# Structural determination of spin label immobilization and orientation: A Monte Carlo minimization approach

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**Keywords**: spectroscopy, spin labels, electron paramagnetic resonance, electron spin resonance, Monte Carlo.

**Running title**: Spin Label Orientation

**Research sponsored by:** National Science Foundation NSF IBN-98-08708, NHMFL In-House grant, American Heart Association GIA-9950424N and Hungarian National Research Foundation 0TKA 034307 and, FKFP 0252/1999 to K.H., and NSF grant MCB98-08202 to K.A.S.

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## Abstract

Electron paramagnetic resonance (EPR) is often used in the study of the orientation and dynamics of proteins. However, there are two major obstacles in the interpretation of EPR signals: (a) most spin labels are not fully immobilized by the protein, hence it is difficult to distinguish the mobility of the label with respect to the protein from the reorientation of the protein itself; (b) even in cases where the label is fully immobilized its orientation with respect to the protein is not known, which prevents interpretation of probe reorientation in terms of protein reorientation. We have developed a computational strategy for determining whether or not a spin label is immobilized and, if immobilized, predicting its conformation within the protein. The method uses a Monte Carlo minimization algorithm to search the conformational space of labels within known atomic level structures of proteins. To validate the method a series of spin labels of varying size and geometry were docked to sites on the myosin head catalytic and regulatory domains. The predicted immobilization and conformation compared well with the experimentally determined mobility and orientation of the label. Thus, probes can now be targeted to report on various modes of molecular dynamics: immobilized probes to report on protein backbone and domain dynamics or floppy probes to report on the extent of steric restriction experienced by the side chain.

# Introduction

Electron paramagnetic resonance spectroscopy (EPR) has been used extensively in the study of *(a)* the reorientation of proteins in ordered macromolecular assemblies (muscle fibers, lipid membranes) and *(b)* protein dynamics. There are two major limitations to taking full advantage of probe based EPR spectroscopy: *(a)* most probes are not fully immobilized by the protein, hence it is difficult to distinguish the mobility of the label within a protein from protein dynamics; *(b)* even in cases where the label is fully immobilized its orientation with respect to the protein axis is not known. Due to these limitations we can easily measure probe dynamics and probe reorientation with respect to the magnetic field, but the interpretation of probe behavior in terms of the protein behavior is more problematic. Thus, knowledge of probe orientation and dynamics within the protein matrix is essential to the interpretation of magnetic resonance experiments in terms of protein behavior.

The increased availability of genetically engineered probe attachment sites places a high demand on finding a probe with the appropriate characteristics for EPR studies of macromolecular orientation and dynamics. The desired probe behavior depends on the information the probe is to report: a well-ordered probe is desired to study backbone and domain dynamics, while a floppy probe is desired to study the extent of steric restriction of the side chain by its local protein environment. The current “hit or miss” strategy, in which various spin labels are tried at a given site until an appropriate probe is found, is very time consuming. Hence, there is strong motivation to develop a predictive method for finding a label and label site combination at which the label will have the desired characteristics.

In the past, several methods have been used to determine the spin label orientation in the molecular frame of reference. For example, we have used electron microscopy to define the orientation of the myosin head and EPR spectra obtained under similar conditions to extract the orientation of the label within the myosin head (1). While successful, this method is limited to cases where the orientation of the macromolecule is known in at least one state.

A more general approach is to determine the position of the probe by X-ray crystallography. Baudet-Nessler *et al*., determined a crystal structure of the protein RNAase A chemically modified with the fluorescent probe AEDANS (2), and crystal structures of spin labeled T4 lysozyme have recently been determined (3). While this approach may be the most accurate predictor of probe orientation, it is far from routine with low expectation of solving a crystal structure for every probe-site combination.

In an effort to develop a tool for screening a set of probes and for determining probe orientations, we propose here a Monte Carlo minimization method (4), for searching the conformational space of a spin label docked to the model structure of a protein. The proposed method is made computationally efficient by limiting the energy calculation to the interactions of the probe and its immediate protein environment, by reducing the degrees of freedom during minimization to those of the probe only and by an implicit treatment of solvent. We show that these zero order approximations provide sufficient detail for screening labels/site combinations and for accurately predicting probe orientation.

# Methods

*Protein coordinates*

The coordinates, including side chains, of chicken myosin sub-fragment 1 (S1) and the C$\alpha$ coordinates of the actomyosin complex were provided by I. Rayment (5,6). Although our experimental model is rabbit muscle, the residues around the binding sites are highly conserved between chicken and rabbit S1. The overall homology between the two species is 90%.

*Spin label topologies and force field parameters*

Spin label structures were built using the InsightII Builder module. Two atom types were added to the CHARMM19 extended atom force field and parameterized as outlined by Barone *et al*., (7,8). Atom types NN and ON were added to represent the nitroxide nitrogen and nitroxide oxygen, respectively. The addition of these atom types allows for the correct parameterization of dihedral and improper angles necessary to mimic the nitroxide moiety topology of the X-ray structures determined by Lajzerowicz-Bonneteau (9). The remainder of the spin label topology was "cloned" against existing residue topologies (i.e. the topology of phenyl groups was modeled after that of phenylalanine). Partial charges were determined using a set of rules described by Barone *et al*., (7,8). Following extensive energy minimization, all bond lengths were within 0.05 Å and all bond angles were within 5° of their crystal structure values for both five and six member nitroxide rings.

*Docking labels*

Spin labels were initially docked to cysteine residues with topologies mimicking typical cysteine side chain linkages. Labels I, II, III, IV, V and VI were docked with a C-S bond length of 1.8 Å and a C-S-C bond angle of ~100°, while label VII was docked with an S-S bond length of ~2.0 Å and a C-S-S bond angle of ~100°.

*Energy Calculations and Minimizations*

All energy calculations were performed using the CHARMM19 extended atom forcefield in vacuum using a constant dielectric of 2.0 for label sites in the protein interior and a distance dependent dielectric for labels on the protein surface. Only the interactions between the spin label and all amino acid residues within a 15 Å sphere of any atom in the spin labeled cysteine were considered. The Powell conjugate gradient minimization algorithm routine as implemented in X-PLOR was used to optimize the structures.

*Conformational searching*

A Monte Carlo minimization algorithm (4,10,11) was used to search the conformational space of the docked spin label. A starting orientation was generated by rotating around all single bonds, starting at the cysteine C$\beta$, by a random amount. At each iteration of the search procedure, one of the single bonds was chosen at random and the distal portion or the spin label was rotated by a random amount. The spin label conformation was then energy minimized with all atoms fixed except for those of the spin label modified cysteine side chain (**Figure 1**). Since our energy minimized nitroxide rings were in close agreement with published X-ray structures, we left the nitroxide rings

intact. If the potential energy of the new conformation was lower than that of the most recently accepted conformation, then the new conformation was accepted; otherwise the Metropolis criterion (12) was applied at 300° K to determine whether to accept or reject the new conformation as the starting point for the next iteration. An arbitrary stopping criterion was chosen on the basis of extensive simulations using label II docked to C707 of myosin S1. In this case, we determined that by the time 10,000 candidate structures had been rejected no new energy minimum conformations were being generated. To completely randomize the energy minimizations ten independent runs with random starting conformations were performed for each label/site combination. The algorithm was originally implemented using the X-PLOR (13) molecular mechanics program and has also been implemented in CHARMM (14).

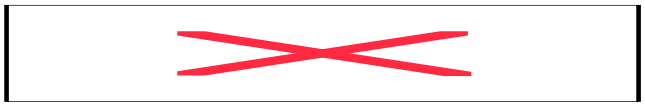
*Labeling with V and VI*

Labeling of S1 with V was accomplished by incubating S1 with a 2 molar excess of V on ice for 6 hours in 120 mM KCl, 10mM MOPS, 2mM EDTA, pH 7.0. For VI, which is less soluble, the label was added in increments over 3 hours. The solution was then incubated on ice overnight and dialyzed to remove unreacted label.

*EPR spectroscopy*

EPR spectra were obtained using a Bruker ECS-106 spectrometer (Bruker Instruments, Billerica, MA). Spectra collected with the muscle fiber parallel to the magnetic field were obtained using a modified $TM_{110}$ cavity, while those taken with the fiber perpendicular to the field were obtained using a $TE_{102}$ cavity.

The maximum splitting in the spectrum taken with the muscle fiber oriented parallel to the magnetic field, $2A_{eff}$, was used to calculate the angle between the magnetic field and the spin probe, □, using the following equation:



where and were found to be 7 G and 35 G, respectively by fitting macroscopically disordered samples.

# Results

*Computational modeling*

Monte Carlo minimizations were carried out on a variety of spin label–protein site combinations. The crystal structure of myosin subfragment 1 and an extensive set of experimental results from a variety of spin labels attached to two sites on S1 provide a suitable set of validation cases. The two chosen sites on S1 represent two distinct label environments: cysteine 707 (C707) is buried within the S1 motor domain and has a solvent accessible surface area of 12 $Å^2$; cysteine 177 (C177) is a solvent-exposed loop site on the essential light chain (ELC) of S1 and has a solvent accessible surface area of 71 $Å^2$. The spin labels used, along with their bonding to cysteine and the bonds around which rotations were allowed during conformational searching, are depicted in Figure 1. These labels were chosen to provide a variety of size, topology, charge distribution and number of bonds tethering the nitroxide moiety to the protein backbone.

*Spin label immobilization by the local protein environment*

Experimentally, a qualitative estimate of whether or not a probe is immobilized is based on the maximum splitting of the spectrum of the isotropically disordered sample: immobilized nitroxides in an aqueous and neutral environment (e.g. protein surface) have a splitting of 70 G, while floppy labels display a maximum splitting of 68 Gauss or less. For ordered samples the presence of motion can be detected by comparing the spectra taken with the magnetic field parallel and perpendicular to the symmetry axis of the sample. In the case of muscle fibers the myosin heads are arranged with helical symmetry around the long axis of the fiber, distributing the nitroxides on the surface of a

cone about the field axis. When the muscle fiber is oriented parallel to the field the average cone angle can be obtained from the splitting and the distribution from the width of the resonances. Rotation of the muscle fiber to a position perpendicular to the magnetic field results in collections of spin labels making angles between 0 and 360° with respect to the field and hence a spectrum with a markedly different lineshape. The difference between the parallel and perpendicular spectra, referred to as tilt anisotropy, is indicative of a defined ordering of the spin label as seen for probe I at the more buried C707 site (**Figure 2***, **lower left***). The anisotropy decreases in two cases: *(a)* in the presence of motion on the timescale of the EPR experiment (0.1-1 ns), which averages the magnetic tensors and *(b)* as the disorder of the label increases, for the ± 90° dispersion about the average angle both the perpendicular and the parallel spectra approach a powder pattern as for label VII at C177 (**Figure 2, *lower right***). These two scenarios can be distinguished, because motion leads to averaging of the maximum splitting while static disorder does not. Thus, the absence of tilt anisotropy indicates disorder, either static or dynamic, whereas decreased splitting for macroscopically disordered samples or ordered samples oriented perpendicular to the field is indicative of dynamic disorder as, for example, with the smaller probe I at the more solvent exposed C177 site (**Figure 2, *lower middle***).

The conformational freedom and the preferred orientation of the nitroxide spin label in the protein environment have been predicted using the Monte Carlo minimization method. As described in the methods section, all torsion angles between the protein backbone and the nitroxide were candidates for random perturbation (**Figure 1**). An iteration of the procedure consisted of randomization of one of the bond torsions

followed by energy minimization. The Metropolis criterion was used to determine whether or not the generated structure was accepted or rejected. The potential energy of the minimized structures was plotted as a function of the angle between the nitroxide 2p$\pi$ orbital (calculated as the average vector product of the CN and NO vectors) and the long axis of actin, (**Figure 2**, ***top***). Four possible outcomes of such modeling are expected: 1) a single well-defined minimum indicative of ***oriented spin labels***; 2) a few, well defined energy minima with little variation around the torsion angles characteristic of labels with ***multiple orientations***; 3) broad multiple minima with no obvious low energy pathway between any two minima (i.e. transformation from one conformation to another requires either large rotation about a single bond or rotation about multiple single bonds) characteristic of ***statically disordered*** labels and 4) multiple minima with clear low energy pathways between them indicative of ***motional disorder***.

Some of the above scenarios were observed in the three cases in which we varied the probe and its protein environment: *(a)* a small iodoacetemide spin label (I) at the buried site C707, *(b)* the same spin label (I) at the surface site C177, and *(c)* a larger spin label (VII) at the same surface site (**Figure 2).**

At the buried site (C707), two clusters of spin label I conformations were found (**Figure 2**, ***left***). The angular spread of the energy profiles was minimal, implying no flexibility within each cluster. This prediction is confirmed by the experimental spectra. The splitting in the spectrum recorded with the muscle fiber perpendicular to the magnetic field is 70 G as for immobilized probes. The parallel spectrum has a much smaller splitting of 36 G. This high degree of tilt anisotropy indicated that I is well ordered at C707.

A different scenario occurs when I is docked at C177 on the protein surface (**Figure 2, *middle***). Spin label I clusters into two families of conformations. The families are not connected (large rotations around several bonds separate them) but display a high degree of flexibility within each family. In particular, the bond along the I side chain most distal to the protein backbone (_6) shows large rotational variability, resulting in the wide spread of angles. In contrast to the large tilt anisotropy of I at C707, the tilt anisotropy is very small. The splitting is 50 G and 40 G for the perpendicular and parallel spectra, respectively. The decreased splitting of the perpendicular spectrum is due to the nanosecond motion, which averages the difference between the minor (7-8 G) and major component (35 G) of the hyperfine splitting. The small residual tilt anisotropy suggests the label is somewhat oriented at this surface site.

The right column of Figure 2 documents the behavior of a large spin label, VII, attached to C177 of myosin light chain 1. For this larger label, one of the conformer families of I is no longer available. There is still a distribution of VII conformers but with tighter angular distribution, especially for the lower energy conformers; moreover, interchange among various conformations within each cluster requires rotation around several bonds including those linking the phenyl groups to the nitroxide. The result is a pattern of several defined angles representing a statically disordered label. These predictions are borne out by the experimental spectra. The perpendicular spectrum has a splitting of 70 G, implying no flexibility capable of tensor averaging. Furthermore, the tilt anisotropy is large, suggesting well defined ordering of the label. The presence of two distinct orientational populations, non-random peaks in the low-field region of the

parallel spectrum, implies two orientational populations, which most likely correspond to the two families found computationally.

The three cases shown demonstrate the utility of our conformational searches as a screening tool for finding label/label site combinations appropriate for EPR studies of macromolecular orientation and dynamics. The data presented for different probes at both buried and surface sites, demonstrate that we can distinguish among probes that are well-ordered (C707 – I) and suitable for studying protein behavior, probes that are dynamically disordered (C177 – I) and probes that are statically disordered (C177 – VII) and suitable for reporting on the local protein environment. As a screening tool, whether a probe is immobilized or floppy can be determined by investigating the width of the isoenergetic angular distributions in the plot of probe orientation versus energy: the wider the spread the less the likely the probe is immobilized.

*Orientation of spin labels*

While the value of the splitting in the spectrum of a macroscopically disordered sample (or the perpendicular spectrum for muscle fibers) is used to validate predictions of probe immobilization, the fidelity of predicting probe orientation requires experimental measurement of the label orientation. In the ideal test case the orientation of the label would be obtained from EPR on single crystals and related to the orientation of the protein in the crystallographic unit cell. In absence of single crystals, a lower symmetry ordered sample such as muscle fibers could be used. There are several approaches to analyze the orientation of the label with respect to muscle fiber axes (15-17), but basically when the muscle fiber is oriented parallel to the magnetic field the splitting is defined by the angle between the nitroxide z-axis and the long axis of actin (**Figure 3**).

This angle can be related to the conformations obtained from the conformational search by taking the cross-product of the vectors between the N, O, and the two C atoms flanking the N in the nitroxide moiety and relating the orientation of that vector to the long axis of the actin filament in the actomyosin model[1].

*Validation*

To validate the Monte Carlo minimization procedure as a tool for predicting spin label orientations we first applied it to a set of spin labels (I, II, III and IV) docked to C707 for which the orientation was previously reported in the literature. We then performed the reverse experiment in which the orientations were first predicted and then verified by experiment using a pair of recently synthesized spin labels, V and VI (18), docked at the same site.

*Label I*

As mentioned in the previous section two clusters of conformers were observed for I on C707. The angle between the z-axis of the nitroxide and the long axis of actin, $\theta^C$, in the first cluster ranged from 65° to 75°, while the second cluster $\theta^C$ ranged from 50° to 55°. These results compare well with those determined by simulation of the spectrum of oriented muscle fibers decorated with I labeled S1 (19). The orientation of the nitroxide was found to be bi-modal, the first component had a mean $\theta$ value of 68° with a Gaussain full-width at half-height (FWHH) of 14°, and the second component had a mean $\theta$ value of 53° with a FWHH of 67° (**Table 1**).

[1] The myosin head crystal structure used in our simulations was transformed into the actomyosin frame of reference (Error! Reference source not found.) using a set of Euler angles and a translation vector found by superimposing the C_ coordinates of S1 onto the C_ coordinates of the actomyosin model obtained from x-ray diffraction.

*Label II*

Conformational searches for II at the same site resulted in a $\Phi^C$ cluster between 50° and 60° and a second cluster with an angle between 70° and 90°. The minimum energy conformation in the 70° to 90° cluster had an angle of $\Phi^C$ = 87°. The lowest energy conformation within the 50° to 60° cluster occurred at $\Phi^C$ = 60° and was approximately 3.5 kT higher in potential than the lowest energy conformation in the other cluster. Experimentally, Fajer *et al*., found angles of 84° (FWHH = 7°) and 76° (FWHH = 17°) in muscle fibers decorated with labeled myosin heads (19).

*Label III*

We have predicted a single family of orientations centered at an angle of 20° with respect to the long axis of actin. Probably, owing to the large size of the label, there is little variation in III conformations: the only variability occurred in _5, which was either 54° or -49°. This variation is simply introduced by flipping of the symmetric and nearly planner nitroxide ring of III. The angle for this label was determined experimentally, using less sophisticated graphical techniques (16), to be 10-12° (20).

*Label IV*

The simulations show a single cluster of conformations spread across a $\Phi^C$ of 80° to 90° with the minimum energy orientation at a predicted angle of 88°. We have shown previously that the orientation of this label is 80° (21), which is within the predicted range of conformers.

*Computational prediction of new spin labels: Label V and Label VI*

As a self-check we performed the reverse experiment of the above by first predicting the orientation(s) of the probe and then measuring the orientation using two recently synthesized spin labels, V and VI (18). Monte Carlo minimizations result in two very narrow clusters of orientations for both labels. The first cluster of VI label conformations had $\theta^C$ ranging from 12° to 14°. The second cluster ranged from 68° to 70° with the lowest energy conformation in this cluster being approximately 2.0 kT higher in energy than the "global" minimum energy conformation. Spectra of myosin heads labeled at C707 with this label and infused into muscle fibers (*not shown*), were characterized by a splitting of ~67 G, yielding a $\theta^E = 16°$. Label V also clustered into two orientations. The first cluster had values of $\theta^C$ between 83° and 85°, and the second cluster ranged over $\theta^C$ values of 72° to 75°. The lowest energy conformer in the first cluster was approximately 3.5 kT lower in energy than the lowest energy conformer in the second cluster. Analysis of experimental spectra resulted in an average angle of $\theta^E = 85°$.

For the six cases studied here excellent agreement between the orientation of the nitroxide predicted from conformational searches and that calculated from the corresponding EPR spectra was achieved (**Table 1**). While in some cases (I, VI) conformational searches uncovered a second less energetically favorable population of orientations, which have not been seen experimentally, the overall correlation among the orientations of the lowest energy conformations and their experimental values is very high (**Figure 4, $R^2 = 0.98$**). This gives us confidence that we can correctly predict probe

orientation, at least in environments allowing for high ordering of the probes in the protein matrix.

## Discussion

We have demonstrated the utility of the Monte Carlo conformational search method as a tool for screening suitable spin probes and labeling sites for EPR studies of protein dynamics and orientation. We have shown for several spin label/protein environment combinations that the lowest energy conformations provide an accurate prediction of the spin label orientation in the protein frame. These results were achieved using methods designed to reduce computation times: the CHARMM19 extended atom forcefield was used to minimize the number of explicit protein atoms by omitting separate treatment of non-polar hydrogen atoms, energies were calculated *in vaccuo* using a constant dielectric at buried sites and a distance dependent dielectric at surface sites and all atoms other than probe atoms were fixed during minimization. Even with such a simplistic model of the spin labeled system, excellent agreement was achieved between predicted and experimentally observed probe immobilization/orientation for a number of probe/site combinations.

The spread of the computed orientation of the nitroxide z-axis and the shape of the energy surface associated with these orientations (**Figure 2**, ***row 1***) provide an estimate of probe mobility. The wider and more shallow the energy minima of the z-axis orientation the more likely the probe is going to be floppy and thus a good candidate for studying the steric effects of local environmental changes. The narrower the range of orientations the more likely the probe is a good candidate for EPR studies of protein orientation and dynamics. At C707, located in a pocket within the myosin head, the lowest energy conformations of Labels I, II, III, IV, V and VI are clustered around narrow angular regions (**Figure 2**, ***row 1***), suggesting the C707 environment immobilizes

these probes at specific orientations. Simulations at a surface site of myosin light chain, C177, show two clusters of orientations with a high degree of flexibility within each cluster for II implying little motional hindrance, while C177 –VII shows several families of orientations with little to no variability within each family suggesting a static disorder of the probes on the protein surface. This was borne out by the experiment; the EPR spectra of VII showed large tilt anisotropy (**Figure 2**, ***right***), while the anisotropy of I was dynamically averaged. Thus, conformational searches distinguish not only between immobilized and mobile probes but also between dynamic and static probe distributions.

The angle between the nitroxide z-axis and the long axis of actin calculated from the minimum energy conformations closely agree with those determined experimentally. This result was validated using six test cases in which the spin label/protein interactions were varied by varying the topology, charge distribution and tether length of the spin labels. For each test case, the angle calculated from the "global" energy minimum conformations are within a few degrees of their experimental values (**Table 1**). Across the six cases there is a 0.98 correlation between calculated and experimental values (**Figure 4**). Thus, conformational searches provide good estimates of nitroxide spin label orientation(s) in the protein frame. The ability to predict spin label orientation(s) within the protein will augment the determination of protein orientation, dynamics and distance measurements from EPR spectra.

*Relationship to other work*

Our motivation was to develop an efficient computational tool for both screening probe mobility and, for immobilized probes, determining the probe orientation in the molecular frame. In most cases the strategy for finding an immobilized probe is that of

"hit-and-miss": a variety spin labels are tested until an appropriate one is found. The computational method developed here takes about a day; whereas, it may take several days/weeks to test just one label experimentally.

Our second aim, determination of probe orientation, was the subject of a number of other studies preceding our efforts. X-ray crystallography was used to determine the structure of spin labeled proteins (3,22,23). Recently, Langen et al., solved the high-resolution crystal structures of T4 lysozyme spin labeled at three solvent exposed sites and at a tertiary contact site (3). In these structures the absence of electron density correlated well with probe mobility (from EPR spectra). Although the immediate protein environment of the four sites did not provide enough steric restraint to immobilize the entire spin labeled side chain, in two cases there were sufficient crystal contacts to do so. As with the conformational search results presented here, in one case the probe was limited to a single conformation, while in another case the same spin label had two distinct conformations. There is little doubt that x-ray structures of spin labeled proteins are the most accurate predictors of probe orientation, but the effort involved in the x-ray structure determination makes it impractical, especially as a screening tool for label/site combinations.

A simpler computational strategy, in which the orientation of the label within the myosin head was determined utilizing information from electron miscroscopy, was previously developed by us (1). In one particular biological state (*rigor mortis*) the myosin head, as visualized by electron microscopy, is tilted with its long axis at a 40° angle with respect to the muscle fiber axis (24). Thus, in the simulation of EPR spectra from such fibers only the spin label to molecular transformation is unknown and can be

solved. Although successful, the method is quite unwieldy and limited to systems for which the macroscopic orientation is known by some other means.

In the past two years several groups have used molecular modeling methods to determine probe orientation. A simple approach based on performing 1000 steps of energy minimization with a constrained spin label tether length was mentioned in the paper of Smith et al., (25). Given the large number of local minima on the energy landscape, methods based on performing a single energy minimization using algorithms such as steepest descent and gradient descent, which are prone to stopping in the nearest local minima, are not likely to uncover the most probable label orientations due to the very limited conformational search of a single energy minimization. This point was emphasized by Persson et al., (26) who used simulated annealing to locate preferred spin label conformations. They note that, even after extensive thermal annealing, probe orientation was strongly dependent on the starting conformation of the spin label modified cysteine side chain. In neither case was the computational method verified against experimental results. This has been done by Root et al., (27) who, using the conformational search method of Chang et al., (11), determined the conformation of a fluorescent probe on RNAse A. Good agreement between the computed conformation and a corresponding x-ray structure was achieved for this single case of a considerably larger and therefore more sterically restrained label. Steinhoff and collaborators pioneered the use of molecular dynamics in the EPR simulations. The MD trajectories were used with success to describe motion of labeled sidechains in bacteriorhodopsin (28,29).

Although, overall there is an excellent agreement between the predicted and observed orientation there are small (< 9°) deviations between the two estimates. Most likely, the difference arises from our treatment of the protein matrix. We assume that the sidechains do not reorient, which if allowed could provide the nitroxide side chain with an energetically more favorable environment. We insisted on this "rigid" matrix model in order to limit conformation search. If all atoms in the local environment of the spin label were included in the search the computational time would increase exponentially. Even if the precision of predicting probe orientation would increase the utility of our method as an efficient screening tool would drastically diminish. Moreover, Langen *et al*., have shown that spin labeled T4 lysozyme structures deviate very little from the wild-type structure at the level of the backbone fold and in only one case did a local side-chain reorient to accommodate the spin label (3).

With few exceptions very little fluctuation was observed around the torsion angles proximal to the protein backbone (_1 and _2 ). The lowest energy structures were within $\pm 15^{o}$ of their canonical values. This suggests that probe flexibility is dominated by fluctuations around more distal bonds. For example, I at C177 has a very narrow distribution of _1 and _2, while _3 has a $90^{o}$ spread and _6 has two regions of nearly $100^{o}$ spread. The result is two families of orientations with high flexibility within each family. This observation is in agreement with the recent studies of the internal motion of 4-substituted pyrroline and pyrrolidine nitroxide methanethiosulfonate spin labels (3,30). Hubbell and collaborators have postulated that the flexibility of these probes is limited to torsional oscillations of the two terminal bonds tethering the nitroxide ring to the protein backbone (_4 and _5). In this _4/_5 model of probe mobility the disulfide group atoms

are fixed in position due to a weak attractive interaction of the S_ of the disulfide-linked labels with the C_H of the cysteine to which the label is attached. This stabilization of the disulfide group fixes the average values of the _1 and _2 dihedral angles. Such interaction, of course, does not exist for other functional groups (e.g maleimide, idoacetamide), but even in the absence of this specific interaction it seems that the mobility about the bonds proximal to the backbone is severely limited.

It is important to note that these findings do not preclude the need to search over all torsion angles of the nitroxide tether. While some torsion angles are limited to narrow ranges, the center of the range is unknown and may occur at a strained value. For example, the lowest energy conformation of II at C707 occurs with strained dihedral values of ($\chi$1, $\chi$2, $\chi$3, $\chi$4, $\chi$5, $\chi$6) = (-82, -50, -103, -95, -16, -57). This strain appears to be offset by two hydrogen bonds: one between the $C_{\beta}$ oxygen and the R714 side chain and the other between the $N_{\beta}H$ and the Q499 side chain.

In summary, the method presented here provides an efficient means for *(a)* screening probe mobility labels and *(b)* estimating the orientation of the probe in the protein frame. Such screening is valuable whenever protein orientation and dynamics are studied as it helps to choose which protein sites to mutagenize to cysteines and which labels to choose. Moreoever, the method might be used in the evaluation of the orientational effects in dipolar interactions between pairs of spin probes. The relative orientation and mobility defines the strength of coupling to the same extent as inter-probe distance (31) and prevents unambiguous interpretation of experimental data.

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Table 1: Comparison of Predicted to Experimental Spin Label Orientations of Spin Labels Attached to C707 in the Catalytic Domain of Myosin Subfragment 1.

| Spin Label | $\phi^C$ | $\phi^E$ | Reference |
|---|---|---|---|
| **I** | 70 * | 68 | (19) |
| | 53 [1] | 53 | |
| **II** | 87 * | 82 | (19) |
| | 60 [2] | 78 | |
| **III** | 20 | 11 | (20) |
| **IV** | 87 | 80 | (21) |
| **V** | 83 * | 85 | This work |
| | 79 [3] | | |
| **VI** | 13 * | 16 | This work |
| | 70 [4] | | |

$\phi^C$ = computed value; $\phi^E$ = experimental value

* "global" energy minima

[1] The conformation of I having this orientation is 4.0 kT higher in energy than the global minima.

[2] The conformation of II having this orientation is 3.5 kT higher in energy than the global minima.

[3] The two orientations of V are isoenergetic.

[4] The conformation of VII having this orientation is 2.0 kT higher in energy than the global minima.

## Figure and Table Captions

Figure 1

Spin labels and their attachment to cysteine. Arrows indicate the single bonds about which rotations Monte Carlo moves where allowed. I (IASL): *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-2-iodoacetamide; II (MSL): *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide; III (InVSL): 2-[(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)methenyl]indan-1,3-dione; IV (IKSL): 3-(2-iodopropionyl)-1-oxyl-2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrole; V: 2,5,5-trimethyl-2-(3-oxobut-1-yn-1-yl)pyrrolidin-1-yloxyl ; VI: 2,5,5-trimethyl-2-(3-phenylpropynone-1-yn-1-yl)pyrrolidin-1-yloxyl ; VII: 1-oxyl-2,5,5-trimethyl-2,4diphenyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl methanethiosulfonate.

Figure 2

Top row: potential energy, in units of kT above the “global” energy minima, of the energetically favored conformations as a function of the orientation of the probe z-axis (see text). Middle row: surface plots showing low energy conformations of the spin label Bottom row: EPR spectra of the labeled fiber oriented parallel (blue) and perpendicular (red) to the magnetic field.

Figure 3

Definition of $\Omega$ as the angle between the nitroxide z-axis the long axis of actin. $\Omega$ is shown in both the actomyosin frame of reference (left) and the nitroxide frame (right).

The actin axis and the magnetic field coincide when the muscle fiber is oriented parallel to the field. The actomyosin structure is that of I. Rayment (5,6).

Figure 4

Comparison of spin label orientations computed from the lowest energy conformations, $\_^{C}$, against experimental values, $\_^{E}$. $R^2 = 0.98$.

Figure 1

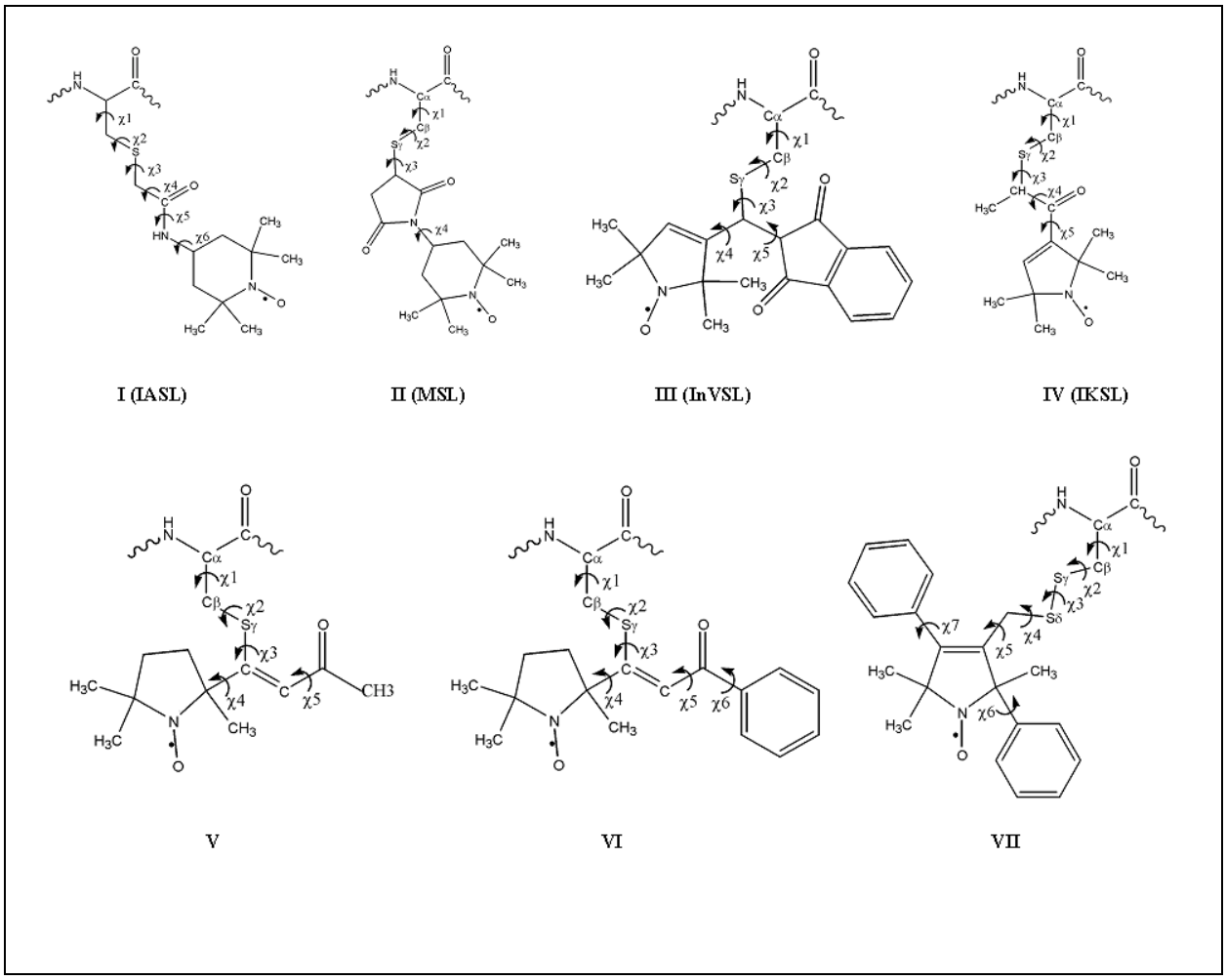

Figure 2

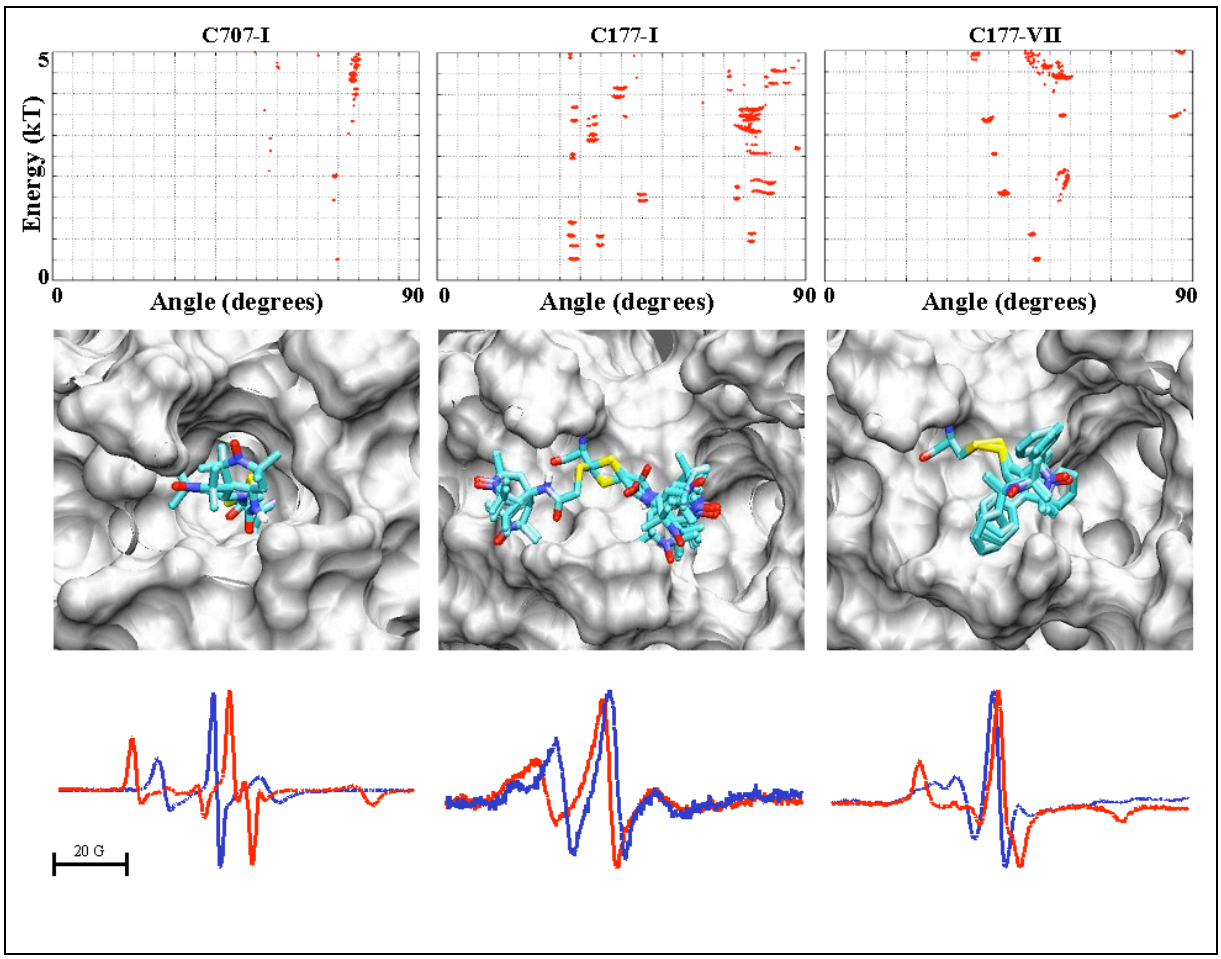

Figure 3

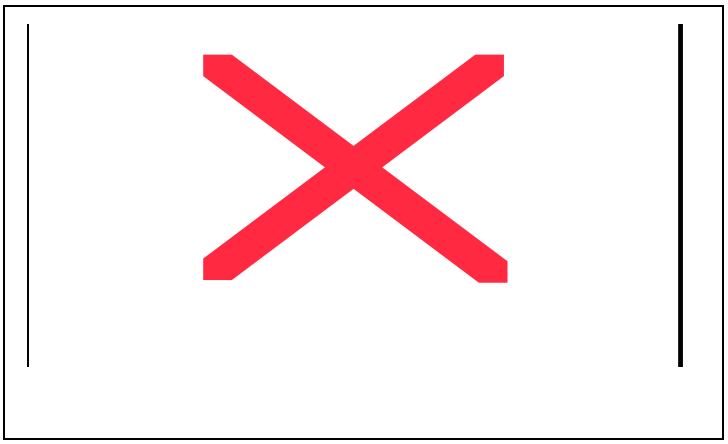

Figure 4