A Spin Label that Binds to Myosin Heads in Muscle Fibers with Its Principal Axis Parallel to the Fiber Axis

Osha Roopnarine and David D. Thomas
Department of Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455 USA

ABSTRACT We have used an indane-dione spin label (2-[-oxyl-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)methenyl]indane-1,3-dione), designated InVSL, to study the orientation of myosin heads in bundles of chemically skinned rabbit psoas muscle fibers, with electron paramagnetic resonance (EPR) spectroscopy. After reversible preblocking with 5,5′-dithiobis(2-nitro-benzoic acid) (DTNB), we were able to attach most of the spin label covalently and rigidly to either Cys 707 (SH1) or Cys 697 (SH2) on myosin heads. EPR spectra of labeled fibers contained substantial contributions from both oriented and disordered populations of spin labels. Similar spectra were obtained from fibers decorated with InVSL-labeled myosin heads (subfragment 1), indicating that virtually all the spin labels in labeled fibers are on the myosin head. We specifically labeled SH2 with InVSL after reversible preblocking of the SH1 sites with 1-fluoro-2,4-dinitrobenzene (FDNB), resulting in a spectrum that indicated only disordered spin labels. Therefore, the oriented and disordered populations correspond to labels on SH1 and SH2, respectively. The spectrum of SH2-bound labels was subtracted to produce a spectrum corresponding to SH1-bound labels, which was used for further analysis. For this corrected spectrum, the angle between the fiber axis and the principal axis of the spin label was fitted well by a Gaussian distribution centered at $\theta_0 = 11 \pm 1°$, with a full width at half-maximum of $\Delta\theta = 15 \pm 2°$. The unique orientation of InVSL, with its principal axis almost parallel to the fiber axis, makes it complementary to spin labels previously studied in this system. This label can provide unambiguous information about axial rotations of myosin heads, since any axial rotation of the *head* must be reflected in the same axial rotation of the principal axis of the *probe*, thus changing the hyperfine splitting. Therefore, InVSL-labeled fibers have ideal properties needed for further exploration of myosin head orientation and rotational motion in muscle.

INTRODUCTION

One of the most elusive events in muscle contraction is the proposed rotation of the myosin head on actin. The commonly accepted hypothesis is that myosin heads rotate from an axial angle of 90° (beginning of the power stroke) to 45° (end of the power stroke) during contraction (Huxley, 1969; Huxley and Simmons, 1971). Orientation-sensitive spectroscopic probes attached to myosin heads are essential tools in the testing of this model, and electron paramagnetic resonance (EPR) of nitroxide spin labels provides the highest orientational resolution (Thomas, 1987). These probes have consistently shown that myosin heads are highly oriented relative to the muscle fiber in rigor (i.e., in the absence of ATP, presumed to represent the end of the power stroke). However, earlier states in the cross-bridge cycle, observed either during active contraction or in the presence of nucleotides designed to trap intermediates, have revealed either an axial probe orientation essentially the same as in rigor or a high degree of dynamic orientational disorder (Thomas, 1987; Fajer et al., 1988; Fajer et al., 1990b; Berger and Thomas, 1994). There are two plausible interpretations: 1) myosin heads do not assume two distinct axial angles in contracting muscle; or 2) the spin labels used previously have not accurately reflected the axial orientations of myosin heads (reviewed by Thomas, 1987).

One possible source of ambiguity in the use of spin labels is that the EPR experiment measures directly the orientation of the probe, not the head, since the orientation of the probe relative to the head is not usually known with much precision. Although the reorientation of any of the three spin label axes relative to the fiber axis will affect the EPR spectrum, the spectrum is sensitive primarily to the orientation of the principal axis. The spin labels that have been used most to probe myosin head orientation (a maleimide spin label [MSL, *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)maleimide], and an iodoacetamide spin label [IASL, 2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide]) have their principal axes oriented approximately perpendicular to the fiber axis (Thomas and Cooke, 1980; Fajer et al., 1990b). Therefore, it is possible that an axial reorientation of the head would result in a much smaller reorientation of the principal axis, making the spectrum relatively insensitive to the head rotation (Ajtai et al., 1989). Thus it is important to investigate other spin labels that may be more suitably oriented on the myosin head to report axial rotations unambiguously.

We have recently shown that an indane-dione spin label (2-[-oxyl-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)methenyl]indane-1,3-dione), designated InVSL, binds rigidly to the reactive thiols of myosin heads in solutions of purified myosin, making it an effective probe of the global orientation and rotational motion of the myosin head (Roopnarine et al.,

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Address reprint requests to Dr. David D. Thomas, Department of Biochemistry, University of Minnesota Medical School, Millard 4–225, 435 Delaware St., SE, Minneapolis, MN 55455. Tel.: 612 625 0957; Fax: 612 624 0632; E-mail: ddt@ddt.biochem.umn.edu. Dr. Roopnarine's current address: Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461.

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1993b). In the present study, we have developed a procedure for attaching InVSL specifically to myosin heads of chemically skinned rabbit psoas muscle fibers. The resulting EPR spectra reveal a resolved spectral component with a hyperfine splitting near the theoretical maximum, indicating that the spin label binds with its principal axis almost precisely parallel to the fiber axis (Roopnarine and Thomas, 1991; Roopnarine et al., 1993a), making it unique among spin labels previously used in muscle (Thomas, 1987). We show that this orientation makes the interpretation of EPR spectra much less ambiguous, because any axial reorientation of the myosin head must cause the same axial reorientation of the principal axis of the probe. These studies establish the feasibility for the use of this probe in the search for new axial head orientations in the contraction cycle.

MATERIALS AND METHODS

Reagents and solutions

The spin label InVSL (Fig. 1) was synthesized as described by Hankovszky et al. (1989) and provided by Kálmán Hideg. Dithiothreitol (DTT) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). The fiber bundles were washed with the following solutions: rigor solution (RS; pH 7.0): 130 mM potassium propionate (KPr), 2 mM $MgCl_2$, 1 mM EGTA, and 20 mM MOPS (pH 7.0); RS (pH 8.0): same as RS (pH 7.0) with MOPS replaced by 20 mM EPPS (pH 8.0); labeling solution (LS): RS (pH 6.5) plus 3 mM $MgCl_2$ and 10 mM potassium pyrophosphate (PP_i). Solutions for myosin and its proteolytic subfragment 1 (S1) preparations were described by Roopnarine et al. (1993b). Unless otherwise indicated, all preparations and labeling procedures were performed at 4°C.

Protein and fiber preparations

Chymotryptic S1 was prepared as described by Roopnarine et al. (1993b). Rabbit psoas muscle fiber bundles were dissected and stored in RS/glycerol (50% v/v) as described by Fajer et al. (1988), except that potassium acetate was replaced with potassium propionate. After 1 month, the fibers were dissected into smaller fiber bundles (<0.5 mm in diameter and ~8 cm long) and tied at both ends on glass rods for labeling experiments. To prepare myofibrils, fiber bundles were washed for 15 min in RS (pH 7.0), minced, and homogenized in RS (pH 7.0) using a tissue homogenizer (Tekmar, Cincinnati, OH).

The concentration of S1 was determined as described in Roopnarine et al. (1993b). Protein concentrations for myofibrils and labeled proteins were determined by the biuret assay (Gornall et al., 1949), using bovine serum albumin as a standard. To calculate the concentration of heads in myofibrils and fibers, it was assumed that 50% of the protein was myosin by weight (Yates and Greaser, 1983).

Spin labeling

Chymotryptic S1 was labeled with InVSL as described by Roopnarine et al. (1993b). The rationale for the development of fiber spin-labeling procedures is described in detail in the Results. Here we describe, for convenient reference, the optimized procedures and the nomenclature used for various labeled and control preparations. Before labeling, fiber bundles were washed with RS (pH 7.0) for 15 min to remove the storage glycerol, and membranous debris was removed by incubating in 0.5% Triton in RS (pH 7.0) for 15 min, followed by a 10-min wash in RS (pH 7.0).

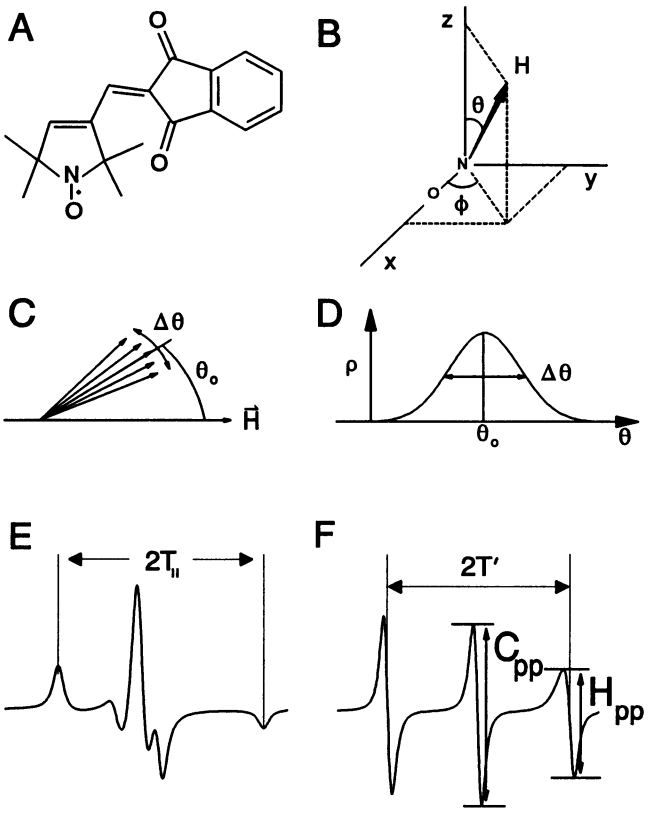

FIGURE 1 (*A*) Chemical structure of InVSL: 2-[-oxyl-2,2,5,5,-tetramethylpyrrolin-3-yl)methenyl]indane-1,3-dione. (*B*) Description of the angles θ and ϕ, which define the orientation of the magnetic field **H** with respect to the principal axes of the nitroxide spin label. These angles determine the orientational dependence of the EPR spectrum. (*C, D*) Angular distribution $\rho(\theta)$ of spin label principal axis **z** relative to **H**, described as a Gaussian distribution centered at θ_0 with a full width at half-maximum of $\Delta\theta$. The distribution $\rho(\phi)$ is defined analogously. (*E*) Simulated spectrum of immobile but randomly oriented (infinite $\Delta\theta$) spin labels (powder spectrum), showing wide peak-to-peak splitting of outer extrema, $2T_{\parallel}$. (*F*) Simulated spectrum of oriented spin labels ($\theta_0 = 0°$, $\Delta\theta = 30°$, $\phi_0 = 0°$, $\Delta\phi = 0°$) showing the spectral parameters (2T′, H_c, and Hpp/Cpp) that are most sensitive to changes in $\rho(\theta)$.

SH1/SH2-labeled fibers

To maximize the specificity of spin-labeling at SH1, fibers were reversibly preblocked with 120 μM DTNB for 2 h in RS (pH 7.0), a solution that decreases SH1 reactivity due to the rigor interaction between the myosin head and actin (Duke et al., 1976). Excess DTNB was removed by washing the fiber bundles in RS (pH 7.0) for 10 min, then 10 min in LS. The fibers were then labeled with 25 μM InVSL in LS for 5 h. The labeling reaction was stopped by transferring the fibers to LS for 1 min and then incubating for 10 min in fresh LS. The fibers were then washed in RS (pH 8.0) for 10 min, and the 5-thio-2-nitrobenzoic acid (TNB) group on the sulfhydryl was removed by incubating in 10 mM DTT in RS (pH 8.0) for 1.5 h. The fibers were washed for 10 min in each of the following: RS (pH 8.0), RS (pH 7.0), and RS/glycerol (50% v/v). The fibers were stored in the latter buffer at −20°C and were used within 1 month. Before EPR or ATPase experiments, fiber bundles were further washed in MgADP as described below (under EPR Experiments), to improve labeling specificity.

In discussions below, fiber samples are defined as follows: native, fibers taken freshly from storage buffer; unlabeled, native fibers taken through the labeling procedure, but omitting DTNB and InVSL; unlabeled/DTT, unlabeled fibers washed with 10 mM DTT in RS (pH 8.0) for 1.5 h at 4°C;

DTNB, native fibers preblocked for 2 h with 120 μM DTNB in RS (pH 7.0); DTNB/DTT, native fibers preblocked with DTNB and then reduced with 10 mM DTT in RS (pH 8.0) for 1.5 h; DTNB/InVSL, native fibers preblocked with DTNB and then labeled with 25 μM InVSL in LS (pH 6.5); DTNB/InVSL/DTT, fibers preblocked with DTNB, labeled with InVSL, then unblocked with 10 mM DTT as above. The latter is the standard preparation for SH1/SH2 labeled fibers, also referred to below simply as labeled fibers.

SH2-labeled fibers

To label SH2 specifically on myosin heads in muscle fibers, we preblocked SH1 groups specifically and reversibly, based on a procedure described previously for myosin (Reisler et al., 1974). Fiber bundles were labeled with 200 μM FDNB in LS for 1 h. Excess FDNB was removed by washing the fiber bundles twice in LS for 5 min each time, then for 10 min in RS (pH 7.0). The SH2 groups were then labeled for 5 h with 25 μM InVSL in RS (pH 7.0) plus 5 mM MgADP, followed by washing for 10 min in RS (pH 7.0) and then 10 min in degassed RS (pH 8.0). The dinitrophenyl (DNP) group on SH1 was removed by incubating the fibers in 50 mM DTT in degassed RS (pH 8.0) overnight. The fiber bundles were washed for 10 min in RS (pH 8.0), 10 min in RS (pH 7.0), and then 15 min in RS/glycerol (50%). The fibers were stored in the latter buffer at −20°C and were used within 1 month.

Fiber samples are defined as follows: unlabeled, native fibers treated with all buffers (omitting FDNB and InVSL) of the labeling procedure; FDNB, native fibers preblocked with 200 μM FDNB in LS (pH 6.5) for 1 h at 4°C; FDNB/DTT, fibers preblocked with FDNB and then washed with 50 mM DTT in RS (pH 8.0) overnight at 4°C; FDNB/InVSL, fibers preblocked with FDNB and then labeled with 25 μM InVSL in RS (pH 7.0) with 5 mM MgADP for 5 h at 4°C; FDNB/InVSL/DTT, FDNB/InVSL fibers washed with 50 mM DTT in RS (pH 8.0) overnight at 4°C. The latter is the standard preparation for SH2-labeled fibers, the spectrum of which was subtracted from that of SH1/SH2-labeled fibers to obtain the spectrum of SH1-labeled fibers, as discussed in Results.

Characterization of labeled proteins and fibers

To determine whether InVSL was covalently bound to the protein, noncovalently bound label was extracted with ethanol (Roopnarine et al., 1993b). ATPase assays were done within 2 days after the labeling procedure. Labeled fibers and control unlabeled fibers were washed in RS (pH 7) plus 5 mM MgADP for 1 h at room temperature, then in RS (pH 7) plus 15 mM EDTA for 15 min. The fibers were washed three times in 10 mM MOPS (pH 7.0) to remove glycerol and Mg^{2+}. The fibers were minced and then homogenized in 10 mM MOPS (pH 7.0). The K/EDTA- and Ca/K-ATPase activities were assayed at 25°C as described by Roopnarine et al. (1993b).

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}):

$$f_{SH} = \frac{K_{UL} - K_L}{K_{UL} - K_\infty} \quad (1)$$

(Roopnarine et al., 1993b; based on Kielley and Bradley, 1956; Yamaguchi and Sekine, 1966), where (K_∞) is the activity of maximally labeled myosin heads in myofibrils obtained by preblocking SH1 with 200 μM FDNB for 1 h in LS as described above in labeling SH2 fibers. After preblocking all SH1 groups specifically with FDNB, resulting in a maximally activated Ca/K-ATPase activity (C_{act}), subsequent labeling at SH2 linearly inhibits this activity (C_L), approaching a limiting value (C_∞) comparable to that of unlabeled myosin (Yamaguchi and Sekine, 1966; Reisler et al., 1974). Therefore, the fraction of heads labeled at SH2 (f_{SH2}) was determined from

$$f_{SH2} = \frac{C_{act} - C_L}{C_{act} - C_\infty} \quad (2)$$

The number of spins 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 heads. The specificity of labeling, defined as the fraction of labels attached to SH1 and/or SH2, was calculated as f_{SH}/f_{SL} (Roopnarine et al., 1993b). 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). The tension (kg/cm^2) of single fibers was measured at 25 ± 2°C as described by Fajer et al. (1988), under the same conditions used for EPR experiments of the labeled fibers in this study.

EPR experiments

Conventional EPR spectra (first harmonic absorption in phase, designated V_1) were acquired with a model ESP 300 spectrometer (Bruker Instruments, Billerica, MA) as described by Roopnarine et al. (1993b), at room temperature (23–26°C), using a Bruker TM_{110} cavity that was modified to hold a capillary either parallel or perpendicular to the magnetic field. The ends of the fiber bundle (<0.5 mm diameter) were tied with silk surgical thread, pulled into a 50-μl glass capillary (length = 6.5 cm, OD = 1 mm), and then placed in the cavity. The ends of the capillary were inserted in Tygon tubing, which fixed the threads so that the fiber bundle was held isometrically during EPR experiments. Spectra of labeled fibers parallel to the magnetic field were acquired with the following instrumental settings: magnetic field modulation frequency, 100 kHz; magnetic field modulation amplitude (peak-to-peak), 2.0 G; microwave power, 20 mW (corresponding to a microwave field intensity, H_1 = 0.144 G); filter time constant, 163 ms; scan time, 163 s; microwave frequency, 9.82 GHz; center field, 3477 G. A field-frequency lock was used to ensure that spectra were accurately aligned for reliable comparison and subtraction.

EPR spectra of labeled fibers perpendicular to the magnetic field were acquired as described by Barnett and Thomas (1989). The tied fiber bundle in the glass capillary was held isometrically by Tygon tubing, then inserted in the center of a quartz tube (length, 15 cm; OD, 5 mm; ID, 3 mm), which was inserted into the cavity, so that the fiber bundle was in the center of the cavity, perpendicular to the magnetic field. The EPR instrumental settings were the same as for fibers parallel to the field, except that the center field was 3466 G and the microwave frequency was 9.78 GHz.

Buffer solutions were flowed through the fibers with a peristaltic pump (LKB Microperpex, LKB-Produkter AB, Bromma, Sweden) at a flow rate of 1.0 ml/min. The labeled fibers were pretreated with buffers in the following sequence at 25 ± 2°C before EPR experiments: 1) RS (pH 7.0) for 5 min to remove storage solution (RS/glycerol); 2) RS (pH 7.0) plus 5 mM MgADP for 1 h; 3) RS plus 15 mM EDTA (pH 7.0) for 15 min to remove MgADP; and 4) RS (pH 7.0) for 10 min. The purpose of washing with MgADP was to improve the specificity of the bound spin labels (discussed in Results), but to ensure proper controls, these washing steps 1–4 were carried out for all samples before ATPase assays.

For InVSL-S1 decorated fibers, labeled S1 (25 mg/ml) in RS (pH 7.0) plus 5 mM MgADP was cycled for 1 h through an unlabeled muscle fiber bundle, which was held in a glass capillary in the cavity as described above for either the parallel or perpendicular orientation of the fiber bundle. The fiber bundle was washed for 30 min with RS (pH 7.0) to remove unbound InVSL-S1, then washed in RS (pH 7.0) plus 15 mM EDTA to remove MgADP, and then in RS (pH 7.0). EPR spectra of myofibrils were acquired as described for fibers, except that the suspension of myofibrils was contained in a sealed capillary.

EPR spectral analysis

The EPR spectra were normalized and their parameters were measured using a program written by Robert L. H. Bennett. Each EPR spectrum was baseline-corrected by subtracting the EPR spectrum of an unlabeled sample, acquired under the same conditions as the labeled sample. The double integral of a baseline-corrected EPR spectrum 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 solution of 0.1 mM spin label in RS (pH 7.0). For spin labels

mobile enough to produce narrow three-line spectra, the concentration was determined more precisely by comparing the product of the amplitude and the square of the peak-to-peak line width of the low-field peak with that of 0.1 mM InVSL. Before further analysis or plotting, each spectrum was normalized to unit concentration by dividing by its double integral value, and the baseline in spectral plots is 100 G wide.

Spectral parameters—line position, line height, and splitting between lines (Fig. 1 *F*)—were measured by fitting spectral features to polynomial functions. For example, the key spectral parameter that characterizes the conventional EPR spectrum of randomly oriented spin labels is the splitting, in gauss (G), between the outer extrema, $2T_{\parallel}'$ (Fig. 1 *E*). An increase in the rate or the amplitude of submicrosecond motion results in a decrease in $2T_{\parallel}'$ (Fig. 1 *E*). In the rigid limit, $2T_{\parallel}' = 2T_{\parallel}$.

Correction for nonspecific labeling

As discussed above, to obtain a spectrum of labels specifically bound to SH1, it was necessary to subtract a spectrum of SH2-bound labels, which corresponded to a highly disordered distribution. Then $(A - xB)/(1 - x) = C$, where A = the original spectrum, B = spectrum of SH2-bound labels, x = fraction of B subtracted, and C = corrected spectrum. The fraction x was determined by well-defined spectroscopic endpoints. Different endpoints were used for spectra acquired from fibers parallel and perpendicular to the magnetic field. For the parallel case, the splitting of the oriented population is so wide that the spectrum almost completely overlaps the spectrum of the disordered SH2-bound spin labels, but the ^{13}C satellite lines of the oriented component are well resolved in the low- and high-field peaks. As spectrum B was subtracted, the satellite lines became well resolved, and the endpoint was reached when the satellite lines of the low- and mid-field peaks of the oriented spin labels were equal in intensity. For the perpendicular case, $2T'$ of the oriented component is narrow so that the low- and high-field peaks of the disordered component are well resolved. Therefore, the endpoint was reached when the low-field peak of the disordered component was reduced to baseline values.

Orientational distributions

EPR spectra of strongly immobilized spin labels were analyzed to determine the spin label orientational distribution relative to the fiber axis, $\rho(\theta, \phi)$ (Fig. 1 *B–D*), by comparing these spectra with simulated spectra, as described by Fajer et al. (1990a). First the orientation-independent parameters were determined by fitting a spectrum of randomly oriented myofibrils. These parameters are components of the g and T tensors, and the Gaussian and Lorentzian line width parameters, Γ_G and Γ_L. Then the spectrum of oriented fibers was analyzed to determine $\rho(\theta, \phi)$. Since the spectra of highly oriented spin labels in this study were found to have very wide splittings and narrow lines, implying that most of the probes had their principal axes approximately parallel to the fiber axis, the spectra were quite insensitive to the angle ϕ. Therefore, a simplified angular distribution $\rho(\theta)$ was used to analyze the spectra, with ϕ_0 and $\Delta\phi$ fixed at 0 to emulate an axially symmetrical system, without significant loss of accuracy. The distribution $\rho(\theta)$ was assumed to be a sum of one or more Gaussian distributions, each centered at θ_0 with a full width $\Delta\theta$ (Fig. 1, *C* and *D*).

These orientational distribution parameters were determined by comparing experimental spectra, corresponding to known θ_0 and $\Delta\theta$, using two complementary approaches, the least squares fitting, and the parametric analysis. In the first approach, the entire spectrum was compared with simulated spectra, and the orientational parameters were varied to minimize χ^2, as described by Fajer et al. (1990a). This method has the advantage that it takes into account the maximum amount of information, but precisely for that reason, it can be unreliable if any part of the spectrum is flawed, for example due to imperfect spectral correction. Therefore, in a complementary approach (described by Barnett et al., 1986), a series of spectra were simulated corresponding to different values of θ_0 and $\Delta\theta$, and selected spectral parameters were plotted against the orientational parameters, thus constructing calibration plots. Then these same spectral parameters were measured for experimental spectra, and the calibration plots were used to determine the orientational parameters. Since only θ was varied, we used those parameters previously shown to be most sensitive to θ_0 and $\Delta\theta$ (Barnett et al., 1986): $2T'$, the splitting in gauss (G) between the zero-crossing points of the low- and high-field lines, and Lpp/Cpp (or Hpp/Cpp), the ratio of the low-field (or high-field) peak-to-peak amplitude relative to the center-field line (Fig. 1 *F*). The $2T'$ value is sensitive almost exclusively to changes in θ_0 (Barnett et al., 1986; Fajer et al., 1990a). Once θ_0 has been determined in this way, the peak ratios Lpp/Cpp or Hpp/Cpp can be used to determine $\Delta\theta$ (Barnett et al., 1986).

RESULTS

Spin labeling specificity

We have developed a procedure for obtaining EPR spectra of an oriented population of InVSL on Cys 707 (SH1) of myosin heads in muscle fibers. This involved three stages: 1) preblocking SH2 sites with DTNB and then labeling the fibers with InVSL, 2) chemical treatment after labeling, and 3) spectral correction to subtract the spectrum of SH2-labeled fibers.

Fibers labeled without DTNB preblocking

The fibers were first labeled with 25 μM InVSL in 5 mM $MgCl_2$ plus 10 mM PP_i in RS (pH 6.5) using the labeling procedure described by Thomas and Cooke (1980) for a maleimide spin label. The EPR spectra showed mostly disordered spin labels with a wide splitting and some weakly immobilized spin labels (Fig. 2, *top*). This spectral heterogeneity is greater than with spin labels previously used in muscle fibers, so we undertook several steps to reduce this heterogeneity by chemical treatment and spectral correction.

Preblocking with DTNB

The labeling specificity was improved by reversible SH-preblocking with DTNB. Since previous success in specific labeling of myosin heads in muscle fibers was obtained by labeling SH1 (Cys 707) (Thomas et al., 1980; Thomas and Cooke, 1980), our strategy was to improve the specificity of labeling SH1 by preblocking SH2 with DTNB. The DTNB concentration and preblocking time were varied before labeling with InVSL to maximize the oriented population of spin labels in the EPR spectrum. The EPR spectrum of InVSL-labeled fibers parallel to the field (Fig. 2, *center*) showed clear evidence for three spin label populations: 1) oriented with a large $2T'$ splitting, implying that the principal axis of the spin label is approximately parallel to the fiber axis; 2) highly disordered; and 3) oriented with a small $2T'$ splitting (* in Fig. 2), implying that the spin label's principal axis is approximately perpendicular to the fiber axis for this population.

Chemical treatment after labeling

In previous experiments with MSL and IASL, the EPR spectrum of the disordered spin labels was decreased selectively by washing the fibers with potassium ferricyanide (Thomas and Cooke, 1980). However, washing InVSL-labeled fibers

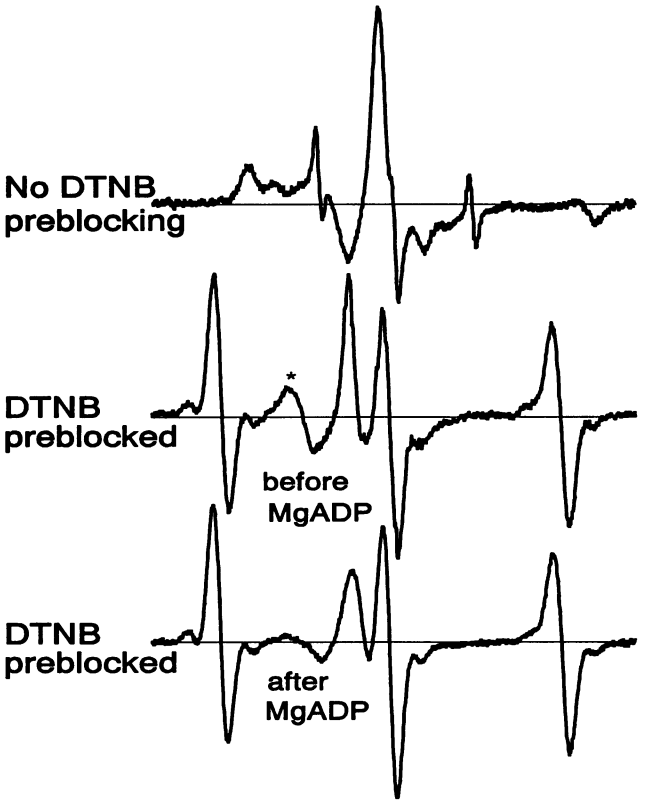

FIGURE 2 Conventional EPR spectra showing the need for DTNB-preblocking before labeling and the removal of non-covalently bound spin labels in labeled fibers. The fibers were aligned parallel to the magnetic field and washed in RS (pH 7.0). (*Top*) Fibers labeled with InVSL without DTNB preblocking. (*Center*) Fibers labeled with InVSL after DTNB preblocking. (*Bottom*) Same sample (*center*), after treatment with RS (pH 7.0) plus 5 mM MgADP for 1 h at room temperature, to remove spin labels contributing to the spectral component marked *. In this and subsequent figures, each spectrum has been normalized to unit concentration by dividing by its double integral, and the baseline is 100 G wide.

with potassium ferricyanide decreased the signals from disordered and oriented spin labels at similar rates, so ferricyanide cannot be used to reduce the fraction of disordered spin labels.

In the absence of flowing buffer, the EPR spectrum of the labeled fibers showed the appearance of a narrow signal characteristic of rapidly tumbling isotropic spin label. When the buffer flow was resumed, this component disappeared, indicating that it corresponded to free spin label. This probably corresponds to the release of spin label due to a retro-Michael reaction, as observed previously for myosin (Roopnarine et al., 1993b). The source of this free label appears to be the oriented component with a small 2T′ splitting (* in Fig. 2), since its intensity decreased as the free spin label was removed. The rate of removal of this component was negligible at 4°C, significant at 25°C, and was fourfold greater in RS (pH 7.0) plus 5 mM MgADP than in RS (pH 7.0). Under the latter conditions (at 25°C), this narrow component was completely removed within 1 h, as indicated by the decrease in intensity to baseline values (Fig. 2, *bottom*). Therefore, in all subsequent EPR experiments, the labeled fiber bundles were prewashed at room temperature with RS (pH 7.0) plus 5 mM MgADP for 1 h or until the removal of this component was complete (Fig. 2, *bottom*), then in RS (pH 7) plus 15 mM EDTA for 15 min to aid in removing bound MgADP. SDS-polyacrylamide gel (12% acrylamide in resolving and 4% acrylamide in stacking gel) electrophoresis of the EDTA-treated fibers showed that there is no loss of light chain 2 or troponin C (data not shown). The EPR spectrum of the MgADP-washed labeled fibers in rigor solution shows two populations of spin labels, disordered and oriented (with a wide splitting). It was observed that after the removal of the oriented component with a small splitting, there was a slow loss in signal intensity of the other two components, suggesting that these spin label populations may also undergo a retro-Michael reaction (Ingold, 1969).

As described previously for myosin (Roopnarine et al., 1993b), ethanol extraction was used to show that after the MgADP wash, >90% of the spin label was covalently bound to the labeled fibers. Selectively washing out 85% of the myosin (quantitated on a 12.5% SDS polyacrylamide gel with a Hoefer densitometer) with Hasselbach-Schneider solution (Yanagida and Oosawa, 1980) removed the oriented and most of the disordered spin labels from the labeled fibers, indicating that most of the label is attached to myosin.

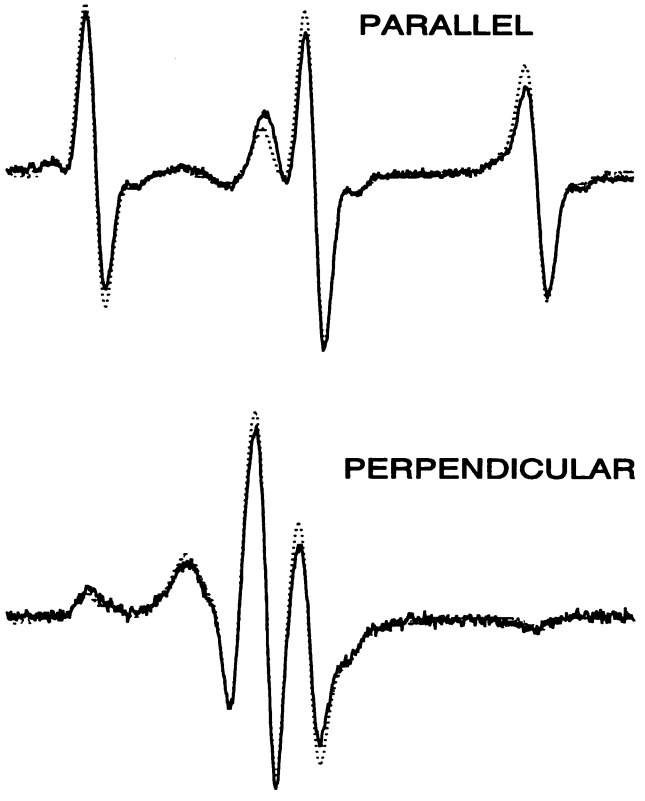

FIGURE 3 EPR spectra of InVSL-labeled (——) and InVSL-S1-decorated (·····) fibers in RS (pH 7.0). (*Top*) Fibers parallel to magnetic field. (*Bottom*) Fibers perpendicular to the magnetic field.

EPR spectra of unlabeled fibers decorated with InVSL-S1

The EPR spectrum of unlabeled fibers decorated with InVSL-S1 is very similar to that of labeled fibers (Fig. 3). This is strong evidence that virtually all of the spin labels in the InVSL-labeled fibers are on the myosin head, and that both the oriented and disordered components correspond to labels on the myosin head. Therefore the orientation of the spin label on the head is determined by the actomyosin bond.

Characterization of SH1/SH2-labeled fibers

Prewashing in MgADP and EDTA (used to accelerate the removal the second oriented component, as described above) did not affect the high-salt ATPase activities (data not shown). Preblocking the fibers with DTNB inhibited the K/EDTA-ATPase activity by 41 ± 4% but did not elevate the Ca/K-ATPase activity significantly (Table 1, DTNB), suggesting that much of the DTNB labeled SH2. Subsequent reaction with InVSL inhibited the K/EDTA-ATPase activity by an additional 33 ± 4% with no significant change in the Ca/K-ATPase activity (Table 1, DTNB/InVSL). After the removal of the TNB groups with DTT (Table 1, DTNB/InVSL/DTT), the K/EDTA-ATPase activity was elevated slightly, but the Ca/K-ATPase activity did not increase significantly, suggesting that much of the InVSL labeled SH2. The f_{SH} due to InVSL labeling was 0.63 ± 0.05 (Table 1, DTNB/InVSL/DTT), indicating that 63% of the heads have InVSL bound to SH1 and/or SH2 in labeled fibers. Control experiments showed that washing the fibers with DTT removed the TNB groups, as indicated by the increase in K/EDTA-ATPase activity of the DTNB-treated fibers to normal values (Table 1, DTNB/DTT). The unlabeled fibers were also treated with DTT, with little or no effect on the K/EDTA-ATPase activity (Table 1, unlabeled/DTT), indicating that virtually all the sulfhydryls that affect the ATPase activity of the unlabeled fibers were fully reduced before labeling. These results indicate that the effects on the ATPase activities in labeled fibers (Table 1, DTNB/InVSL/DTT) are due solely to InVSL modification.

Double integration of EPR spectra of myofibrils (made from DTNB/InVSL/DTT fibers) indicated that there was 0.57 ± 0.03 mole of spin label per mole of myosin head (f_{SL}). The specificity of labeling is defined by the fraction of spin labels that are either on SH1 or SH2, f_{SH}/f_{SL} = 0.63/0.57 = 1.11 ± 0.13 (Table 1). This correlation between the number of spins and the decrease in K/EDTA activity clearly indicates that virtually all the spin labels were attached to myosin heads at either SH1 or SH2, but not both, as observed previously for InVSL labeling of myosin (Roopnarine et al., 1993b). The actual fraction of spin labels on either SH1 or SH2 cannot be determined directly from these ATPase values, but is determined from EPR spectra as discussed under EPR spectra of SH1-labeled fibers. The assignment of EPR spectral components (either oriented or disordered) specifically to SH1 or SH2 is discussed below under *Characterization of SH2-labeled fibers* and *EPR spectra of SH2-labeled fibers*.

The active tension of unlabeled fibers varied between 1.5 and 2 kg/cm^2, and the resting tension was always less than 10 ± 2% of the active tension. The active and resting tensions of the SH1/SH2-labeled fibers at 25°C were 88 ± 5% and 75 ± 4% of the values for unlabeled fibers, respectively. Thus labeling 63% ± 3% of the heads at SH1/SH2 decreases the active tension by only 12 ± 5%. If the tension varies linearly with the fraction of modified heads, the force produced by a labeled head is 81 ± 8% of the force produced by an unlabeled head. This is consistent with previous reports on the effect of labeling on these mechanical measurements, in which it was determined that labeling up to 50% of the SH1 sites did not affect mechanical properties of the labeled fibers (Crowder and Cooke, 1984; Fajer et al., 1988; Matta et al., 1992).

Characterization of SH2-labeled fibers

Analysis of the oriented population of spin labels of the labeled fibers was facilitated by removing the signal due to the disordered population. It was not possible to remove this signal by chemical methods. Therefore, it was removed by computer subtraction of an EPR spectrum due only to SH2-bound spin labels, which was obtained by spin labeling after preblocking SH1, as described in Materials and Methods.

TABLE 1 High-salt ATPase activities of labeled fibers

Sample (myofibrils)	K/EDTA	Ca/K	f_{SH}
Unlabeled	1.0 ± 0.04	1.0 ± 0.05	
Unlabeled/DTT	0.97 ± 0.04	1.1 ± 0.01	
DTNB	0.59 ± 0.04	1.68 ± 0.04	0.41 ± 0.04
DTNB/DTT	1.07 ± 0.07	0.93 ± 0.07	0.07 ± 0.01
DTNB/InVSL	0.26 ± 0.05	0.92 ± 0.07	0.77 ± 0.06
DTNB/InVSL/DTT	0.40 ± 0.03	0.79 ± 0.08	0.63 ± 0.03

ATPase activities are reported as μmol P_i/min/mg. Each sample is named according to its chemical modification. The ATPase values are the average of three labeling preparations. The ATPases were normalized to 1 by dividing by the ATPase values of the unlabeled samples, which were 1.04 ± 0.02 (K/EDTA) and 0.07 ± 0.01 (Ca/K) μmoles P_i/mg/min, respectively. The fraction of heads with modified SH1 and/or SH2 (f_{SH}) was determined from Eq. 1, using a value of 0.04 μmol P_i/mg/min for K_∞, as determined for FDNB-labeled fibers in Table 2. The EPR spectra for fibers labeled with DTNB/InVSL/DTT are shown in Fig. 4, *top row*.

TABLE 2 High-salt ATPase activities of SH2-labeled fibers

Sample	K/EDTA	Ca/K	f_{SH2}*
Unlabeled (UL)	1.0 ± 0.01	1.0 ± 0.01	
FDNB	0.04 ± 0.02	14.33 ± 0.01	1.0 ± 0.02
FDNB/DTT	1.02 ± 0.03	1.67 ± 0.04	
FDNB/InVSL	0.03 ± 0.02	8.5 ± 0.04	0.41 ± 0.03
FDNB/InVSL/DTT	0.70 ± 0.04	1.5 ± 0.03	

ATPase activities are reported as μmol P_i/min/mg. The samples are named according to its chemical modification. The ATPase values are the average of three labeled preparations. The ATPase values were normalized to 1 by dividing by the value for the unlabeled samples. Typical values for K/EDTA- and Ca/K-ATPase activities of unlabeled fibers are given in the legend to Table 1. The EPR spectra of fibers labeled with FDNB/InVSL/DTT are shown in Fig. 4, *center row*.

* The fraction of heads with modified SH2 only (f_{SH2}) was determined from Ca/K-ATPase activity using Eq. 2, using a value of 0.07 ± 0.01 μmol P_i/mg/min for C_∞, obtained from unlabeled fibers.

Preblocking SH1 with FDNB inhibited the K/EDTA-ATPase by 96 ± 2% and activated the Ca/K-ATPase by a factor of 14.33 ± 0.01, indicating that FDNB specifically labels SH1 (Table 2, FDNB) under these conditions, as previously shown for myosin in solution (Bailin and Bárány, 1972; Reisler et al., 1974). Subsequent labeling with InVSL decreased the FDNB-activated Ca/K-ATPase activity (C_{act} in Eq. 2) by 60 ± 3% (Table 2, FDNB/InVSL), but had no additional effect on the K/EDTA-ATPase activity, indicating that InVSL labeled SH2 as previously shown for myosin in solution (Yamaguchi and Sekine, 1966). The fraction of SH2-labeled myosin heads determined from Eq. 2 was f_{SH2} = 0.41 ± 0.03. After treating these fibers with DTT to remove the DNP from SH1, the K/EDTA-ATPase (Table 2, FDNB/InVSL/DTT) increased to 70 ± 4% of the activity of unlabeled fibers, while the Ca/K-ATPase activity decreased to almost normal values, consistent with SH2 labeling of myosin heads (Yamaguchi and Sekine, 1966; Reisler et al., 1974). Double integration of the EPR spectrum showed that the number of spin labels per myosin head for FDNB/InVSL fibers was f_{SL} = 0.43 ± 0.1, so the specificity of SH2 labeling, f_{SH2}/f_{SL} = 0.41/0.43 = 0.95 ± 0.2, indicating that most of the spin labels were on SH2. As discussed below, the EPR spectrum of SH2-labeled fibers consists entirely of disordered spin labels.

EPR spectra of labeled fibers in rigor

The EPR spectrum of InVSL-labeled fibers in rigor shows both an oriented and a disordered population of spin labels (Fig. 4, *top row*). The high- and low-field regions of the spectrum of disordered spin labels overlaps the spectrum of the oriented spin labels for the spectrum acquired parallel to the magnetic field, resulting in a decrease in the apparent hyperfine splitting component. The wide splitting, 2T′, of the oriented spin labels indicates that the principal axes of the spin labels are approximately parallel to the fiber axis.

The EPR spectrum of fibers perpendicular to the magnetic field has a much narrower splitting for the oriented population of spin labels than the spectrum of fibers aligned parallel to the magnetic field (see Table 3 and Fig. 4, *top row, right column*). This large difference indicates that the oriented population has a narrow angular distribution. The small 2T′ of the oriented population indicates that the principal axis of the spin label is approximately perpendicular to the magnetic field, confirming that it is approximately parallel to the fiber axis. The low-field peak due to the disordered spin labels is well resolved from that of the oriented spin labels in the spectrum of the fibers perpendicular to the magnetic field (Fig. 4, *top row, right column*).

EPR spectra of SH2-labeled fibers

The EPR spectra of the SH2-labeled fibers show highly disordered spin labels in rigor (Fig. 4, *middle row*). The splitting between the outer extrema is 70.04 ± 0.03 G and is 70.3 ± 0.03 G for parallel and perpendicular fiber orientations, respectively. The similarities between these splitting values and the spectra indicates that the spin labels are almost completely disordered. These spectra are unaffected by 5 mM MgATP or 5 mM MgADP.

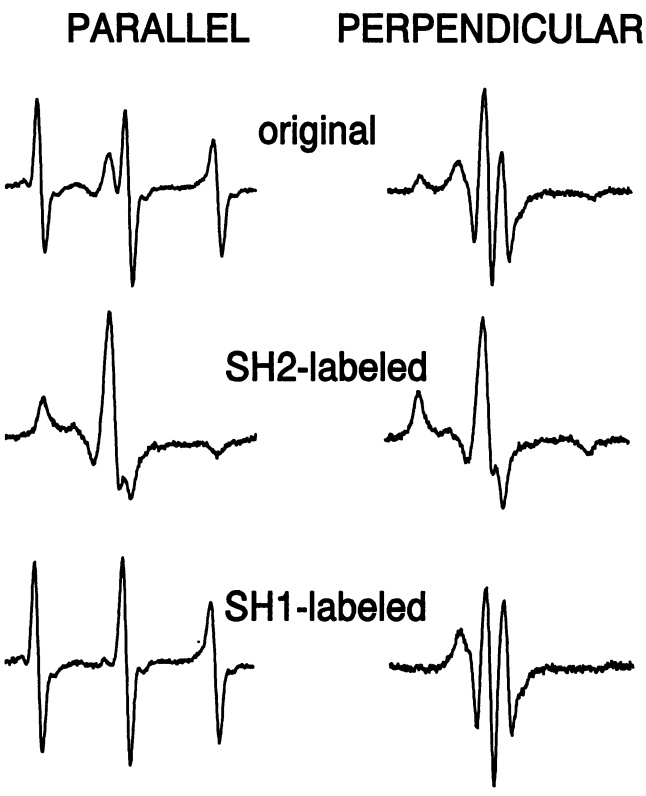

FIGURE 4 Conventional EPR spectra of InVSL-labeled fibers parallel (*left column*) and perpendicular (*right column*) to magnetic field, in RS (pH 7.0). (*Top row*) Fibers labeled by the standard procedure (resulting in some heads labeled at SH1, others at SH2). (*Center row*) SH2-labeled fibers. (*Bottom row*) Corrected spectra of SH1-labeled fibers, obtained by digital subtraction of 60 ± 1% of the SH2 spectrum (*center row*) from the corresponding spectrum (*top row*).

TABLE 3 Spectral parameters of labeled fibers parallel and perpendicular to the magnetic field

Sample	2T′ (G) (parallel)	2T′ (G) (perpendicular)
Labeled fibers	70.66 ± 0.04	15.07 ± 0.03
Corrected labeled fibers	70.89 ± 0.05	15.09 ± 0.03
InVSL-S1-decorated fibers	70.37 ± 0.02	14.72 ± 0.03

Spectral parameters of labeled fibers parallel and perpendicular to the magnetic field. 2T′ is the splitting between the zero-crossing points of the low- and high-field lines of the oriented population of spin labels of labeled fibers, defined in Fig. 1 *F*. The corrected EPR spectrum of labeled fibers (Fig. 4, *bottom*) corresponds to the spectrum of labels bound to SH1.

EPR spectra of SH1-labeled fibers

The spectrum of SH2-labeled fibers (Fig. 4, *center*) was subtracted from the spectrum of SH1/SH2-labeled fibers (Fig. 4, *top*) to obtain a spectrum of InVSL specifically bound to SH1 (Fig. 4, *bottom*). These subtractions for both parallel and perpendicular fibers were in good agreement, despite different

endpoint criteria, indicating that approximately 60 ± 1% of the spin labels are disordered and bound to SH2 while the remaining 40 ± 1% are oriented and bound to SH1. For fibers oriented parallel to the field, this correction increased 2T′ by approximately 0.23 G (Table 3, *row 3*). The large splitting (2T′) when the fibers are parallel to the field (Fig. 4, *bottom left*) and the small splitting when they are perpendicular (Fig.4, *bottom right*) indicates that the principal axis of the SH1-bound spin label is approximately parallel to the fiber axis. The ratio of the high- and center-field peaks, Hpp/Cpp, of the corrected spectrum of the labeled fibers is 0.72 ± 0.02 (Fig. 4, *left column, bottom row*), indicating that SH1-bound spin labels have a narrow orientational distribution (Barnett et al., 1986).

EPR spectra of myofibrils

The EPR spectra of myofibrils prepared from labeled fibers were independent of sample orientation and were typical of completely disordered but rigidly immobilized spin labels (Fig. 5). The splitting $2T_{\parallel}$ between the outer extrema of the EPR spectra parallel and perpendicular to the field was 72.07 ± 0.01 G and 72.07 ± 0.02 G. This agreement indicates that the labeled myofibrils are completely randomly oriented, and the large splitting strongly suggests that the spin labels are rigidly immobilized on the myosin heads. This is further supported by saturation transfer EPR spectra, showing that these labels are immobile on the microsecond time scale (Roopnarine et al., 1993b). The myofibril spectrum was corrected using a spectrum of SH2-labeled myofibrils, as described above for labeled fibers. The $2T_{\parallel}$ of the corrected spectrum increased to 73.1 ± 0.02 G. Therefore, this spectrum was used to determine the orientation-independent tensors and line width parameters (Table 4) of SH1-bound InVSL, as described by Fajer et al. (1990a). The simulated spectrum for labeled myofibrils is an excellent fit to the experimental spectrum, as shown in the residual spectrum (Fig. 6).

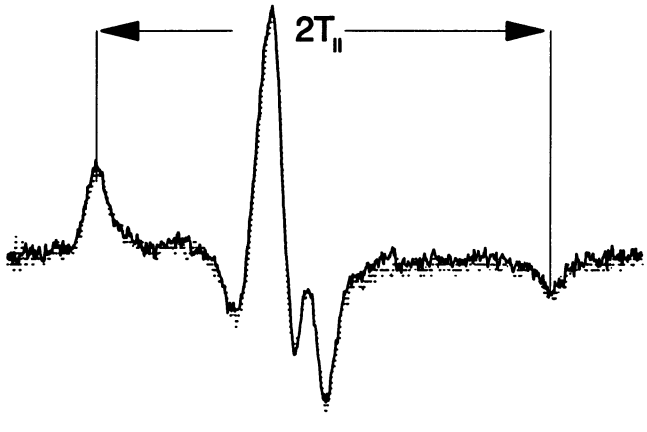

FIGURE 5 Conventional EPR spectra of myofibrils in RS (pH 7.0). (——) Sample cell parallel to the magnetic field. (·····) Sample cell perpendicular to magnetic field.

TABLE 4 Magnetic parameters obtained by fitting simulated powder spectra to the EPR spectrum of InVSL-labeled myofibrils

Parameter	Calculated value	SEM
g_x	2.00819	0.000035
g_y	2.00687	0.000028
g_z	2.00309	0.0000084
T_x	7.17	0.0463
T_y	6.05	0.1275
T_z	36.55	0.0438
Γ_G	1.6	0.206
Γ_L	2.29	0.0304

The parameters are the average of five best fits from nine independent searches. Γ_G and Γ_L are the orientationally independent Gaussian and Lorentzian line width values (full width at half-maximum), respectively. The T values and line widths are in gauss and the g values are unitless.

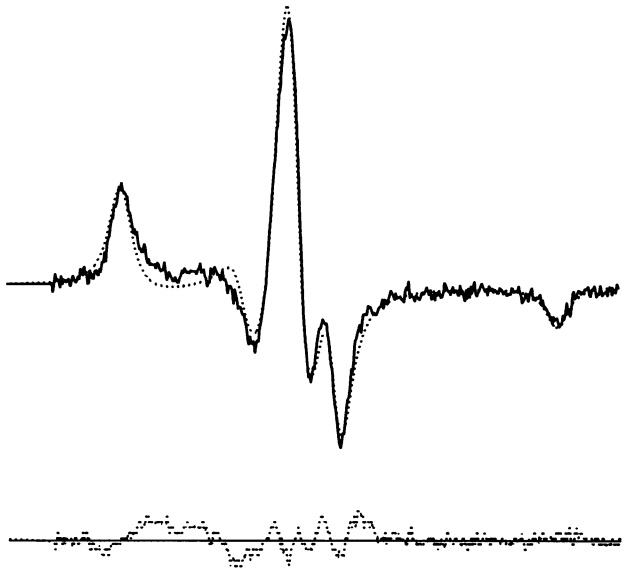

FIGURE 6 Comparison of experimental (——) and best-fit simulated (·····) spectra of labeled myofibrils. The spectrum was simulated using the parameters in Table 4, which were the result of the least-squares fitting procedure. The residual (·····) is shown at the bottom.

Analysis of orientational distribution of SH1-bound spin labels

The parameter values in Table 4 were used to simulate EPR spectra of oriented spin labels in fibers aligned parallel to the magnetic field, and Fig. 7 shows the sensitivities of the spectral parameters 2T′ and Hpp/Cpp to changes in θ_0 and $\Delta\theta$ (Fig. 7). These plots were used to determine the angular parameters of the SH1-bound spin label population from the corrected spectrum (Fig. 4, *bottom left*). The 2T′ and Hpp/Cpp of the oriented population of spin labels from the labeled fibers parallel to the magnetic field are 70.89 ± 0.1 G and 0.72 ± 0.05 G, respectively. A first estimate of the range for θ_0 was determined from the value of 2T′ (Fig. 7 *A*, heavy dotted line at 2T′ = 70.89 G), which was used to determine the range for $\Delta\theta$ (Fig. 7 *B*, heavy dotted line at H_{pp}/C_{pp} = 0.72). The range for $\Delta\theta$ was used to further constrain the range for θ_0 in the final determination. Values were

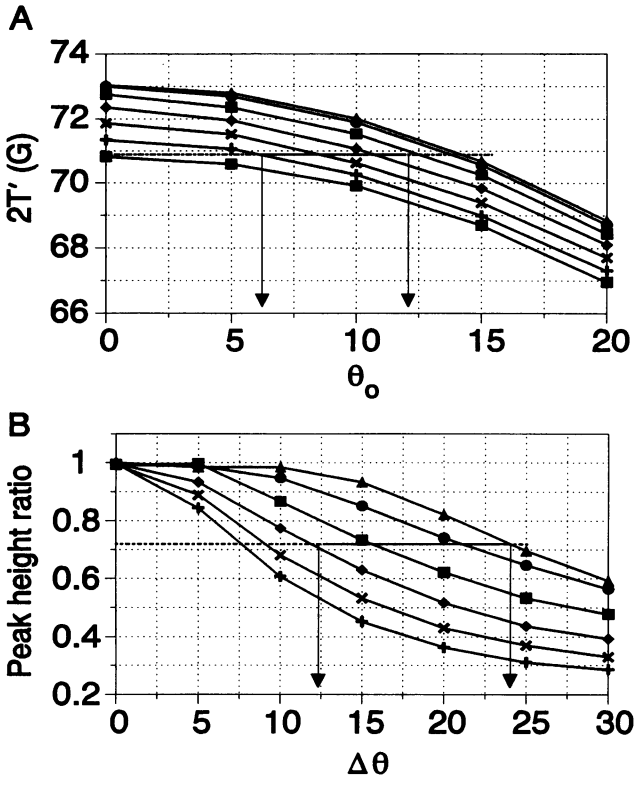

FIGURE 7 EPR spectral parameter plots used to determine the spin label orientational distribution. Spectra of known θ_0 and $\Delta\theta$, for fibers parallel to the field, were simulated using parameters in Table 4. (*A*) Dependence of the spectral splitting of simulated spectra on the 2T′ vs. θ_0. Angles of $\Delta\theta$: 0° (▲), 5° (●), 10° (■), 15° (♦), 20° (×), 25° (+), 30° (■). (*B*) Dependence of the ratio of the peak-to-peak heights of the high and low lines, Hpp/Cpp, of simulated spectra on the orientational distribution of the spin labels, $\Delta\theta$. Angles of θ_0: 0° (▲), 5° (●), 10° (■), 15° (♦), 20° (×), 25° (+). The heavy horizontal lines are the observed spectral values, which were used to obtain initial estimates for the possible angle values (vertical arrows).

considered acceptable only if the simulated spectra had values of the main hyperfine splitting (2T′, which is sensitive primarily to θ_0) and the ratio of the high-field peak to the center-field peak (Hpp/Cpp, which is sensitive mainly to $\Delta\theta$) that were within experimental error of the observed values. The acceptable fits were within the range θ_0 = 10–12° and $\Delta\theta$ = 13–17°. Fig. 8 shows that the simulated spectrum (θ_0 = 11°, $\Delta\theta$ = 15°) is a good fit to the experimental spectrum.

Essentially equivalent results were obtained by fitting the entire EPR spectrum, using the procedure of Fajer et al. (1990a). However, that procedure is not necessary in this case, since the orientation of InVSL near θ_0 means that only two parameters must be varied, making the graphical procedure feasible. In addition, the fitting procedure was less reliable, since the spectral corrections introduced some spectral artifacts that misled the fitting program. Most important of these is the presence of the ^{13}C satellite lines, which are not included in the simulations and therefore dominate the difference between the experimental and simulated spectra (Fig. 8). This problem could be minimized by either including the ^{13}C satellite lines in the simulation or excluding these spectral regions from the fits.

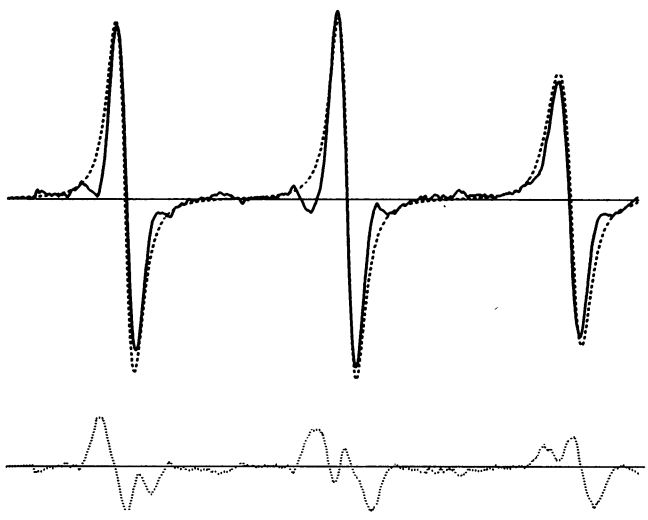

FIGURE 8 Comparison of experimental (——) and best-fit simulated (·····) spectra for spin labels on SH1, for fibers parallel to the field (Fig. 4, bottom left). The spectrum was simulated using the parameters in Table 4 and for values of θ_0 = 11° and $\Delta\theta$ = 15°. The residual (·····) is shown at the bottom.

DISCUSSION

This study describes a new spin label for studying the rotational dynamics and orientational changes of myosin heads in muscle fibers. The use of an extrinsic probe on a protein to measure the global rotational dynamics and orientation of the protein requires that the following criteria be met: the probe should be rigidly immobilized on the protein; the labeling should be orientation-specific (probably requiring site-specificity); and the probe should not affect the function of the protein. When a spin label is to be used for monitoring the global orientation and motion of the protein, the label should not report local structural changes that may be induced by substrate binding. We have shown that InVSL is rigidly immobilized on myosin both in the presence and absence of ATP (Roopnarine et al., 1993b).

Development of labeling procedure

We were successful in developing a labeling procedure for obtaining oriented spin labels on myosin heads of muscle fibers. It was necessary to preblock the fibers with DTNB to decrease heterogeneity of labeling (Fig. 2, *center*). The EPR spectra of labeled fibers, parallel to the magnetic field, show three different populations of spin labels: oriented with a wide splitting; disordered (wide angular distribution); and oriented with a small splitting. The latter population of spin labels was irreversibly removed as free spin label after prolonged incubation at room temperature. The rate of release was increased fourfold in the presence of MgADP (Fig. 2, *bottom*), possibly as a result of a structural change due to

MgADP binding to the myosin head. It is possible that some of the spin labels bind at or near the ATP binding site and are released by binding of MgADP.

Location of InVSL on labeled fibers

Ethanol extraction showed that >90% of the spin label is covalently bound in labeled myosin or myofibrils (made from labeled fibers). The EPR spectra of MgADP-washed InVSL-labeled fibers show two populations of spin labels, oriented with a narrow angular distribution and disordered with a wide angular distribution (Fig. 2, *bottom*). The EPR spectra of MgADP-washed S1-decorated fibers also show similar spin label populations, indicating that virtually all of the spin labels are on the myosin heads of the labeled fibers (Fig. 4). This conclusion is supported by the large decrease in the EPR signal upon washing out 85% of the myosin in labeled fibers with Hasselbach- Schneider solution. Having established that the spin labels are on myosin heads, we used high-salt ATPase assays to further determine the location of the spin labels. High-salt ATPase activity is only indirectly related to the labeling of SH1 and SH2 on myosin heads. A direct analysis would require the isolation, sequencing, and quantitation of peptides. This would be very difficult and would probably not result in the needed precision. Therefore, our analysis is based on previous studies of purified myosin, in which the protein chemistry and ATPase activities have been shown to correlate remarkably well. Unequivocal specific labeling of SH1 is characterized by nearly complete inhibition of K/EDTA-ATPase activity and strong activation of Ca/K-ATPase activity (Kielley and Bradley, 1956; Sekine et al., 1962; Sekine and Kielley, 1964). Subsequent labeling of SH2 decreases the elevated Ca/K-ATPase (Yamaguchi and Sekine, 1966; Seidel, 1969; Reisler et al., 1974). Myosin with only SH2 labeled has inhibited K/EDTA-ATPase activity and near or sub-normal Ca/K-ATPase activity (Yamaguchi and Sekine, 1966; Reisler et al., 1974).

The excellent correlation between the number of spins and the decrease in K/EDTA-ATPase activity clearly indicates that virtually all the spin labels were either on SH1 or SH2, but does not quantitate the fraction of spin label at either site (Table 1). The lack of elevation of the Ca/K-ATPase activity (Table 1) is consistent with at least partial labeling of SH2 (Yamaguchi and Sekine, 1966). We further assigned the spectral components to SH1 and SH2 based on the EPR results from SH2-labeled fibers. Since FDNB completely and specifically blocks the SH1 sites (Table 2), and also completely eliminates the oriented spectral component (Fig. 4, *center*), the oriented spectral component must correspond to SH1 sites and the disordered spectral component must correspond to mostly SH2 sites. However, we cannot rule out the possibility that a small fraction of the disordered spectral component comes from labels on SH1. A small fraction of the disordered spin label could be attached to actin, but it must be true either that this fraction is negligible or that the orientational distribution of this actin-bound fraction would have to be indistinguishable from that of the myosin-bound fraction. In the absence of direct chemical information about the sites of InVSL reaction, our conclusions about the sites of InVSL labeling are not strictly proven. However, the key conclusion of our study—an S1-bound spin label with a unique orientation—does not depend on the specific labeling sites.

Correction of EPR spectra

Previous EPR studies with MSL and IASL-labeled fibers showed a disordered fraction of spin label, which was selectively reduced by treatment with potassium ferricyanide (Thomas and Cooke, 1980). However, treatment of In VSL-labeled fibers with potassium ferricyanide reduced both oriented and disordered spin labels. Fluorescence studies with IAEDANS (5-[[2-[(iodoacetyl)amino]ethyl]amino] naphthalene-1-sulfonic acid) also suggested a large fraction of disordered probes in rigor, at least 50% in labeled fibers and 26% in IAEDANS-S1 decorated fibers (Wilson and Mendelson, 1983). The fraction of disordered labels on muscle fibers appears to be related to reactivity of the spin labels. The least reactive spin labels, IASL (Thomas and Cooke, 1980) and IPSL (*N*-[3-(iodoacetyl)amino-2,2,5,5-tetramethylpyrrolidine-1-oxyl[(iodoacetamido)proxyl]) (Ajtai et al., 1990), are the most specific for SH1 and have the highest fraction of oriented spin labels. The most reactive spin label, InVSL, has the lowest SH1 specificity (reacts more with SH2 than SH1) and show the largest population of disordered spin labels. The increased reactivity of InVSL is due to two strong electron withdrawing groups (Esmann et al., 1990).

We were able to obtain corrected EPR spectra of these oriented spin labels by subtracting the spectrum of SH2-labeled fibers (Fig. 4). Although we used different methods to define the subtraction endpoint for the spectra acquired parallel or perpendicular to the magnetic field, both corrections showed that 60 ± 1% of the spin labels are bound to SH2 and are disordered. The corrected spectra show that the oriented spin labels have very different splittings when the fibers are aligned differently in the magnetic field (Fig. 4, *bottom row*), indicating that the oriented spin labels have a narrow angular distribution.

Interpretation of data: spin label rotation

The main goal for these orientational studies is to test whether or not the myosin head rotates axially between the two distinct angles of 90° and 45° during the power stroke (Huxley, 1969; Huxley and Simmons, 1971). The only orientational information that can be derived directly from EPR spectra is about the orientation of the probe's axes relative to the long axis of the muscle fiber. Precise definition of the spin label's axes relative to the myosin head requires EPR spectroscopy of spin-labeled S1 crystals, which should be feasible now that the procedure for crystallizing S1 has been published (Rayment et al., 1993a).

The present study shows an orientation of spin labels on rigor myosin heads that is different from those of previously

studied spin labels, MSL, IASL (Thomas and Cooke, 1980), and IPSL (Ajtai et al., 1990). The narrow splittings observed for MSL or IASL on myosin indicate that principal axis is near 90°, and it has been shown quantitatively by Thomas and Cooke (1980) that MSL is oriented at $\theta_0 = 82°$ and IASL at $\theta_0 = 68°$. In contrast, InVSL has a very wide splitting, near the theoretical maximum, indicating that θ_0 is near 0°. This unique orientation of InVSL on the myosin head makes InVSL complementary to the other labels, because it provides a different angular perspective for monitoring the changes in head angles. In fact, the 0° orientation provides a particular advantage in removing ambiguity of interpretation of axial orientational changes, as shown in Fig. 9. If the myosin head rotates axially, the principal axis of InVSL (**z** in Fig. 1) must rotate axially by the same amount, because the near 0° value of θ_0 implies that its principal axis must lie in the plane of axial head rotation. This axial rotation would change θ_0, which would directly be seen in the EPR spectrum as a change in the splitting 2T′ (Fig. 1).

Alternatively, as in the case for MSL-labeled fibers, when the probe's principal axis is nearly perpendicular to the fiber axis (Fig. 9, *right*), the detection of the head rotation is ambiguous, because the probe's axis relative to the axis of head rotation is unknown. There are two limiting possibilities: 1) The probe axis could be approximately perpendicular to the rotation axis, in the plane defined by the rotating head and the fiber axis (shown by the arrow in Fig. 9, *right*). In this case, any axial head rotation would result in an axial rotation of the spin label, changing θ_0 by the same angle, which would change the splitting (2T′ in Fig. 1) in the EPR spectrum. 2) The probe axis could be approximately parallel to the rotation axis, perpendicular to the plane of head rotation (shown by ⊙ in Fig. 9, *right*). In this case an axial head rotation would have a negligible effect on θ_0 and 2T′. This ambiguity in the orientation of the principal axis of MSL relative to the myosin head rotation axis has led to some controversy about the amplitude of myosin head rotation in response to nucleotides (Ajtai et al., 1989; Fajer et al., 1990b), and can be avoided best by having a spin label that binds with its principal axis parallel to the fiber axis, like InVSL.

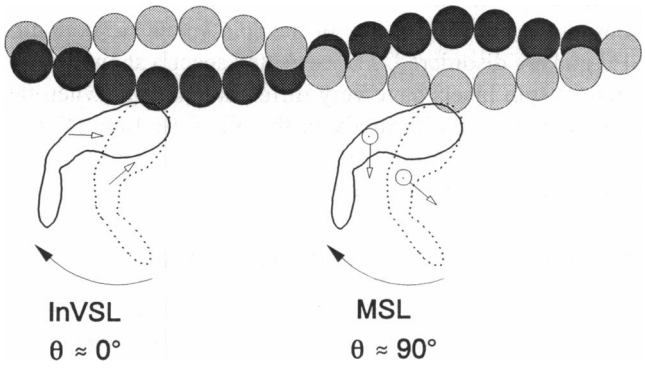

FIGURE 9 Model for the orientation of the principal axis (**z** in Fig. 1, shown here as an arrow fixed to the head) of InVSL (or any other label having **z** approximately parallel to the fiber axis) and MSL (**z** approximately perpendicular) bound to SH1 on the myosin head, illustrating the sensitivity of each label to a hypothetical 45° axial rotation of the head on actin. The solid head is the known rigor (post-power-stroke) orientation, the dotted head is a proposed pre-power-stroke orientation, and the curved arrow shows the rotation expected for a 45° rotation between these two states. As discussed in the text, this (or any other) axial head rotation would be unambiguously detected by InVSL as a change in the hyperfine splitting 2T′, while the effects on the MSL spectrum are less predictable and the interpretation in terms of head rotation would thus be more ambiguous.

Relationship to other work

The other extensively studied fiber spin labels, IASL (Thomas and Cooke, 1980) and IPSL (Ajtai et al., 1990), have θ_0 values of 68° and 45°, respectively. The spectra of these probes have sensitivity to θ_0, but the principal axes of these probes may not be in the plane of axial head rotation, so a similar ambiguity as for MSL applies. More importantly, IASL becomes mobile relative to the head in the presence of ATP, and IPSL is mobile even in the absence of ATP, so neither probe is a reliable reporter of head rotation. The different orientations of the spin labels MSL, IASL, IPSL, and InVSL on the myosin heads indicate that the nitroxide groups of these spin labels are in different environments, although the probes are probably all attached covalently to SH1. Although InVSL is uniquely capable of detecting any and all axial rotations of the *entire* head, even InVSL (on SH1) would fail to detect a rotation involving the bending of the head, with the SH1-containing domain remaining in the rigor orientation (Cooke et al., 1982; Cooke, 1986; Thomas, 1987; Rayment et al., 1993b).

CONCLUSIONS

We report the labeling of muscle fibers with a new spin label, InVSL. We have shown that the spin label is covalently bound to the myosin head in muscle fibers. InVSL does not specifically label a single sulfhydryl, but the label specifically attached to SH1 is resolved spectrally due the high orientational resolution of EPR. The labeled fiber has an oriented population of spin label on SH1 and a disordered population of spin label on SH2. We have also shown that MgADP and MgATP does not alter the EPR spectral line shape of the disordered population of spin labels in SH2-labeled fibers. Therefore, a change in the EPR spectral line shape must reflect changes in the oriented population of spin labels at the SH1 site. The spin label is oriented with its principal axis almost parallel to the fiber axis, which allows it to directly and unambiguously report axial rotations of the myosin head. This study establishes the feasibility of using InVSL to study myosin head rotation in various physiological states.

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