arising from a population of probes undergoing rapid but restricted rotational motion resembles the signal arising from a population of probes oriented at the average angle of the mobile population. This effect, known as motional narrowing, contributes to the narrowness of the lines observed in Figure 2A. In addition, the narrow line widths require that the average orientations of the spin probes are restricted to a small range of angles. For a population of immobilized spin probes the angle between the principal axis of the probe and

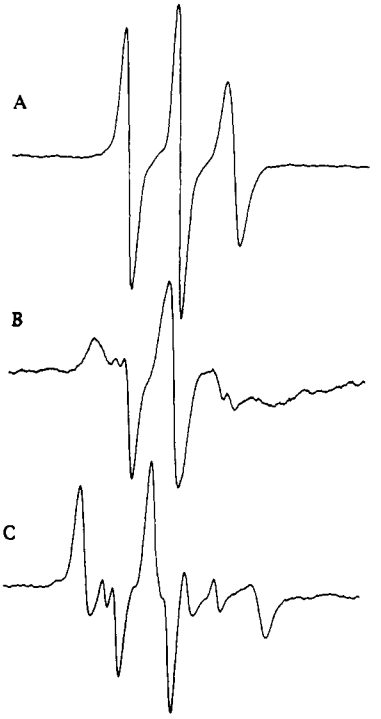

FIGURE 2: EPR spectrum of F-actin gels (70 mg/mL F-actin) labeled with 6sSLADP and oriented in 0.5-mm capillaries. (A) The capillary was oriented parallel to the magnetic field. (B) The capillary was oriented at 20° to the magnetic field. (C) The capillary was oriented perpendicular to the magnetic field.

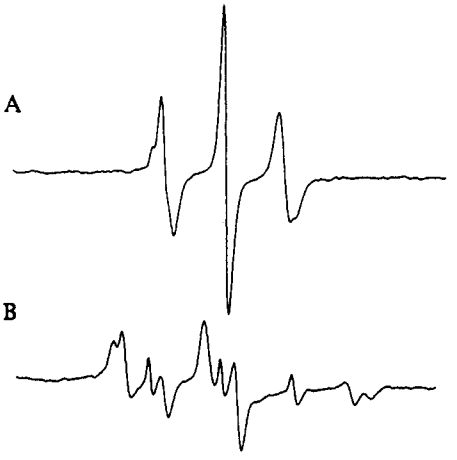

FIGURE 3: EPR spectrum of F-actin gels (approximately 100 mg/mL F-actin) labeled with 6nSLADP and oriented in 0.5-mm capillaries. (A) The capillary was oriented parallel to the magnetic field. (B) The capillary was oriented perpendicular to the magnetic field.

the magnetic field can be determined from the splitting between the three sharp lines seen in Figures 2A and 3A [see Barnett et al. (1986) for discussion]. The splitting between the crossover points for the high and low field peaks is 2.9 ± 0.04 mT in Figure 2A and 2.7 ± 0.03mT in Figure 3A. When these values are used with Figure 2A of Barnett et al. (1986), they lead to values for the average orientation of the principle axis of 70° for the 6nSLADP and 67° for the 6sSLADP. In the above, we have assumed that motional narrowing has produced a signal appropriate for an immobilized probe at the axis of the cone of motion. This approximation will introduce some error into the exact value of the angle measured, making the estimated angle slightly larger than the actual angles. However, the conclusion that the average angles of the probes have a narrow orientational distribution is not

changed by the approximation used to estimate the value of the average angles.

Although 6nSLADP displayed two distinct components in spectra of randomly oriented actin, as well as in the spectrum of the gel oriented perpendicular to the magnetic field, two components were less obvious when the gels were oriented parallel to the field. An indistinct shoulder on both the low field and the high field peaks can be seen; however, these are due to a small amount of free analog in the sample. Thus the average splitting for the two components differs, at most, by about 0.1 mT, indicating that the average angles of the two populations are similar. The observed splitting was independent of the protein concentration in the capillary. When 20 mM P_i was diffused into capillaries of oriented actin, or when 20mM P_i was mixed with a labeled F-actin pellet, and the mixture was incorporated into capillaries, neither spin label showed any change in spectral splittings.

DISCUSSION

EPR spectra are capable of providing information on both the orientation and the rotational mobility of the spin probes. The spectra of both spin probes show that they are not immobilized on the protein surface but are undergoing rapid nanosecond motions. The spectra of samples of oriented actin depend dramatically on the orientation of the magnetic field relative to the actin filament, showing that this mobility is due to rapid subnanosecond rotations within a restricted angular region. Because the amplitude of this motion is determined by the interaction of the probe with the protein surface, a change in the conformation of this surface would alter the observed signal.

Although the region available for movement is most probably not a simple cone of revolution, calculations that do assume a cone provide some insight into relative degrees of rotational mobility (Griffith & Jost, 1976). For instance a cone of half-angle 30 ± 1° implies that the probe motion is restricted to only 7% of the total 4π solid angle available in isotropic movement. A cone of 42 ± 1.5° implies motion within 13% of the total available solid angle. The uncertainty of <0.1 mT in the observed splitting would imply an uncertainty of 1–1.5° in the half-angle of the cone corresponding to a change in the region of rapid rotations of less than 1%. Thus any change in the conformation of the protein surface in the vicinity of the probe that altered the effective region available for motion by more than 1% should be observed in the spectrum. As can be seen by this calculation, the splitting between high and low field peaks provides a very sensitive measure of the interaction between the probe and the protein surface.

The structure of actin provides a framework for discussing the implications of the EPR spectrum. The structure of the actin monomer is composed of 4 subdomains. Subdomains 3 and 4 are found at low radius, where they form the core of the actin filaments. Subdomains 1 and 2 are at higher radius, where subdomain 1 forms the major site for interaction with myosin. Subdomains 1 and 2 are separated from subdomains 3 and 4 by a deep cleft which splits the molecule into two regions of approximately equal mass. This cleft is traversed in only two places by the polypeptide chain. Such a tenuous connection suggests the possibility that actin could undergo structural changes involving domain movements associated with opening or closing of the cleft. The nucleotide binds near the bottom of this cleft, with the sixth position of the adenine base extending outward and exposed to the solvent. From the structure it is easy to visualize why spin probes attached to the sixth position of the adenine ring would be undergoing rapid motion, and why this motion would be restricted. The probe site is found at the bottom of a shallow depression in the molecular surface between domains 3 and 4. Probes attached here would extend into the solvent, allowing them to undergo rapid Brownian rotations, but these rotations would be restricted by residues on either side of the nucleotide adenine site. In particular, the motion would be restricted by residues around 213–214 in subdomain 4 and by residues 303–306 in subdomain 3. Motion would also be hindered by lysine 336, which is found in one of the two segments of polypeptide chain which bridge between subdomain 3 and subdomain 1. Thus, the spin probes attached to the sixth position of the nucleotide would be ideally situated for observing movements between subdomain 3 and subdomain 4. They would also sense motion between subdomain 1 and subdomain 3 as one of the two links between these two subdomains would be close to the probe. However, the nucleotide is situated close to the center of the actin monomer and thus would not be directly sensitive to monomer–monomer interactions in the filament.

The primary function of the nucleotide appears to be regulation of actin polymerization [for review, see Carlier (1989)]. In vivo, actin monomers containing bound ATP add to the growing end of an actin filament, with hydrolysis of ATP occurring subsequent to the addition of the monomer. While it is technically not feasible for us to contrast spectra of actin filaments containing ATP with those containing ADP, we have been able to look at the effect of rebinding phosphate to the nucleotide site on the actin. Phosphate rebinds to the filament with a dissociation constant of about 1.5 mM, making it simple to saturate the filament with phosphate (Carlier & Pantaloni, 1988). The data shown in Table 1 demonstrate that rebinding of phosphate to the actin filament has negligible effect on the spectra of probes bound to the nucleotide, implying that there is little change in the actin structure. Thus, the rebinding of phosphate, which is known to stabilize the F-actin filament, must exert its effect through a rather subtle change in the actin structure.

The myosin head binds tightly to F-actin in a complex thought to resemble that which occurs at the end of the power stroke. If the structure undergoes dramatic structural alterations during the powerstroke, one might expect that the binding of myosin in the rigor complex might alter the actin structure. In fact, a variety of spectroscopic data have suggested that the binding of myosin produces local changes at a variety of sites in the actin structure but that it does not produce global changes [for review, see Cooke (1986) and DosRemedios et al. (1987)]. Our data are entirely consistent with this view. Binding of myosin causes a modest change in the populations of the two states seen by the 6nSLADP probe but causes no change in the restriction of the motion of either population, while also causing no change in the spectrum of 6sSLADP.

DNase-I forms a very tight bond with G-actin that prevents both the nuclease activity of the DNase-I and the polymerizability of the actin (Lazaridies & Lindberg, 1974). The association constant for this complex is high, implying that it is due to a specific interaction. The function of this interaction remains unknown, although its purpose may be to inhibit any errant DNase-I that enters the cell, thus protecting the genetic material from a potentially toxic enzyme. The structure of actin was determined in the complex with DNase-I, prompting the question of whether the tight association between the two proteins has altered the structure of the actin. The binding of DNase-I caused no change in the rotational mobility of the probes but altered the equilibrium between

the two spectral components seen for 6nSLADP. This suggests that the protein structure in the vicinity of the nucleotide is similar for F-actin and for the actin–DNase-I complex.

Both the high field and low field peaks seen in Figure 1 have split into doublets for the 6nSLADP, showing that the probe exists in two populations, which most likely differ by the degree to which their rotational motion is restricted by the protein surface. Several results suggest that the two populations arise because the actin structure has two different conformations, each of which has a slightly different ability to restrict the motion of the probe. One result is that the binding of either DNase-I or S1 changes the relative sizes of the two components without affecting the degree of mobility of either of them. Both these proteins bind at some distance from the probe, where they would not be expected to interact directly with it. If they were to interact directly with the probe, one would expect that it would change the degree to which the probe motion is restricted, instead of simply just changing the sizes of the two different populations. This observation is most easily explained by assuming that the actin has two slightly different global conformations, and that the binding of S1 shifts the equilibrium between these two conformations. Another result also argues that actin may have two conformations. Spin probes attached to two additional sites, to Cys-374 and to phalloidin bound to the filament, also have two spectral populations (Naber et al., 1993, 1994). The observation of two populations at three spatially distant sites is most easily explained by two conformations of actin.

Actin filaments are relatively easily oriented, and a number of studies involving spectroscopic probes or X-ray diffraction have investigated the structure of these oriented filaments. Early attempts at orienting actin filaments achieved only low degress of orientation, and, more recently, highly aligned samples were achieved by Popp et al. (1987). This latter method was used to orient actin filaments for studies of X-ray diffraction, leading to a model of the F-actin filament (Holmes et al., 1990). While X-ray diffraction patterns are compatible with filaments that are very well aligned, X-ray diffraction is not sensitive to highly disordered populations. Thus it is of interest to use a complementary method to ascertain whether disordered fractions exist. Recently, Ostap and Thomas (1992) oriented actin by continous flow, observing the orientation of a spin probe attached to Cys-374. They found that there was a large disordered population, along with a highly ordered population of probes, and interpreted the disordered population as arising from disordered actin filaments. Naber et al. (1994) observed the same probe attached to actin filaments oriented using the method of Popp and co-workers, and they also observed two populations, one ordered and the other disordered, the two populations having approximately equal sizes. Although X-ray diffraction of these oriented gels showed only an ordered population, it was not simple to rule out the possibility of a disordered population in these oriented gels. It is important to rule out such a possibility, because the presence of a disordered component could influence the diffraction pattern, leading to errors in the model structure of the F-actin filament. We show here that actin filaments [aligned using the methods of Popp et al. (1987)] are highly aligned, with no evidence of a disordered population. As shown by Figure 2B, in which the axis of the capillary was shifted by only 20°, the presence of a population of disordered actin filaments would produce dramatic changes in the observed spectrum. Thus, we conclude that actin filaments oriented by the method of Popp et al. do not contain a disordered population of filaments, and that the disordered population of probes seen for MSL arises from a population of probes that are disordered on oriented actin filaments. This conclusion is also supported by our recent work using a spin-labeled phalloidin, whose spectrum in oriented gels was identical to that on muscle fibers (Naber et al., 1993).

In summary, actin is involved in a number of interactions with other proteins that could induce conformational changes. We have measured possible conformational changes in the vicinity of a deep cleft in the actin structure. The presence of two spectral components for one of the probes suggests that actin has two different conformations in this region, which differ slightly in their ability to restrict the motion of this probe. Although the motion of the probes should be sensitive to even subtle changes in the protein structure, we found that interactions with DNase-I, myosin, or phosphate do not induce new spectral components, although small shifts in the population of existing components is observed. In addition, the spectra show that the probes are both highly oriented in samples of oriented actin gels, and that this orientation is not changed by binding of phosphate at the nucleotide site.

ACKNOWLEDGMENT

We thank Dr. A. Murphy for a gift of 6-mercapto-ATP. This work was supported by a grant from the USPHS (AR30868).

REFERENCES

Ando, T., & Hiroshi, A. (1979) *J. Mol. Biol. 129*, 265–277.
Barnett, V. A., Fajer, P., Polnaszek, C. F., & Thomas, D. D. (1986) *Biophys. J. 49*, 144–146.
Bender, N., Fasold, H., Kenmoku, A., Middelhoff, G., & Volk, K. E. (1976) *Eur. J. Biochem. 64*, 215–218.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Burtnick, L. D. (1984) *Biochim. Biophys. Acta. 791*, 57–62.
Carlier, M.-F. (1989) *Int. Rev. Cyt. 115*, 139–170.
Carlier, M.-F., Pantaloni, D. (1988) *J. Biol. Chem. 263*, 817–825.
Cooke, R. (1986) *CRC Crit. Rev. Biochem. 21*, 53–118.
Cooke, R., & Duke, J. (1971) *J. Mol. Biol. 246*, 6360–6369.
dos Remedios, C. G., Masao, M., & Barden, J. A. (1987) *J. Muscle Res. Cell Motil. 8*, 97–117.
Fujime, S., & Ishiwata, S. (1971) *J. Mol. Biol. 62*, 251.
Gaffney, B. J. (1976a) in *Spin Labeling: Theory and Applications* (Berliner, L. J., Ed.) pp 183–238, Academic Press, New York.
Gaffney, B. J.(1976b) Practical considerations for the calculation of order parameters for fatty acid or phospholipid spin labels in membranes, *Spin Labeling, Theory and Applications* (Berliner, L. J., Ed.) pp 567–571, Academic Press, New York.
Griffith, O. H., & Jost, P. C. (1976) *Spin Labeling: Theory and Applications* (Berliner, L. J., Ed.) pp 453–523, Academic Press, New York.
Hanson, J., Lednev, V., O'Brien, E. J., & Bennett, P. M. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 311–318.
Hegyi, G., Premecz, G., Sain, B., & Muhlrad, A. (1974) *Eur. J. Biochem. 44*, 7–12.
Holmes, K. C., Popp, D., Gebhard, W., & Kabsch, W. (1990) *Nature 347*, 44–49.
Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., & Holmes, K. C. (1990) *Nature 347*, 37–44.
Lazaridies, E., & Lindberg, U. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4742–4746.
Lehrer, S. S., & Elzinga, M. (1972) *Fed Proc. 31*, 502.
Miki, M., & Wahl, P. (1984) *Biochim. Biophys. Acta 786*, 188.
Miki, M., dos Remedios, C. G., & Barden, J. A. (1987) *Eur. J. Biochem. 168*, 339–345.

Naber, N., Ostap, E. M., Thomas, D. D., & Cooke, R. (1993) *Proteins: Struct., Funct., Genet. 17*, 347–354.
Naber, N., Lorenz, M., & Cooke, R. (1994) *J. Mol. Biol.* (in press).
Ostap, E. M., & Thomas, D. D. (1991) *Biophys. J. 59*, 1235–1241.
Ostap, E. M., Yanagida, T., & Thomas, D. D. (1992) *Biophys. J. 63*, 966–975.
Oosawa, F. (1983) in *Actin Structure and Function in Muscle and Non-Muscle Cells* (dos Remedios, C. G., & Barden, J. A., Eds.) p 69, Academic Press, Sydney.
Popp, D., Lednev, V. V., & Jahn, W. (1987) *J. Mol. Biol. 197*, 679–684.
Prochniewicz-Nakayama, E., Yanagida, T., & Oosawa, F. (1983) *J. Cell Biol. 97*, 1663.
Schutt, C. E., & Lindberg, U. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 319–323.
Spudich, J. A., & Watt, S. J. (1971) *J. Biol. Chem. 246*, 4866–4871.
Sutoh, K. (1982) *Biochemistry 21*, 3654–3661.
Thomas, D. D. (1987) *Annu. Rev. Physiol. 49*, 891–909.
Thomas, D. D., & Cooke, R. (1980) *Biophys. J. 32*, 891–906.
Thomas, D. D., Seidel, J. C., & Gergely, J. (1979) *J. Mol. Biol. 132*, 257–273.
Yanagida, T., & Oosawa, F. (1978) *J. Mol. Biol. 126*, 507.
Yanagida, T., & Oosawa, F. (1980) *J. Mol. Biol. 140*, 313–320.