Saturation transfer electron parametric resonance of an indane-dione spin-label

Calibration with hemoglobin and application to myosin rotational dynamics

Osha Roopnarine, Kálmán Hideg,* and David D. Thomas

Department of Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455 USA; and
*Central Laboratory for Chemistry, University of Hungary, Pécs, Hungary

ABSTRACT We have used a recently synthesized indane-dione spin label (2-[-oxyl-2,2,5,5,-tetramethyl-3-pyrrolin-3-yl)methenyl]indane-1,3-dione (InVSL) to study the rotational dynamics of myosin, with saturation-transfer electron paramagnetic resonance (ST-EPR). To determine effective rotational correlation times (τ_r^{eff}) from InVSL spectra, reference spectra corresponding to known correlation times (τ_r) were obtained from InVSL-hemoglobin undergoing isotropic rotational motion in aqueous glycerol solutions. These spectra were used to generate plots of spectral parameters vs. τ_r. These plots should be used to analyze ST-EPR spectra of InVSL bound to other proteins, because the spectra are different from those of tempo-maleimide-spin-labeled hemoglobin, which have been used previously as ST-EPR standards. InVSL was covalently attached to the head (subfragment-1; S1) of myosin. EPR spectra and K/EDTA-ATPase activity showed that 70–95% of the heads were labeled, with ≥90% of the label bound to either cys 707 (SH1) or cys 697 (SH2). ST-EPR spectra of InVSL-S1 attached to glass beads, bound to actin in myofibrils, or precipitated with ammonium sulfate indicated no submillisecond rotational motion. Therefore, InVSL is rigidly immobilized on the protein so that it reports the global rotation of the myosin head. The ST-EPR spectra of InVSL-myosin monomers and filaments indicated τ_r^{eff} values of 4 and 13 μs, respectively, showing that myosin heads undergo microsecond segmental rotations that are more restricted in filaments than in monomers. The observed τ_r^{eff} values are longer than those previously obtained with other spin labels bound to myosin heads, probably because InVSL binds more rigidly to the protein and/or with a different orientation. Further EPR studies of InVSL-myosin in solution and in muscle fibers should prove complementary to previous work with other labels.

INTRODUCTION

Saturation-transfer electron spin resonance (ST-EPR)[1] specroscopy is a powerful technique for studying the slower rotational motions (μs to ms) of spin-labeled proteins (Thomas et al., 1976). This technique has great sensitivity to decreased saturation caused by rotational motion. The effect of rotational motion is directly observed in the intensity and shape of the EPR spectrum. ST-EPR is particularly well suited for the study of protein rotational dynamics in biological assemblies such as muscle (Thomas, 1987) and membranes (Thomas, 1986). The rotational motions of spin-labeled myosin have been studied primarily with two spin labels, an iodoacetamide derivative of tempo (IASL) and a maleimide derivative of tempo (MSL) (Thomas et al., 1975, 1980). ST-EPR measurements of myosin subfragment-1 (S1; a proteolytic fragment of myosin, containing the "head" region), labeled with either MSL or IASL, indicated that the myosin head tumbles freely in solution with an effective rotational correlation time (τ_r^{eff}) of ~0.2 μs at 20°C. This rotational motion is restricted in myosin monomers (τ_r^{eff} = 0.4 μs) and is even more restricted in myosin filaments (τ_r^{eff} = 10 μs) (Thomas et al., 1975, 1980). The spin-labeled myosin heads are rigidly immobilized ($\geq 10^{-3}$ s) when they are attached to actin. The quantitative description of myosin's rotational motion is crucial to most models of muscle contraction (Cooke, 1986), so improvements in myosin spin-labeling technology are particularly important to this field.

An important requirement for studying the global rotational motion of large proteins is that the probe should be rigidly immobilized on the protein. A weakly immobilized spin label (i.e., one a with nanosecond rotational correlation time relative to the protein) will distort the EPR spectrum, making it difficult to determine unambiguously the slower effective rotational correlation time of the labeled protein (Squier and Thomas, 1986). MSL and IASL are strongly immobilized ($\tau_r^{eff} > 0.1$ μs) on myosin in the absence of nucleotide, but IASL becomes weakly immobilized upon nucleotide binding. MSL remains strongly immobilized in the presence of ATP, but it is slightly mobilized by the binding of ADP and vanadate (Barnett and Thomas, 1987). It would be desirable to have a spin label that remains more rigidly fixed to the protein under all conditions. Another motivation for a new spin label is that the sensitivity to anisotropic rotational motion (the type of motion that must occur in such asymmetric molecules as myosin and S1) depends the orientation of the spin label axes relative to the protein's axes of rotation. MSL and IASL have similar orientations on S1 (Thomas and Cooke, 1980), so it is worth searching for a label having a substantially different orientation.

Address correspondence to David D. Thomas and Osha Roopnarine.

[1] Abbreviations used in this paper: EPR, electron spin resonance; ST-EPR, saturation transfer EPR; TE_{102} cavity, transverse electric cavity; TPX, tetramethylene polymer plastic; V_1, first harmonic absorption in-phase conventional EPR spectrum; V_2', second harmonic absorption out-of-phase ST-EPR spectrum.

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A recently synthesized indane dione nitroxide spin label (InVSL) has been reported to be strongly immobilized on proteins (Esmann et al., 1990; Horváth et al., 1990; Roopnarine et al., 1990). InVSL was designed to have high reactivity with SH groups in proteins (Hankovszky et al., 1989; Esmann et al., 1990). The reactive site in InVSL is the diene (see Fig. 1), and strong electron withdrawing groups in the indane dione moiety give this diene the potential to react with nucleophilic groups on proteins in a Michael-type addition (Hankovszky et al., 1989). It has been proposed that InVSL reacts specifically with SH groups, but this has not been documented (Esmann et al., 1990; Horváth et al., 1990). Furthermore, the double bond in the five-membered ring (pyrroline) decreases the flexibility of the nitroxide bond, and the hydrophobic character of the label increases the probability that it will insert itself deeply into the protein. Therefore, InVSL has the potential for both high reactivity with cysteines and rigid immobilization on proteins.

InVSL has been previously used to label the sodium-pump protein (Na,K-ATPase) (Esmann et al., 1990) and the calcium-pump protein (Ca-ATPase) (Horváth et al., 1990). The ST-EPR spectrum of the Ca-ATPase protein suggested slower motions for InVSL than for MSL (Horváth et al., 1990). The effective rotational correlation times for InVSL-labeled Ca-ATPase were determined by comparison with reference spectra of MSL-labeled hemoglobin (Horváth et al., 1990). Because the structure around the nitroxide group is significantly different for MSL and InVSL, more accurate results might be obtained by using an InVSL-based reference system.

The present study reports ST-EPR studies of rotational dynamics of myosin and its subfragments using InVSL. As a first step, we use the model system of spin-labeled hemoglobin to compare the effects of isotropic rotational motion on the ST-EPR spectra of two different spin labels, InVSL and MSL, thus establishing reference spectra that can be used to analyze rotational motions of proteins labeled with InVSL or MSL. We then show that InVSL is strongly immobilized on the myosin head, making it an accurate reporter of the rotational motion of the labeled proteins. We also demonstrate that InVSL does not react specifically with SH groups on myosin. We then use the reference spectra to analyze the rotational dynamics of isolated myosin heads (S1) in solution, myosin monomers, myosin filaments, and actin-bound myosin.

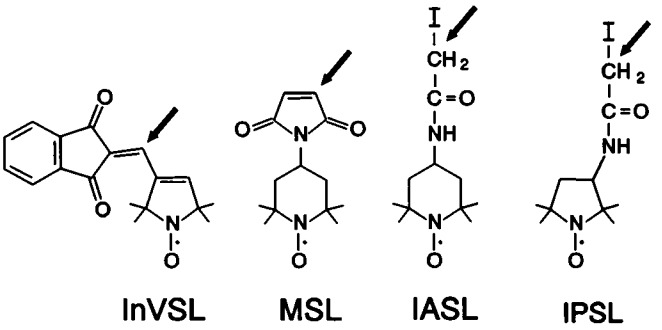

FIGURE 1 Chemical structure of InVSL and other spin labels used to study myosin rotational dynamics. Arrows indicate the proposed sites of reaction with SH groups, showing that the number of flexible bonds between the labeling site and the nitroxide group is minimized in InVSL.

METHODS

Reagents and solutions

InVSL (Fig. 1) was synthesized by Hankovszky et al. (1990). Other spin labels (Fig. 1) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Creatine kinase and DTT were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). ATP, creatine phosphate, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Glycerol was obtained from EM Science (Gibbstown, NJ).

The following solutions were used in this study: myosin solution (MS): 0.5 M KCl, 0.1 mM EDTA, 5 mM MOPS (pH 7.0); myosin-labeling solution (MLS): 50 mM KCl, 0.1 mM EDTA, 10 mM MOPS (pH 6.5); S-1 solution (S1S): 120 mM KCl, 1 mM EDTA, 10 mM MOPS (pH 7.0); rigor solution (RS): 130 mM KPr, 20 mM MOPS (pH 7.0), 2 mM $MgCl_2$, 1 mM EGTA; ATP-regenerating solution (ARS): 5 mM MgATP, 10 mM K_2PO_4, 40 mM creatine phosphate, 750 U/ml creatine kinase in RS (pH 7.0) (KPr omitted to maintain ionic strength). Unless otherwise indicated, all preparations and procedures were performed at 4°C.

Protein preparations and assays

Glycerinated myosin was prepared from rabbit fast-twitch skeletal muscle as described by Eads et al. (1984). Myosin was dialyzed in S1S at 10 mg/ml overnight to precipitate myosin filaments. The myosin filaments were digested with α-chymotrypsin for 10 min as described by Eads et al. (1984). Actin was prepared as described by Pardee and Spudich (1982). Human oxyhemoglobin was prepared as described by Squier and Thomas (1986).

Protein concentrations for myosin and S1 were determined spectrophotometrically from the expression: $[(A_{280} - A_{320})/E_{280}(1\ mg/ml)]$, using $E_{280}(1\ mg/ml)$ values of 0.53 for myosin and 0.74 for S1 (Margossian and Lowey, 1982), and for actin the expression was $[(A_{290} - A_{320})/E_{290}(1\ mg/ml)]$, using $E_{290}(1\ mg/ml) = 0.63$ (Margossian and Lowey, 1982). Molar concentrations were determined using the following molecular weights: myosin 480,000 (Tonomura et al., 1966), chymotryptic-S1 109,000, and actin 42,500 (Margossian and Lowey, 1982). Protein concentrations of myofibril suspensions were determined by the biuret assay (Gornall et al., 1949), using bovine serum albumin as a standard.

Hemoglobin (1.0 mM) was labeled overnight at 4°C with InVSL or MSL (2.0 mM) (Squier and Thomas, 1986). Excess spin label was removed by dialysis against 0.1 M sodium phosphate buffer (pH 7.0). In the case of MSL, this procedure has been shown to result in specific labeling of both β-93 sulfhydryls (Ohnishi et al., 1966; Thomas et al., 1976). The spin-labeled hemoglobin was concentrated in concentrators (Centricon; Amicon Corp., Danvers, MA) (molecular weight cutoff = 10,000) to 5 mM. Model system samples, corresponding to known rotational correlation times, were prepared from this labeled hemoglobin solution by dilution in different glycerol/buffer solutions (vol/vol), as described by Squier and Thomas (1986). The rotational correlation time was determined as described previously (Hyde and Thomas, 1973) from the Stokes-Einstein-Debye equation: $\tau_r = V\eta/kT$, where V is the molecular volume of a sphere (hemoglobin is as-

sumed to be a sphere of radius 29 Å), η is the solution viscosity in poise, k is Boltzmann's constant, and T is the temperature in °K, so $V/k = 7.6 \times 10^{-4}$. EPR spectra of InVSL-hemoglobin in different glycerol concentrations were acquired at various temperatures. Reference spectra corresponding to no detectable motion (rigid limit) were obtained from two samples: labeled hemoglobin precipitated in 70% ammonium sulfate (Thomas et al., 1976) and labeled hemoglobin immobilized by covalent attachment to glass beads, as described below for labeled S1.

Myosin or S1 was diluted to 1.5–2.0 mg/ml in MLS plus 2 mM $MgCl_2$ and 2 mM K_4PP_i, and then labeled with InVSL (the ratio of spin label/S1 head = 2:1) on ice with gentle stirring. After 90 min on ice, an equal volume of MLS was added to the reaction mixture, which was centrifuged at 10,000 *g* for 30 min in a centrifuge tube to pellet myosin or in Centricon (Amicon Corp.) concentrators (molecular weight cutoff = 30,000) to concentrate S1. Excess free spin label was removed by diluting the labeled protein in MLS and centrifuging again. This was repeated (at least four times) until the free spin label was negligible in the supernatant (monitored by EPR). Labeled myosin filaments and S1 were dialyzed against RS (pH 7.0), whereas myosin monomers were dialyzed against MS (pH 7.0) for EPR experiments.

To determine whether InVSL was rigidly immobilized on S1, EPR spectra were recorded from immobilized S1, which was prepared by four different procedures: (*a*) Labeled S1 (30 mg/ml) was mixed with actin (22 mg/ml) in a 2:1 ratio in RS (pH 7.0). (*b*) Myofibrils were prepared by homogenizing minced fiber bundles in RS (pH 7.0) using a tissue homogenizer (Tekmar Co., Cincinnati, OH). Labeled S1 (30 mg/ml) was gently stirred with myofibrils (25 mg/ml) in RS (pH 7.0) for 8 h. The myofibrils were washed three times in RS (pH 7.0) to remove unbound InVSL-S1 and resuspended in RS (pH 7.0) for EPR experiments. (*c*) Labeled S1 was covalently attached to glass beads (preactivated isothiocyanato-controlled pore glass beads; model G4893, 500 Å average pore size, 20–40 μmol isothiocyanate/g of glass (Sigma Chemical Co.) essentially as described previously (Thomas et al., 1980). The glass beads (25 mg) were washed three times in 50 mM EPPS (pH 8.0) and then incubated with labeled S1 (100 μl of 16 mg/ml) in 50 mM EPPS (pH 8.0), gently shaking overnight. Unreacted S1 was removed by washing the beads with the following solutions in this sequence: 50 mM EPPS (pH 8.0), 0.1 M sodium acetate (pH 5.5), 0.5 M KCl (pH 7.0), and RS (pH 7.0). (*d*) Labeled S1 (30 mg/ml) was precipitated in 60% saturated $(NH_4)_2SO_4$ and then centrifuged to remove the supernatant. The precipitated S1 was used for EPR experiments.

Characterization of labeled proteins

To determine whether InVSL was covalently bound to the protein, noncovalently bound spin label was extracted with ethanol (Eads et al., 1984). An equal volume of absolute ethanol was added to labeled protein (myosin, S1, or myofibrils) at room temperature. After 5 min at room temperature (with occasional mixing), the ethanol–protein mixture was centrifuged in a tabletop centrifuge for 5 min at 3,000 *g*. The protein extracted in the supernatant was <1% as determined by the BCA (Bicinchoninic acid) protein assay from Pierce Chemical Co. (Rockford, IL). An EPR spectrum of the supernatant was acquired (described below) to determine the concentration of extracted spin label.

ATPase assays were done within 2 d after the labeling procedure. Myosin and S1 were dialyzed in MS and S1S, respectively, to remove Mg^{2+} before assays (Mg^{2+} inhibits the K/EDTA-ATPase activity. ATPase activities were assayed at 25°C in the following solutions: K/EDTA (0.6 M KCl, 10 mM EDTA, 5 mM ATP, 50 mM MOPS, pH 7.5), Ca/K (0.6 M KCl, 10 mM $CaCl_2$, 5 mM ATP, 50 mM MOPS, pH 7.5), and Mg (124 mM KPr, 25 mM MOPS, 7 mM $MgCl_2$, 5 mM ATP, 1 mM EGTA, 25 mM MOPS, pH 7). The reaction was initiated by the addition of ATP, and aliquots were taken at 1-min intervals over a 5-min period. Quenching and measurement of inorganic phosphate were carried out as described previously (Lanzetta et al., 1979; Eads et al., 1984). The fraction of heads labeled at SH1 and/or SH2 (f_{SH}) was determined from the fractional inhibition of the K/EDTA-ATPase activity of the labeled sample (K_L) relative to the unlabeled controls (K_{UL}): $f_{SH} = 1 - [(K_L - K_\infty)/(K_{UL} - K_\infty)]$, where K_∞ = activity of completely labeled myosin (Kielley and Bradley, 1956). The number of spin labels per head (f_{SL}) in labeled proteins was determined from the concentration of spin labels (determined by double integration of the conventional EPR spectrum, as described below) divided by the concentration of S1 heads. The specificity of labeling, defined as the fraction of labels attached to SH1 and/or SH2, was calculated as f_{SH}/f_{SL} (Eads et al., 1984). A value of 1.0 indicates complete specificity in labeling, with all labels attached to myosin heads at either SH1 or SH2 (not both). A value < 1 indicates that some heads have either labels at both SH1 and SH2 or labels at other sites (nonspecific labeling).

EPR experiments

The EPR spectra were acquired with a spectrometer (model ESP 300; Bruker Instruments Inc., Billerica, MA) using a TE_{102} cavity (model ER4102 ST; Bruker Instruments Inc.). The instrumental settings for conventional EPR (first harmonic absorption in-phase, designated V_1) spectra were magnetic field modulation frequency = 100 kHz, magnetic field modulation amplitude (peak-to-peak) = 2.0 G, microwave field frequency = 9.37 GHz, microwave power = 20 mW (corresponding to a microwave field intensity, H_1 = 0.144 G), filter time constant = 163 ms, scan time = 163 s, and center field = 3,315 G.

ST-EPR spectra (second harmonic absorption out-of-phase, designated V_2') were acquired as described by Squier and Thomas (1986). In the V_2' experiments, the reference phase on the 100 kHz phase-sensitive detector was set at 90° from the conventional EPR setting. The instrumental settings were: field modulation frequency = 50 kHz, magnetic field modulation amplitude = 5.0 G, microwave field intensity (H_1) = 0.25 G, filter time constant = 655 ms, scan time = 325 s, and center field = 3,315 G. The protein samples were placed in a 25-μl gas-permeable tetramethylene polymer plastic (TPX) capillary (1.0-mm internal diam) and positioned in the center of the TE_{102} cavity containing a quartz dewar. The temperature was controlled using a variable temperature controller (model ER411; Bruker Instruments Inc.), and the temperature of the sample was monitored with a digital thermometer (model BAT-12; Sensorek Co., Clifton, NJ) using a thermocouple probe immersed in the top end of the sample capillary in the cavity. All EPR spectra of spin-labeled proteins were acquired with 1,024 points per 100 G scan. The EPR spectra of spin label concentration standards were acquired with 4,096 points per 100 G scan to ensure adequate resolution for these narrow-line spectra.

Spectroscopic data analysis

The EPR spectra were analyzed with a computer program written by Robert L. H. Bennett. Each EPR spectrum was baseline corrected by subtracting an EPR spectrum of an unlabeled sample, acquired under the same conditions as the labeled sample. In addition, some InVSL spectra showed evidence of a small population of rapidly tumbling (unbound) spin label, presumably because of the retro-Michael reaction (Ingold, 1969). In these cases, spectra were corrected by subtracting a spectrum of the same sample that contained a larger fraction of unbound label. The key spectral parameters that characterize the conventional EPR spectrum of randomly oriented spin labels are the splitting, in gauss (G), between the outer extrema, $2T_\parallel'$ (see Fig. 5). An increase in the rate or the amplitude of submicrosecond motion results in a decrease in $2T_\parallel'$, whereas an increase in the local polarity of the nitroxide bond results in an increase in $2T_\parallel'$ (Griffith and Jost, 1976; Thomas et al., 1979).

The double integral of a baseline-corrected EPR spectrum (acquired with nonsaturating power) is proportional to the concentration of spin labels, independent of the line shape. The concentration of spin labels was determined by comparing the double integral with that of a standard 0.1 mM spin label (InVSL or MSL, which gave the same results)

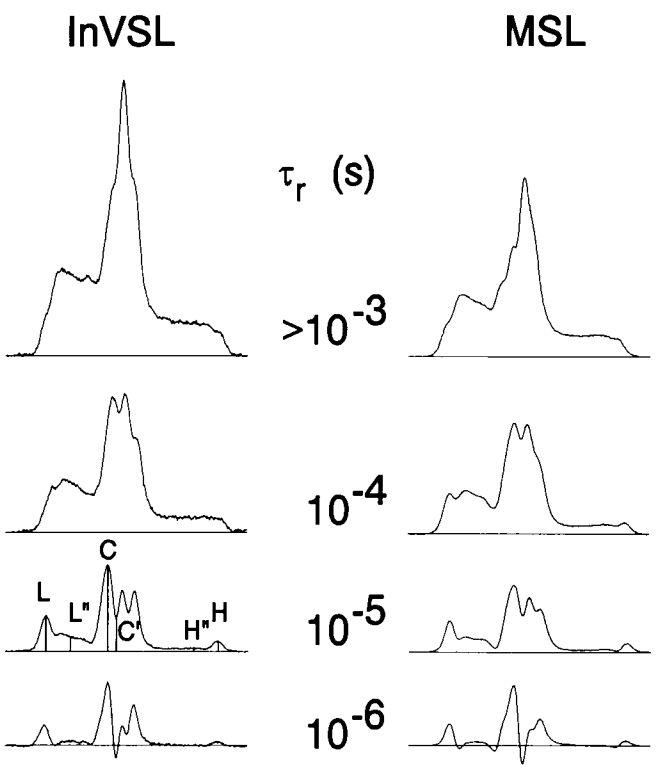

FIGURE 2 Reference ST-EPR spectra of spin-labeled hemoglobin demonstrating the effect of isotropic rotational motion on EPR spectral line shapes. Spin-labeled hemoglobin (InVSL in left column and MSL in right column) was immobilized on glass beads or diluted in the following glycerol concentrations: 90% (vol/vol) at −8.0°C, 70% (vol/vol) at −10°C, 50% (vol/vol) at −8.0°C. The spectra are normalized to the same spin concentration (dividing by $\int\int V_1/H_1$ (0.032 G). The horizontal axis is the resonance field and the baseline is 100 G wide. The line-shape parameters L″/L, C′/C, and H″/H are shown.

in the same buffer solution as the labeled protein. Alternatively, for spin labels mobile enough to produce narrow three-line spectra, we found that a more precise concentration measurement was obtained by computing the product of the amplitude and the square of the peak-to-peak line width of the low-field peak, using 0.1 mM InVSL as a standard.

ST-EPR spectra were analyzed as described by Squier and Thomas (1986). In some cases, small spectral contributions from unbound spin labels were observed and were removed by spectral subtraction. The effective rotational correlation time (τ_r^{eff}) of labeled myosin and its subfragments was determined by comparing spectral parameters with standard calibration plots of these parameters from InVSL-hemoglobin in aqueous glycerol solutions, as described previously for MSL-hemoglobin (Squier and Thomas, 1986). The ST-EPR spectra were normalized to unit concentration by dividing by the double integral of the low-power (H_1 = 0.032 G) V_1 EPR spectrum and multiplying by H_1 (0.032 G). The spectral line-shape parameters of ST-EPR spectra that are sensitive to protein rotational motion are the line-height ratios L″/L, C′/C, and H″/H (Fig. 2). The integrated intensity parameter ($\int V_2'$) was determined from the integral of the V_2' spectrum, normalized to the rigid-limit (no detectable motion) value, as described by Squier and Thomas (1986). For hemoglobin, the rigid-limit sample was labeled hemoglobin immobilized on glass beads. For S1 and myosin, the rigid-limit value was determined from the average of values obtained for InVSL-S1 immobilized on glass beads and precipitated with ammonium sulfate.

RESULTS

ST-EPR reference spectra

Reference ST-EPR spectra corresponding to known rotational correlation times τ_r, for InVSL-hemoglobin and MSL-hemoglobin in aqueous glycerol solutions, are shown in Fig. 2. EPR spectra corresponding to no detectable motion (rigid-limit spectra) were obtained for hemoglobin immobilized on glass beads (Fig. 2, *top*) and precipitated with ammonium sulfate (similar spectra, not shown). As τ_r decreases, the overall intensity of the spectrum (as measured by the integrated intensity, $\int V_2'$) decreases, and particular spectral positions (e.g., L″, C′, H″) decrease more than others (e.g., L, C, H). These changes are shown quantitatively in plots of spectral parameters vs τ_r (Fig. 3). The MSL-hemoglobin spectra and parameter plots are in good agreement with those of Squier and Thomas (1986).

The spectra of the two spin labels show similar sensitivities to rotational diffusion, but significant differences are observed in the spectral intensities and line shapes (Figs. 2 and 3). For each τ_r value, the shape and intensity of the InVSL-hemoglobin spectrum is similar to one for MSL but corresponding to a longer correlation time. Thus, a spectrum analyzed with the InVSL-Hb reference spectra would be assigned a shorter effective correlation time τ_r^{eff} than if it were analyzed with the MSL-hemoglobin reference spectra. Perhaps the most striking difference is the greater overall intensity of InVSL spectra for τ_r values $> 10^{-5}$ s, which is shown quantitatively in the plot of the integrated intensity parameter $\int V_2'$ (Fig. 3, *lower right*). For the rigid-limit samples, $\int V_2'$ is 50% greater for InVSL than for MSL. However, this discrepancy for $\int V_2'$ is greatly diminished if the InVSL-hemoglobin curve is normalized to its own rigid-limit value (Fig. 3). Possible explanations for the differences between InVSL and MSL are analyzed in the Discussion section. In any case, these results suggest that the most accurate determination of τ_r^{eff} from ST-EPR requires reference spectra obtained with the same spin label as that attached to the protein of interest.

The line heights L″, C, and C′ are affected by the presence of free or weakly immobilized spin labels. Therefore, the L″/L and C′/C line-shape parameters are not reliable parameters for determining the rotational correlation times of samples that contain free or weakly immobilized spin labels (Squier and Thomas, 1986). This is especially important for InVSL, because it tends to undergo a retro-Michael reaction, resulting in the conversion of bound to free spin labels. Although the line-shape parameter H″/H is not affected much by free or weakly immobilized spin labels (Squier and Thomas, 1986), it loses sensitivity to rotational motion when the correlation time becomes $<10^{-5}$ s (Fig. 3). Therefore, the integrated intensity parameter ($\int V_2'$) is often the

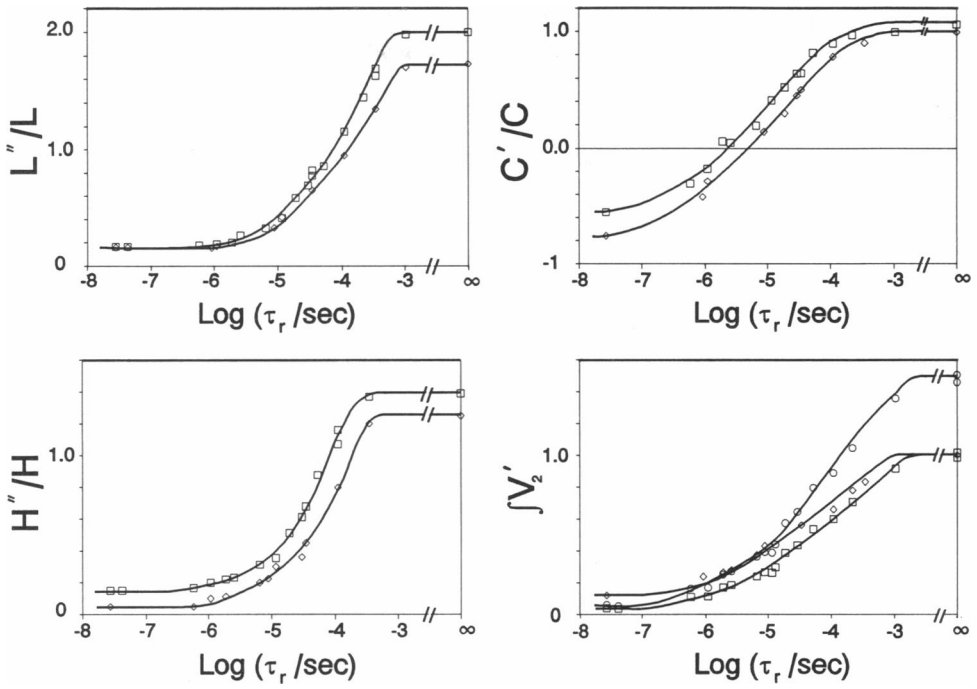

FIGURE 3 Dependence of ST-EPR parameters on microsecond rotational motion. (□) InVSL-hemoglobin; (◇) MSL-hemoglobin. The parameters are defined in Methods and in Fig. 2. The integrated intensity parameter $\int V_2'$ shows three plots: the squares are the InVSL–hemoglobin values, normalized to their own rigid limit, which were used to analyze the data on InVSL-labeled myosin heads. The diamonds are the MSL-hemoglobin values, also normalized to their own rigid limit. The circles are the InVSL-hemoglobin data, normalized to the MSL-Hb rigid limit, to illustrate the relative intensities of the InVSL and MSL spectra.

most reliable for determining the rotational correlation time of InVSL-proteins.

Reaction of InVSL with myosin SH groups

Myosin has over 40 thiol residues; the two most reactive are cys 707 (called SH1) and cys 697 (called SH2) (Reisler, 1982). The extent of sulfhydryl modification during the labeling reaction was determined by the inhibition of the K/EDTA-ATPase activity and activation of the calcium Ca/K-ATPase activity. It has been shown that the fractional inhibition of K/EDTA-ATPase activity is a reliable measure of the fraction of heads labeled at SH1 and/or SH2 (Kielley and Bradley, 1956; Sekine and Kielley, 1964). In addition, labeling of SH1 activates the Ca/K-ATPase activity by a factor of at least 4 (Sekine and Kielley, 1964), whereas labeling of only SH2 (Reisler et al., 1974) or both SH1 and SH2 (Yamaguchi and Sekine, 1966; Reisler et al., 1977) produces near normal (or slightly inhibited) activity of the Ca/K-ATPase. Myosin filaments (1.75 mg/ml) in MLS plus 2 mM $MgCl_2$ and 2 mM K_4PP_i were labeled with 14.6 μM InVSL (InVSL/myosin head, 2:1) at 4°C. Aliquots from the labeling reaction were quenched with a 10-fold excess (over the spin-label concentration) of DTT at various time intervals, and the K/EDTA- and Ca/K-ATPase activities were assayed. The K/EDTA-ATPase activity of unlabeled myosin filaments was 2 μmol P_i/mg per min, but was 0.38 μmol P_i/mg per min in $MgPP_i$ (the ATPase activity of myosin is low in the presence of Mg^{2+}) and was unaffected by the addition of DTT. Within 5 min of reaction with InVSL, the K/EDTA-ATPase activity was inhibited by 55% (Fig. 4), indicating that 45% of the heads were modified at SH1 and/or SH2 by InVSL. Maximum labeling was achieved after

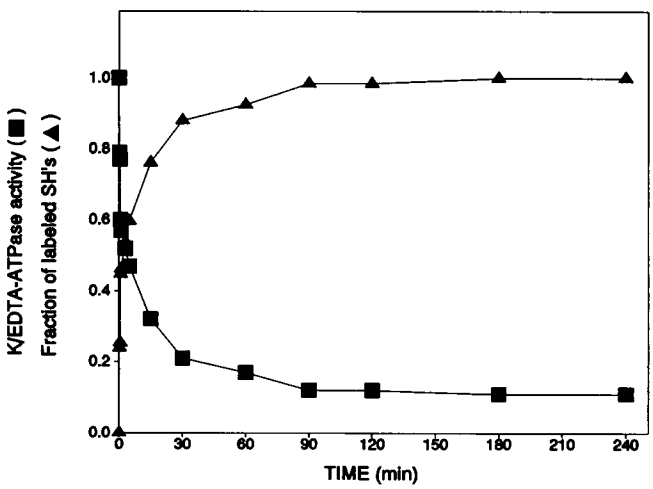

FIGURE 4 Labeling kinetics of myosin with InVSL. (■) K/EDTA-ATPase activity of labeled myosin (K_L), normalized to the activity of unlabeled myosin (K_{UL}). (▲) f_{SH}, the fraction of modified SH1 and/or SH2, calculated from $[1 - (K_L - K_\infty)/(K_{UL} - K_\infty)]$. The activity of maximally labeled myosin (K_∞) after 4 h of labeling was 0.04 μmol P_i/mg per min.

1.5 h, after which time the K/EDTA-ATPase was inhibited by 88%. The Ca/K-ATPase activity was unmodified by labeling, suggesting that most of the spin label reacts with SH2. In a similar labeling experiment, the reaction was monitored by EPR for 5 h. As the reaction progressed, the intensity of the sharp three-line spectrum with narrow splitting (corresponding to free spin label) decreased, and the intensity of the broad spectrum (corresponding to strongly immobilized spin label, presumably bound to the protein) increased.

Myosin filaments or S1 were subsequently labeled with InVSL at a ratio of 2.0 labels per head for 90 min at 4°C as described in Methods. The f_{SH} (fraction of heads with SH1 and/or SH2 labeled) ranged from 0.70 to 0.95 and the f_{SL} (number of spin labels bound per head, determined by double-integration of EPR spectra) was consistently within ±10% of the f_{SH} value. The specificity of labeling (f_{SH}/f_{SL}) was 0.97 ± 0.10, indicating that each labeled head had only one label and essentially all the spin labels were on the myosin head at either SH1 or SH2.

The Mg-ATPase activities of InVSL–myosin and InVSL–S1 were the same as that of unlabeled protein (0.03 μmoles P_i/mg per min), which is typical of labeling SH2 (Daniel and Hartshorne, 1974). It has been shown that the Mg-ATPase activity under physiological conditions (in the presence of millimolar Mg^{2+}) is inhibited for unlabeled myosin, activated four- to fivefold for SH1-labeled myosin (Burke et al., 1973) and remains unchanged when myosin is labeled under conditions that modifies SH2 (Daniel and Hartshorne, 1974).

Covalent attachment of InVSL to myosin

The EPR spectrum of InVSL–myosin monomers at 4°C indicates strongly immobilized spin labels (see Fig. 6, *top row, left column*). However, at room temperature, a narrow three-line spectrum, typical of isotropically tumbling free spin label, overlays the central region of the spectrum. The intensity of this population of spin labels is constant at 4°C but slowly increases continuously at room temperature. Addition of 5 mM MgADP accelerates this apparent release of spin label from the protein. This temperature-dependent slow release of the spin label was also observed in previous studies with Na-K/ATPase protein (Esmann et al., 1990). This phenomenon is irreversible when the temperature is reversed.

The labeled myosin monomers were dialyzed against RS (pH 7.0) to form filaments. The effect of temperature on the spectrum was similar to that observed for monomers. The labeled myosin filaments were sedimented by centrifugation in an Eppendorf centrifuge for 5 min at 4°C. The EPR spectrum of the free spin label in the supernatant showed a narrow three-line spectrum, comparable in line shape and intensity to the narrow component in the myosin filaments spectrum before sedimentation.

To determine whether InVSL was covalently bound to the myosin, an equal volume of ethanol was added to labeled myosin filaments, and the sample was incubated for 5 min at room temperature. This treatment has been shown to denature myosin and release noncovalently bound probes (Eads et al., 1984). The EPR spectrum of the supernatant showed three sharp lines similar to those of isotropically tumbling free spin label. The ethanol extracted 10% of the total spin label, therefore, 90% of the spin label is covalently attached to myosin. Thus, 10% of the spin label was noncovalently bound to myosin, despite being immobilized before ethanol extraction. These results suggest that this population of spin labels either was never covalently bound to myosin or was initially covalently bound, but underwent a retro-Michael reaction in which the covalent bond between the spin label and myosin was reversed (Ingold, 1969). Consistent with the known properties of retro-Michael reactions (Ingold, 1969), the release of InVSL from myosin was accelerated by increasing temperature or decreasing pH.

In previous EPR studies with MSL and IASL, the signal from weakly immobilized spin labels on myosin was selectively decreased by potassium ferricyanide (Graceffa and Seidel, 1980; Thomas et al., 1980). Labeled myosin in MS was mixed with 4 mM potassium ferricyanide for 5 min and dialyzed against MS until the yellow color of potassium ferricyanide was removed. The EPR spectrum showed that the intensity due to the weakly immobilized spin labels decreased by 75%, but the intensity due to the desired strongly immobilized spin labels also decreased by 50%. Therefore, ferricyanide was not used in the preparation of labeled myosin. Instead, the labeled myosin and S1 samples were washed extensively as described in Methods.

Myosin's reactivity with InVSL, MSL, and IASL

Myosin filaments were labeled separately with InVSL, MSL, and IASL. Myosin filaments (4.8 mg/ml) in MLS (containing 2 mM $MgPP_i$) were labeled with 0.01 mM spin label (spin label/myosin head, 1:1) at 4°C for 30 min. The reaction was stopped as described in Methods. The f_{SH} values for InVSL-, MSL-, and IASL-myosin samples were 0.67, 0.46, and 0.33, respectively. These results show that the reactivity of the spin labels with myosin's most reactive SH groups increases in the order InVSL > MSL > IASL. This is consistent with previous results, in which a comparative study of the reactivity of a series of vinyl ketones (including InVSL) and maleimides showed that InVSL had the highest reactivity with Na-K/ATPase protein (Esmann et al., 1990).

EPR spectra of InVSL-S1

To obtain reliable ST-EPR spectra for determining rotational correlation times of spin-labeled proteins, the spin

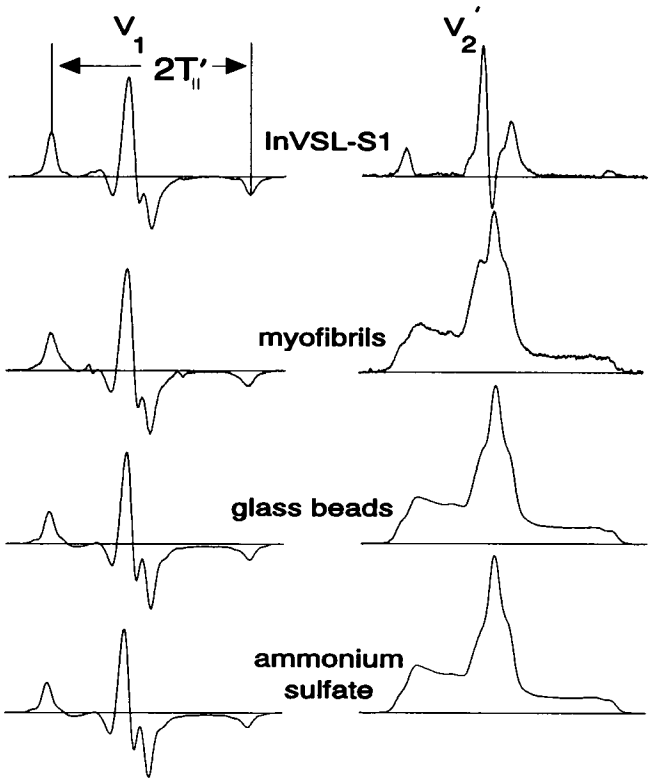

FIGURE 5 (*Left*) Conventional EPR spectra (V_1). (*Right*) ST-EPR spectra (V_2') of InVSL–S1 in solution, immobilized on myofibrils, immobilized on glass beads, and immobilized by ammonium sulfate precipitation. All samples were in RS (pH 7.0), except the precipitate, which contained 40% RS (pH 7.0) and 60% saturated ammonium sulfate. The spectra were recorded at 4°C; the baseline is 100-G wide. The ST-EPR spectra are plotted so that their peak-to-peak intensities are the same. The effective rotational correlation times are shown in Table 1.

label should be rigidly attached so that it undergoes no submillisecond motion relative to the protein. This requires that the EPR spectra of the immobilized protein show no evidence of rotational motion. Because we are interested in detecting the rotational motion of the myosin head (S1), we performed these control EPR experiments on InVSL–S1. The conventional (V_1) EPR spectrum of InVSL–S1 (Fig. 5, *top left*) has a "powder" lineshape characteristic of strongly immobilized spin label, with a very large hyperfine splitting ($2T_\parallel' = 72.35 \pm 0.01$ G), indicating little or no submicrosecond motion. Very similar V_1 spectra, with only slightly greater splittings, were observed for immobilized InVSL-S1, produced either by covalent binding to glass beads or precipitation with ammonium sulfate (Fig. 5).

The ST-EPR spectra of immobilized InVSL-S1 (on actin [not shown], myofibrils, glass beads, or ammonium sulfate precipitated) show that the spin label is rigidly immobilized on S1, undergoing no submillisecond rotation relative to S1 (Fig. 5, Table 1), therefore, InVSL should be an accurate reporter of submillisecond rotational motion of myosin heads.

The addition of 5 mM MgATP (in ARS solution) to S1 immobilized on glass beads did not affect the line shape of the ST-EPR spectrum (Table 1), indicating that the spin label did not report any local or global submicrosecond motion that may be induced by MgATP binding. There was also no effect of adding 5 mM MgADP and 5 mM orthovanadate (data not shown). The Mg-ATPase activity of the immobilized InVSL–S1, obtained under the same EPR conditions, was not affected by the binding of the protein to glass beads. This suggests that although S1 is immobilized on glass beads, it still undergoes the normal conformational transitions of the ATPase cycle. This is an important control, because IASL does undergo nanosecond rotation relative to S1 in the presence of ATP, both in solution (Seidel et al., 1970) and when S1 is bound to glass beads (Thomas et al., 1980). Thus, InVSL will be a useful probe of global myosin head rotation in future studies involving ATP.

The effective rotational correlation times for InVSL-labeled myosin and S1 were determined from calibration plots of the spectral parameters L″/L, C′/C, H″/H, and $\int V_2'$ of InVSL-hemoglobin (Fig. 3). Only the C′/C lineshape parameter is useful for S1 tumbling freely in solution, because the other parameters have little or no sensitivity to submicrosecond rotational motion. An effective rotational correlation time (τ_r^{eff}) of 0.7 ± 0.1 μs was determined for labeled S1 (Table 1). The rotational motion of S1 is decreased by the binding of S1 to pure actin (70-fold increase in τ_r^{eff}) and to the actin in myofibrils (225-fold increase in τ_r^{eff}) (Table 1).

EPR spectra of InVSL–myosin

The peak-to-peak splitting $2T_\parallel'$ of the conventional EPR spectrum (Fig. 6, *left column*) was 72.11 ± 0.04 G for myosin filaments and 72.2 ± 0.08 G for myosin monomers, confirming that the spin label is rigidly immobilized. This $2T_\parallel'$ splitting is greater than previously observed for other spin labels on myosin filaments, such as MSL and IASL (68.3 ± 0.35 and 69.75 ± 0.39 G, respectively, [Barnett and Thomas, 1987]), and IPSL on S1 (63.84 ± 0.2 G, [Ajtai et al., 1990]), indicating either that InVSL is more rigidly immobilized and/or that its nitroxide bond is in a more polar environment. The V_1 spectrum of InVSL-myosin filaments was unchanged by the addition of 5 mM MgATP (spectrum not shown), implying that no nanosecond rotational motion is induced by ATP binding or hydrolysis.

ST-EPR shows that InvSL-labeled heads are less rotationally mobile in myosin filaments than in myosin monomers (Fig. 6, *right column, top and middle*). Effective rotational correlation times for myosin monomers and filaments were 4.0 ± 0.3 and 12.4 ± 0.3 μs, respectively. The ST-EPR spectrum of actomyosin (Fig. 6, *right column, bottom*) shows that myosin binding to actin slows the rotational motion of the labeled myosin

TABLE 1 **ST-EPR parameters of InVSL attached to myosin heads**

Sample	L″/L	C′/C	H″/H	$\int V'_2$	Mean τ_r^{eff}
					μs
S1					
Parameter	0.12 ± 0.01	−0.25 ± 0.02	0.14 ± 0.01	0.11 ± 0.02	
τ_r^{eff} (μs)	—	0.66 ± 0.05	—	0.72 ± 0.07	0.70 ± 0.05
acto-S1					
Parameter	1.26 ± 0.04	0.83 ± 0.05	1.01 ± 0.02	0.43 ± 0.08	
τ_r^{eff} (μs)	126 ± 4	66 ± 4	79.4 ± 3	32 ± 5	73 ± 4
S1-decorated myofibrils					
Parameter	1.40 ± 0.04	1.00 ± 0.02	1.17 ± 0.02	0.65 ± 0.24	
τ_r^{eff} (μs)	158 ± 7	178 ± 5	100 ± 4	141 ± 5	159 ± 6
S1-ammonium sulfate ppt					
Parameter	2.05 ± 0.14	1.10 ± 0.04	1.13 ± 0.02	1.01 ± 0.06	
τ_r^{eff} (μs)	>1,000	>1,000	100 ± 4	>1,000	>1,000
S1-glass beads					
Parameter	1.99 ± 0.03	1.06 ± 0.01	1.22 ± 0.02	0.99 ± 0.11	
τ_r^{eff} (μs)	>1,000	>1,000	141 ± 5	>1,000	>1,000
S1-glass beads + MgATP					
Parameter	1.77 ± 0.03	1.06 ± 0.02	1.25 ± 0.02	ND	
τ_r^{eff} (μs)	>1,000	>1,000	175 ± 6		>1,000
Myosin monomers					
Parameter	0.29 ± 0.02	0.16 ± 0.04	0.29 ± 0.02	0.19 ± 0.02	
τ_r^{eff} (μs)	4.0 ± 0.3	4.1 ± 0.3	4.5 ± 0.2	4.1 ± 0.2	4.1 ± 0.3
Myosin filaments					
Parameter	0.50 ± 0.02	0.41 ± 0.01	0.40 ± 0.02	0.30 ± 0.02	
τ_r^{eff} (μs)	12.6 ± 0.3	12.0 ± 0.2	12.6 ± 0.2	10.0 ± 0.3	12.4 ± 0.3
Actomyosin					
Parameter	1.10 ± 0.05	0.82 ± 0.01	0.81 ± 0.02	0.52 ± 0.06	
τ_r^{eff} (μs)	100 ± 6	63 ± 2	56 ± 3	66 ± 4	71 ± 4

Spectral parameters were defined as described by Squier et al. (1986). The integrated intensity parameter $\int V'_2$ was normalized by dividing by the rigid limit value for immobilized InVSL–S1 (the average of the values for S1 on glass beads and ammonium sulfate-precipitated S1). τ_r^{eff} is the effective rotational correlation time calculated from the given spectral parameter, using the calibration plots for IASL-hemoglobin in Fig. 4. The mean τ_r^{eff} is the average value for that sample (row). The uncertainty for a parameter is the range of values obtained for two to five observations, and the uncertainty in τ_r^{eff} was propagated directly from the parameter uncertainty. Thus, the τ_r^{eff} uncertainty reflects precision but not accuracy, which is appropriate, because τ_r^{eff} is useful mainly for comparison of one sample with another, not for estimating actual correlation times (Howard et al., 1993).

heads, as reported previously for IASL (Thomas et al., 1975) and MSL (Thomas et al., 1980).

DISCUSSION

Summary of results

This study describes a new spin label for studying rotational dynamics in myosin and its subfragments. The label binds covalently to myosin, reacting with high specificity to the fast-reacting SH groups (SH1 and/or SH2) of the myosin head (S1). Control ST-EPR experiments with immobilized S1 (Fig. 5) show that the label binds rigidly to the myosin head, so that the ST-EPR spectrum of InVSL provides a reliable measure of the global rotational dynamics of the myosin head. The quantitative analysis of these spectra is made more accurate by the use of reference spectra based on InVSL-hemoglobin (Figs. 2 and 3).

Hemoglobin reference spectra

Although the ST-EPR spectra of InVSL- and MSL-hemoglobin have similar sensitivity to rotational motion, there are significant differences in the line shapes and parameter plots (Figs. 2 and 3). In general, the InVSL spectra have greater intensity and have line shapes suggesting slower motion compared with MSL spectra corresponding to the same correlation time. A possible explanation for these differences is that InVSL is more rigidly immobilized on hemoglobin. The number of potentially flexible bonds between the nitroxide group and the cysteine attachment is smaller for InVSL than for MSL (Fig. 1), and the greater hydrophobicity of InVSL might promote its insertion into the core of S1. Alternatively, the differences could be due to motion-independent physical properties of InVSL, such as relaxation times (T_1 or T_2) or hyperfine interaction values. This is plausible, because the structure of InVSL in the vicinity of the nitroxide group is quite different from that of MSL. Because it is virtually impossible to determine the causes of these spectroscopic differences, the most prudent approach is to use the InVSL-hemoglobin spectra, rather than the MSL-hemoglobin spectra, in analyzing the EPR spectra of InVSL bound to myosin or other proteins. Thus the parameter plots in Fig. 3 should be

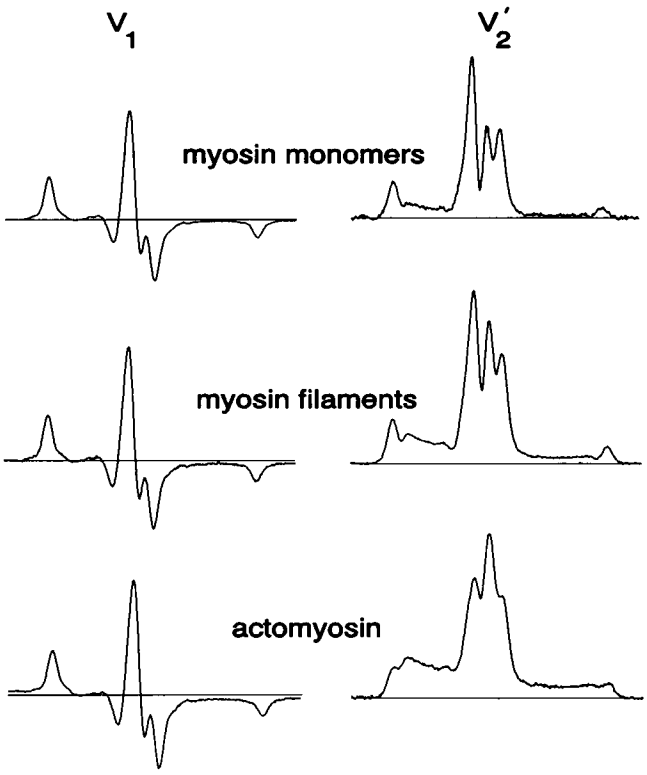

FIGURE 6 Different rotational motions of myosin heads. (*Left*) Conventional EPR spectra (V_1). (*Right*) ST-EPR spectra (V_2'). All samples were in RS (pH 7.0) except myosin monomers, which were in MS. The myosin samples contained 25 mg/ml of myosin. Actomyosin contained 38 mg/ml myosin and 21 mg/ml actin, corresponding to a ratio of 1:5. The spectra were recorded at 4°C in the TE_{102} cavity and the baseline is 100-G wide. The ST-EPR spectra are plotted so that their peak-to-peak intensities are the same. Effective rotational correlation times are determined from ST-EPR spectra and are given in Table 1.

useful to other investigators using InVSL (or MSL) to study rotational dynamics with ST-EPR.

Reaction with SH groups

The agreement of the K/EDTA-ATPase inhibition with the total spin label incorporation indicates that virtually every labeled myosin head has one spin label attached to either SH1 (cys 707) or SH2 (cys 697), but not to both. This is the first confirmation of the prediction that InVSL has high specificity for SH groups (Hankovosky et al., 1989; Esmann et al., 1990). Because the Ca/K-ATPase activity of myosin is not significantly activated by InVSL, it appears that the reaction is more specific for SH2 than for SH1. However, it is unlikely that only SH2 is labeled, because our EPR spectra of muscle fibers decorated with InVSL-S1 (or directly labeled with InVSL) resolve two different populations of spin labels (Roopnarine and Thomas, 1991). Since the V_1 spectrum of labeled S1 or myosin shows no nanosecond motion, we conclude that the spin label is strongly immobilized at both sites, and the overall protein motion will be reported by spin labels at either site on the myosin head.

Myosin high-salt ATPase results show that IASL has very high specificity for SH1 compared with SH2 (Thomas et al., 1975), whereas MSL has SH1 specificity similar to InVSL (Thomas et al., 1980). Comparison of the reactivity of myosin with InVSL, MSL, and IASL showed that InVSL is the most reactive spin label, whereas IASL is the least reactive. It seems that the more reactive the spin label, the less specific it is for myosin's SH1 compared with SH2. The apparent preference of InVSL for SH2 should prove useful for the purpose of comparing signals from these two distinct sites on myosin, and InVSL's high reactivity should be useful when labeling proteins with a single cysteine that has low reactivity, such as some myosin light chains (Roopnarine et al., 1993).

Rigid immobilization on myosin

The conventional EPR spectrum of InVSL-myosin filaments (Fig. 6) has an unusually large splitting between the outer extrema ($2T_\parallel' = 72.2 \pm 0.3$ G, Table 1), indicating that the spin labels are strongly immobilized on the myosin head, undergoing no significant nanosecond motion. ST-EPR spectra of InVSL-S1 immobilized on myofibrils, glass beads, and by ammonium sulfate (Fig. 5, Table 1) indicate that InVSL is immobile on myosin even on the millisecond time scale, thus making it a reliable reporter of global myosin head rotations in the microsecond time range. Therefore, InVSL is so strongly immobilized on myosin that it will report only global rotational motions of the myosin head. This degree of immobilization of InVSL on the myosin head is greater than observed previously for other spin labels. None of the other labels, MSL, IASL (Seidel et al., 1970; Thomas et al., 1975, 1980; Barnett and Thomas, 1987), and IPSL (Ajtai et al., 1990), have $2T_\parallel'$ values > 70 G on myosin. This could be due to greater nanosecond mobility of these other labels. However, $2T_\parallel'$ is affected not only by nanosecond motion, but also by the polarity near the nitroxide group, which can affect the rigid-limit value $2T_\parallel$ (Griffith and Jost, 1976; Thomas et al., 1979), so values of $2T_\parallel'$ as low as 68 G are consistent with rigid probe binding. IASL–myosin and MSL–myosin satisfy this requirement, but the value observed for IPSL (63.8 ± 0.2 G; Ajtai et al., 1990) clearly indicates nanosecond rotation of the probe relative to the protein. The ST-EPR spectra of IASL–S1 and MSL–S1 immobilized on glass beads indicate effective correlation times on the order of 100 μs, supporting the use of these labels for faster head rotations (Thomas et al., 1980). However, InVSL–S1 is even more strongly immobilized when bound to S1 attached to glass beads ($\tau_r > 1$ ms), indicating that InVSL is a more reliable reporter of submillisecond head rotations.

Another important requirement for a myosin head spin label is that the label should remain rigidly bound to the protein even in the presence of ATP. The ST-EPR spectrum of MSL-myosin is unaffected by ATP, but the

ST-EPR spectrum of MSL-S1 immobilized on glass beads suggests a slight submillisecond mobilization by MgATP (Thomas et al., 1980), and the conventional EPR spectrum of MSL-myosin shows a slight submicrosecond mobilization in the presence of MgADP plus orthovanadate (Wells and Bagshaw, 1984; Barnett and Thomas, 1987). Conventional EPR of IASL shows that the probe undergoes substantial submicrosecond rotational motion in the presence of ATP (Barnett and Thomas, 1987), even when S1 is immobilized on glass beads (Thomas et al., 1980). The EPR spectra of IPSL-labeled S1 show that this probe has submicrosecond mobility both in the presence and absence of ATP (Ajtai et al., 1990). However, in the present study we have shown that neither the conventional nor ST-EPR spectrum of InVSL S1 is affected by MgATP or MgADP plus orthovanadate, even when S1 is immobilized on glass beads, indicating that the spin label does not undergo any submillisecond rotational motion induced by MgATP binding or hydrolysis. Because only InVSL is strongly immobilized on myosin in the presence and absence of ATP, it is a superior spin label for accurately reporting the global rotational motion of myosin heads in the presence and absence of nucleotides.

Rotational dynamics of myosin and S1

The effective rotational correlation time (τ_r) of InVSL-S1 is 0.70 μs (Table 1), which is almost an order of magnitude greater than predicted (77 ns) for a sphere of the same molecular weight (115,000) (Thomas et al., 1975). This slower motion requires not only that InVSL be immobilized on S1, but that S1 is a highly asymmetric protein (Thomas et al., 1975). This is consistent with the 25-Å resolution structure of crystallized S1 (Winkelmann et al., 1991), which shows that the myosin S1 is shaped like a bent tadpole. The τ_r^{eff} value for myosin monomers is much greater ($\sim$4 μs), indicating that the joining of the two heads to the tail restricts the head rotation substantially. The formation of myosin filaments restricts the motion further, resulting in τ_r^{eff} = 12 μs, which is unaffected by ATP. However, the observed value is still $\geq$10 times that expected for the fastest rotation predicted for a rigid myosin filament (Thomas et al., 1975), indicating that the filament is not rigid and that we are probably detecting motion of the myosin head relative to the filament. The binding of myosin heads to actin causes a further increase in τ_r^{eff} (to $\sim$70 μs). This residual rotational motion is probably due to the flexibility of the actin to which the heads are bound (Thomas et al., 1979). This microsecond motion is further restricted when InVSL-S1 binds to actin in myofibrils (Table 1), indicating that actin is less flexible in the myofibrillar lattice than in solution.

For most of the samples in Table 1, there is a significant dependence of τ_r^{eff} on the spectral parameter measured. As pointed out previously, this is probably due to the presence of anisotropic rotational diffusion (Robinson et al., 1985), but present theoretical methods for simulation of EPR spectra are not sufficiently accurate to analyze these motions quantitatively and to distinguish different models for the motion (Howard et al., 1993).

These results for InVSL are similar to those previously reported for IASL (Thomas et al., 1975) and MSL (Thomas et al., 1980; Berger and Thomas, 1991), although the τ_r^{eff} values are significantly greater, especially for myosin monomers. This could be due to a slightly greater immobilization of InVSL on the myosin head, as suggested by the greater immobilization of InVSL on immobilized S1 (Fig. 5). Alternatively, the different correlation times for different spin labels could be due to different orientations of the probes' principal axes relative to the myosin head. Because the heads are undergoing anisotropic rotation, τ_r^{eff} depends on the orientation of the probe relative to the diffusion axes of the protein (Thomas et al., 1975). Conventional EPR studies of labeled fibers indicate that the principal axis of the nitroxide group of InVSL is oriented almost parallel to the fiber axis (Roopnarine and Thomas, 1991), whereas MSL and IASL are approximately oriented at 82 and 68°, respectively (Thomas and Cooke, 1980; Fajer et al., 1990). Thus, InVSL has a very different orientation relative to the myosin head, and this could explain the different τ_r^{eff} values.

A recent theoretical study of ST-EPR (Howard et al., 1993) shows that a restriction in the angular amplitude of rotational motion can increase the observed effective rotational correlation time τ_r^{eff}. Therefore, the τ_r^{eff} values derived from ST-EPR spectra in this study must be considered upper bounds for the actual correlation times. However, the angular amplitude (full width) of rotation for myosin heads in monomers or filaments has been estimated to be $\geq$80° (Eads et al., 1984); for amplitudes this large, the effective correlation times are accurate (Howard et al., 1993). A more quantitative analysis in terms of specific models for anisotropic motion requires more accurate spectral simulations than have been reported (Robinson et al., 1985; Howard et al., 1993).

Other ST-EPR studies with InVSL

In a previous study with spin-labeled Ca-ATPase, effective rotational correlation times for InVSL–Ca-ATPase and MSL–Ca-ATPase were both determined from calibration plots of H″/H for MSL-hemoglobin, resulting in τ_r^{eff} values of 65 and 22 μs, respectively (Horváth et al., 1990). However, the present study shows that this apparent discrepancy is eliminated if the appropriate reference spectra are used. We have shown above (Fig. 3) that the use of MSL reference spectra to analyze InVSL data can result in overestimated correlation times. Fig. 3 (H″/H) indicates that if the actual value of τ_r^{eff} is 65 μs, using the InVSL-hemoglobin reference spectra, the use of the MSL-hemoglobin reference spectra would result in underestimating τ_r^{eff} by a factor of 2.2. Thus, the apparent

discrepancy between the correlation times reported by InVSL and MSL for the Ca-ATPase (Horváth et al., 1990) would be eliminated if the appropriate reference spectra were used.

Conclusion

We have shown that the differences between the ST-EPR spectra of model systems of InVSL- and MSL-hemoglobin are significant, especially with respect to the intensity of the V'_2 spectrum. Therefore, it is important to obtain reference spectra of hemoglobin labeled with the same spin label as attached to the protein of interest. This study also reports on the labeling of myosin with InVSL and the characterization of the labeled protein. The spin label is covalently bound to SH groups on the myosin head. Most of the labels are attached specifically to SH2. Conventional and ST-EPR spectra showed that the spin label is rigidly immobilized on the protein and is sensitive to global rotational motions of the myosin head. ST-EPR spectra of myosin filaments indicate that myosin heads rotate relative to the filament backbone on the microsecond time scale, but that this motion is more restricted than in myosin monomers. The effective rotational correlation times reported by InVSL for S1 and myosin are longer than previously calculated by MSL or IASL. Because InVSL is the only spin label that has been shown to undergo no submillisecond rotational motion relative to the myosin head, even in the presence of nucleotides and nucleotide analogs, it should prove valuable in future studies of myosin head rotation in solution and in muscle fibers.

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