Determination of spin-label orientation within the myosin head

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ABSTRACT Current methods of analyzing EPR spectra of spin-labeled muscle fibers allow the determination of spin-label orientation within the fiber, rather than the orientation of the myosin head itself. In order to describe the orientational distribution of spin-labeled myosin heads within the muscle fibers, the orientation of the spin label within the myosin head must be known. The iodocacetamide label orientation in the myosin head was determined to be (16.8°, 28.3°, 4.2°) or (16.6°, 72.0°, 4.3°). These Eulerian angles were obtained from the analysis of EPR spectra of fibers decorated with labeled myosin heads in the absence of ATP, with the assumption that the head's tilt angle is 40°, as observed in a recent EM study [Pollard, T., Bhandari, D., Maupin, P., Wachtstock, D., Weeds, A. & Zot, H. (1993) Biophys. J. 64, 454-471]. Knowledge of spin-label orientation will allow for quantitative determination of myosin head orientation in the various states of the contractile cycle.

Since the initial formulation of the rotating crossbridge theory (1), much effort has been expended on finding direct evidence for reorientation of the myosin head interacting with actin during muscle contraction. Rotation of the head is postulated to result in strain between the actin and myosin filaments, which is then relieved by the sliding of the filaments past each other. Structural studies have involved electron microscopy (EM), x-ray diffraction, and a variety of spectroscopic techniques sensitive to rotational motion. Each of these approaches has different limitations due to the complexity of data interpretation and artifacts due to sample preparation. Spectroscopy, magnetic, or optical, has the major advantages of superior sensitivity to orientational changes and of site specificity—i.e., the ability to probe a single site on the protein. There is, however, a problem in translating spectroscopic signals into molecular terms. The problem can be broken into three separate topics: (i) development of simulation algorithms, (ii) optimization techniques to fit simulations to the observed spectra, and (iii) translating the orientational distributions of probes into the orientation of the molecules.

In electron paramagnetic resonance (EPR) spectroscopy, spectral simulations have been developed by a variety of approaches, including polar coordinates (2, 3), spherical harmonics (4), and Eulerian transformations (5). Simulations can be fitted to experimental spectra employing Marquardt-Levenberg least-squares routines (6) or the SIMPLEX algorithm (7). Alternatively, the orientational distribution of the spins can be determined by direct deconvolution of the spectral lineshape, rather than by fitting (8).

Little attempt has been made in the area of relating the spin distribution to the molecular orientation. The stumbling block is the prerequisite of knowing the probe orientation within the molecule. Sometimes it is possible to determine the probe orientation from a single crystal of a protein by correlating the spin axis with the crystallographic axes (9, 10). Since the myosin head is difficult to crystallize, a different approach was taken here. Instead of a single crystal, we used samples for which partial information of protein orientation was known. Numerous EM studies of actomyosin complexes in the absence of ATP show the myosin head to be inclined at 40°-50° with respect to actin filaments (11-13). This information was used to analyze the EPR spectra of equivalent samples in terms of the orientation of the spin probe within the myosin head. The analysis was accomplished by adaptation of a simulation algorithm based on Eulerian transformations with explicit distributions of (i) probe within the myosin head, (ii) head within the muscle fiber, and (iii) muscle fiber within the external magnetic field (5, 14). Spectra simulated with this algorithm were compared with the experimental spectra of spin-labeled myosin heads decorating actin filaments in a muscle fiber. Varying the fiber tilt angle and assuming the myosin head to be inclined at 40° with respect to the actin filament axis, as observed in a recent quantitative EM study (13), allowed determination of the orientation of the probe within the head. The optimization of the fits was done by means of SIMPLEX procedures developed earlier (7).

METHODS

The EPR spectra of perdeuterated N-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (IASL)-labeled muscle fibers and IASL-labeled myosin subfragment 1 (S1) infused into glycinated muscle fiber bundles were obtained in an ESP-106 spectrometer (Bruker, Billerica, MA) as described (15).

The EPR simulations were accomplished by using the solution of a Hamiltonian in the laboratory frame (16). The resonance field is given by $H_{res} = (hv/gz) + m_i(A_{zz}^z + A_{zz}^{2z} + A_{zz}^{3z})$, where the subscripts denote the $ij$th element of the $g$ and $A$ tensors in the laboratory frame of reference. The magnetic tensors were transformed sequentially from the nitroxide frame of reference to the myosin head frame, to the muscle fiber coordinates, and finally to the laboratory frame (Fig. 1). The transformations were accomplished by using the matrices $L_{NM}$, $L_{MS}$, $L_{SL}$ defined in Eq. 1 and the orientational distribution in each frame of reference, $\Omega_{NM}$, $\Omega_{MS}$, $\Omega_{SL}$. The value of a tensor $A$ after transformation is: $A' = L'AL'$, where $L'$ is a transpose of cosine matrix $L$.

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L = \begin{bmatrix}
(\cos \beta \cos \alpha \cos \gamma) & - (\sin \alpha \sin \gamma) & (\cos \beta \sin \alpha \cos \gamma) + (\cos \alpha \sin \gamma) & - \sin \beta \cos \gamma \\
(\cos \beta \cos \alpha \sin \gamma) & - (\sin \alpha \cos \gamma) & (- \cos \beta \sin \alpha \sin \gamma) + (\cos \alpha \cos \gamma) & \sin \beta \sin \gamma \\
\sin \beta \cos \alpha & \sin \beta \sin \alpha & \cos \beta
\end{bmatrix}
\]

[1]

Abbreviations: S1, subfragment 1; IASL, perdeuterated N-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide.

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Thus, the simulation of EPR lineshape reduces to the calculation of tensors in the laboratory frame of reference \((g_{ab} \text{ and } A_{LAB})\) and to evaluating the expression for the resonance field given above. Each Eulerian transformation \(\Omega\) is defined by a set of three angles \(\alpha, \beta, \gamma\), defining nine variables in total; however, some of the angles are degenerate, simplifying the number of variables. Eulerian angles represent the path taken to transform one set of \(x, y, z\) coordinates to another. The first angle \(\alpha\) represents the rotation about the \(z\) axis creating a new set of \(x'\) and \(y'\) axes, \(\beta\) is the rotation about the \(y'\) axes tilting \(z'\) to \(z''\), and \(\gamma\) is a twist about the \(z''\) axis (17). In this work the rigor head torsion is defined to be zero \((\alpha_{MS} = 0^\circ)\); i.e., the \(x\) axis of the myosin head lies in the plane of the projection while the \(y\) axis is normal to that plane. When so defined, the \(\beta_{MS}\) angle corresponds to the head tilt angle with respect to the filament axis for \(\alpha_{MS} = 0^\circ\). If \(\alpha_{MS} \neq 0^\circ\), then \(\beta_{MS}\) encompasses both slew and tilt angles of the head. A Gaussian disorder of the myosin head is allowed with a half-width at half-height \(\Delta\), about the average positions of \(\alpha_{MS}\) and \(\beta_{MS}\).

To assure adequate coverage of the head position, angles between \(\alpha_{MS} = 3\Delta_{MS}\) and \(\alpha_{MS} + 3\Delta_{MS}\) were taken into calculations at \(2^\circ\) intervals. The same sampling was used for \(\beta_{MS}\). The angles \(\gamma_{MS}\) and \(\gamma_{SL}\) reflect the helical arrangement of the heads on the surface of the thick filaments, and the rotational disorder of the thick filaments about the fiber axis, respectively, and thus are degenerate. \(\alpha_{SL}\) was varied between 0 and \(2\pi\) in \(2^\circ\) steps to reflect rotational disorder of the filaments. \(\beta_{SL}\) is defined by experimental geometry. For fibers oriented parallel to the magnetic field, \(\beta_{SL} = 0^\circ\), while for fibers oriented perpendicular to the field, \(\beta_{SL} = 90^\circ\). The angle \(\gamma_{SL}\) is ignored, since it represents reorientation about the field direction, which does not affect EPR spectra.

The values for the \(g\) and \(A\) tensors and the intrinsic linewidth were obtained from the spectra of randomly oriented myosin heads in a suspension of myofibrils by using a modified algorithm of Siderer and Luz (7, 18). The results are shown in Fig. 2. The values for magnetic tensors for IASL-labeled heads were determined to be \(g_x = 2.00872, g_y = 2.00592, g_z = 2.00237, A_x = 7.55 \text{ G}, A_y = 7.96 \text{ G}, A_z = 35.23 \text{ G}, \) homogeneous linewidth \(\Gamma_L = 0.5 \text{ G}, \) nuclear spin-independent inhomogeneous broadening \(\Gamma_G = 1.5 \text{ G}, \) and nuclear spin-dependent linewidth \(\Gamma_{M0} = 0.24 \text{ G}. \) The errors of the tensor values are less than \(\pm 0.00005\), which corresponds to an accuracy of 0.08 G. The errors of hyperfine tensor elements and on the linewidth are \(\pm 0.05 \text{ G}\) as estimated from the scatter of repeated optimizations, each starting from randomly chosen initial values.

All parameters used for simulations were optimized using the SImplex algorithm (7). The figure of merit \(\chi^2\) describing quality of fit was defined as the sum of squared differences normalized to spectral noise and number of points. For the global optimization in which two spectra were optimized simultaneously, the figure of merit was defined as the sum of \(\chi^2\) for each spectrum normalized to the minimum \(\chi^2\) for the spectrum in question. This normalization avoids undue weighting of one or the other spectrum.

**RESULTS**

Tilt Series. The spectra of fibers oriented parallel to the magnetic field (Fig. 3) show the three-line pattern characteristic of well-ordered myosin heads as first noted by Thomas and Cooke (19). These spectra can be analyzed in terms of a single Gaussian distribution of spin label with respect to the magnetic field (20). The cylindrically symmetric arrangement of the myosin heads within a muscle fiber places the \(z\) tensor axis of the probe on different heads on the surface of a cone with angle \(\theta_0\) defined by the probe \(z\) axis and the axis of muscle fiber. Since the symmetry axis of each cone is coincident with the magnetic field direction, the contribution of the tensor elements remains constant \((\cos^2\theta_0)\) for all the heads. The signals originating from the probes on different heads are identical.

Perpendicular spectra, \(\beta_{SL} = 90^\circ\), display a certain similarity to the powder spectrum, inasmuch as the intensity is spread across the full spectral range. Similar spectra were

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**Fig. 1.** Four frames of coordinates in a muscle fiber experiment. The nitroxide frame of reference is defined by the geometry of the spin label; the molecular frame is defined from the EM micrographs with the long axis of myosin head as \(z\) axis and the \(x\) axis in the plane of the head image. The sample coordinates are those of the muscle fiber, with fiber axis defining the \(z\) direction, while the \(\gamma\) axis of the laboratory frame is parallel to the direction of the static magnetic field.

**Fig. 2.** Overlay of EPR spectra of myofibrils decorated with IASL-labeled S1 heads (solid) and powder lineshape simulation (dotted). The spectrum was simulated with \(g_x = 2.00872, g_y = 2.00592, g_z = 2.00237, A_x = 7.55, A_y = 7.96, A_z = 35.23, \Gamma_L = 0.5 \text{ G}, \Gamma_G = 1.5 \text{ G}, \text{ and } \Gamma_{M0} = 0.24 \text{ G}.\)
although the probes on the heads on opposite sites of the cone still subtend the same angle with the magnetic field.

Spectra of partially tilted fibers, $\beta_{SL} = 21^\circ$, are different from both the perpendicular and parallel spectra. The spectral degeneracy imposed by the heads arrangement and filament disorder breaks down even further. The angle between the field and the axes of the probes attached to heads lying on opposite sites of the $\theta_h$ cone is different [e.g., $\cos^2(\beta_{SL} + \theta_h)$ and $\cos^2(\beta_{SL} - \theta_h)$] for the nearest and furthest extremes. Spectral simulations of fibers tilted at larger angles suggest no significant advantage over a $21^\circ$ fiber tilt angle.

**Amoeba and Grid Searches.** The varying degeneracy in tilted spectra is exploited in the search of probe orientation. Fig. 4 shows projections of the best fits on various $\Omega_{NM}$ planes for SIMPLEX. First, consider the spectra from fibers oriented parallel to the magnetic field (light stippling in Fig. 4). There are many sets of $(\alpha, \beta, \gamma)_{NM}$ angles for which the simulations fit equally well the experimental spectra. The figure of merit for any of the solutions within the contours of Fig. 4 is within a factor of 2 from the best fit for each of the spectra (the best fits had $\chi^2$ values of 1.3, 0.7, and 0.8 for $0^\circ$, $21^\circ$, and $90^\circ$ tilts, respectively, reflecting the high quality of the fits). For reference, the best-fitting sets of angles are grouped in five regions: A at $(10^\circ, 30^\circ, \gamma)$; C at $(10^\circ, 70^\circ, \gamma)$, mirror image $A'$ and $C'$ regions at $(180^\circ - \alpha)$, and a large arc-like region $B$ at $(100 - 140^\circ, 60 - 25^\circ, \gamma)$ (Fig. 4A). There are also numerous small regions at $\alpha_{NM} \approx 160^\circ$. Inspection of the projection on the $(\alpha, \gamma)_{NM}$ plane limits $\gamma_{NM}$ to $0 - 30^\circ$ for the A and C sets. Two arc-like regions with $\alpha_{NM} > 90^\circ$ correspond to the B, $A'$, and $C'$ regions. To ensure that no solutions in random searches were missed, grid simulations were performed in a $\Omega_{NM}$ space encompassing all possible orientations of the nitroxide probe within the myosin head and varying disorder of the head. The mesh used in a grid search is necessarily coarse ($2.5^\circ$). The same regions of $\Omega_{NM}$ space are found by both SIMPLEX and grid searches (data not shown).

Obviously, the spectra of the fibers oriented parallel to the magnetic field are not sufficient to identify uniquely the

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**FIG. 3.** EPR spectra of IASL-labeled S1 decorating unlabeled muscle fibers tilted at $0^\circ$, $21^\circ$, and $90^\circ$ with respect to the external magnetic field.

observed by Thomas and Cooke (19). In a cylindrically symmetrical sample oriented perpendicular to the field, more angles of the nitroxide probe and the field are represented. The contribution of the $x$, $y$, $z$ tensors' elements varies between 0 and $\cos^2(90^\circ - \theta_h)$. Cylindrical weighting accounts for the difference between perpendicular and powder spectra, the latter having spherical distribution. The degeneracy of different heads in parallel spectra is greatly broken,

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**FIG. 4.** Contours of $\chi^2$ of SIMPLEX searches to fit tilt series spectra: $0^\circ$, light stippling; $90^\circ$, medium stippling; $21^\circ$, dark stippling. Projections of the SIMPLEX path are shown on the $\alpha_{NM}$-$\beta_{NM}$ plane (A), the $\alpha_{NM}$-$\gamma_{NM}$ plane (B), and the $\beta_{NM}$-$\gamma_{NM}$ plane (C). Contour cutoff at $\chi^2 = 2\chi^2_{min}$ for each of the tilt angles. Regions A, A', B, C, and C' are discussed in the text.
orientation of the nitroxide probe within the protein. It has been suggested that the spectra of fibers tilted 90° to the field increase the orientational content (8, 19). Unexpectedly, the areas of the best fits were nearly coincident with those obtained for 0° tilt. Thus, the perpendicular spectra provide little additional information.

Finally, consider the intermediate fiber tilt (dark stippling in Fig. 4). The 21° tilt spectra resulted in a limited number of solutions. Only solutions A, A’, and C gave satisfactory fits. As before, $\beta_{NM} = 30°$–70° but $\gamma_{NM}$ was limited to 0–30° (Fig. 4C) and $\Omega_{NM} = 0°$–15° or 150°–180°. Solution B and the arc-like areas shown in Fig. 4B and C yielded lineshapes qualitatively different from the spectrum in Fig. 3, discounting these areas as possible solutions. Thus, the intermediate tilt of $\beta_{BL} = 21°$ broke the degeneracy present in both parallel and perpendicular fiber spectra.

Cross-Correlation and Global Fits. The next level of optimization was accomplished by fitting two of the tilt spectra, $\beta_{BL} = 0°$ and 21°, at the same time. Perpendicular spectra were abandoned because the information content was the same as in the parallel spectra, and the perpendicular spectra took 45 times longer to simulate. Two different strategies were used: cross-correlation and global optimization. In the first approach, SIMPLEX fitting was performed only on 0° tilt spectra and the answer for $\Omega_{NM}$ was then used in the simulation of 21° spectra. The final chi-squared was calculated from the total of the two spectra. This strategy is called cross correlation because it performs a fit to the parallel spectra as in Fig. 4 and checks the answer against the 21° tilt. Although this technique is computationally faster, the optimization is biased toward the 0° spectrum. The global strategy performs SIMPLEX optimization on a pair of spectra for each iteration. The simultaneous optimization avoids the bias toward one or the other spectrum. The two methods were combined by performing simultaneous optimization of 0° and 21° tilts and cross correlation with 90° tilt. This strategy allowed the elimination of solution $A'$ and narrowed the $\Omega_{NM}$ to two symmetric solutions: $A = (16.8°, 28.3°, 4.2°)$ and $C = (16.6°, 72.0°, 4.3°)$. The best fits are shown in Fig. 5. The errors as estimated from the standard deviation on repeated optimizations are 2° on $\alpha$ and $\gamma$ angles and 0.5° on $\beta$. The half-width of myosin-head Gaussian spread was $\Delta_{MS} = 7°$ ± 2° and $\Delta_{BS} = 6° ± 2°$ for both probe orientations.

**DISCUSSION**

This work developed a method to determine spin-probe orientation within the myosin head. The simulations algorithm developed for planar lipid membranes (14) was modified for samples with cylindrical symmetry as appropriate for muscle fibers. The algorithm uses a form of Hamiltonian derived by Liberti and Griffith (3), which yields itself to convenient transformations from one system of coordinates to another (5). A similar approach has been proposed for fluorescence signals (21). Three Eulerian transformations have been used to simulate the spectra. The first one, spin probe to myosin head, was unknown; the second, head to fiber, was modeled by using EM information about head tilt; and the third, fiber to lab, was defined experimentally by tilting the fiber axes with respect to the magnetic field. The spectra were then simulated with five unknown parameters: the $\alpha_{NM}$, $\beta_{NM}$, and $\gamma_{NM}$ angles describing probe orientation and the $\Delta_{MS}$ and $\Delta_{BS}$ describing a half-width of Gaussian disorder of the head. The myosin head was modeled as a cylinder of elliptical cross section inclined at 40° to the fiber axes as was shown in recent EM micrographs (13). The major axis of the ellipse was defined to be in the plane of the EM projections.

SIMPLEX optimization of the simulated spectra identified a number of five-parameter sets which were consistent with the experimental spectra of IASL-labeled myosin heads infused into fibers placed parallel to the magnetic field. The number of satisfactory solutions was limited by tilting the fibers by 21° to the magnetic field. The solutions for tilted fibers were a subset of the solutions for parallel or perpendicular oriented fibers. Forcing simultaneous fits to the spectra of fibers tilted at 0° and 21° to the field yielded two possible solutions of (16.8°, 28.3°, 4.2°) and (16.6°, 72.0°, 4.3°). Within experimental accuracy and the inadequacies of Gaussian modeling, the two sets of parameters result in nearly identical spectra. Since the head orientation in one half of the sarcomere is related by symmetry with the heads of its other half, the spectra at $(\alpha, \beta, \gamma)_{NM}$ are very similar to $(\alpha, 45° + \beta, \gamma)_{NM}$. Head disorder and the rotational disordering of the myosin filaments further diminish small existing differences, but within the constraints of the model the two solutions are too similar to choose between them. Additional tilt angles are not capable of reducing this degeneracy.

**Relation to Other Work.** Tilting of the sample axes with respect to the magnetic field in order to obtain orientational information is a well-known technique in EPR spectroscopy of lipid membranes. The orientation of the magnetic tensor in the spin-label analogs of lipids or cholesterol is easily predicted from the molecular structure. Tilting of the infused membranes was extensively used to determine the orientation of lipids with respect to the bilayer normal (22, 23). Thomas and Cooke (19) also used parallel and perpendicular spectra of oriented muscle fibers to demonstrate the well-preserved order of myosin heads in rigor. In the present work, the problem was reversed. A certain molecular distri-
for axial myosin 5.0°—i.e., as high accuracy), IASL spin with mines projection. Once from result with nitroxide suggestions (27), there visualized cell solutions. One its solute. The same analysis was succeeded here is not report of the head. This The probe orientation determined here is not absolute. Its frame of reference is the image of myosin head as visualized by EM of actomyosin complexes (13). The image is a two-dimensional projection of the three-dimensional structure with a low resolution. For the study described here, an x axis of the myosin head was defined in the plane of this projection. Once the relationship between the EM image and the molecular structure is known, it will be trivial to redefine γNM or αMS with respect to some molecular feature, because γNM + αMS = 4°. The uncertainty in the determination of head tilt from EM does have an impact on the values for probe orientation. To determine the extent of possible error, the same analysis was performed for heads tilted at 45°. The corresponding solutions were (14.8°, 23.5°, 6.6°) and (16.2°, 67.0°, 5.0°) —i.e., the changes were mainly in βMS, which decreased by 5°. To a first approximation, βNS + βMS = 68°, the result of the small αMS (4°) that separates the two rotations. Interestingly, a small value of αNS means that the nitroxide z axis lies approximately in the same plane as the long axis of the head (Fig. 6). Thus, axial rotation of the head will result in the reorientation of the principal (z) axis of the IASL label. Since the orientation of the probe z axis determines spectral splitting (the parameter easiest to measure with high accuracy), IASL spin label is a very sensitive probe for axial movement of the myosin head, contrary to previous suggestions (27).

A rather arbitrary aspect of the model is its treatment of the head and probe disorder. Comparison of 9—13° disorder of the heads as observed in EM (figure 9 A and B of ref. 13) and the 8° disorder of spin label (20) suggests that all disorder observed in EPR can be ascribed to head disorder. This allowed the disorder of the label orientation with respect to the myosin head to be ignored. The head disorder itself was modeled as a Gaussian which might result in errors. The resolution of the electron micrographs is, however, too low to quantify the axial or torsional disorder.

The Eulerian approach alleviates the problem of dealing with a complex molecular shape. As pointed out by Mendelson and Wilson (28), since the head has an irregular shape, modeling reorientation in terms of axial and torsional motion might not be appropriate. However, βMS represents the tilt angle only if αMS = 0°. For any other torsional angle, with αMS ≠ 0°, βMS is a complex mixture of head tilt and torsion. Thus αMS and βMS are general enough to describe the head orientation. The only mode of head reorientation which will remain undetected by EPR is the rotation about the long axis of the filament. The helical arrangement of the heads on the myosin filament surface precludes the sensitivity to this rotation. The EPR signal is defined in great measure by the g tensor, which does not have degenerate anisotropy (gx ≠ gy, unlike the anisotropy of the hyperfine tensor (Ax ≈ A2). At 9 GHz, where most EPR spectroscopy on muscle is conducted, the g anisotropy translates into a spectral resolution of 5 G between the minor axes, while experimental accuracy is at least 0.1 G. Apart from rotation about the filament axis, any other head reorientation will modulate either this anisotropy or the even greater anisotropy of the major axes. Unlike fluorescent probes, whose signal is dipolar in nature, there is no "bad" orientation for spin probes which would render the signal insensitive to change of orientation.

In conclusion, a strategy has been developed to determine spin-probe orientation within the myosin head. Knowledge of spin orientation will allow the determination of the myosin head orientation during muscle contraction and thus will identify the mode of head reorientation during force generation.

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