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Molecular origin of EPR lineshapes on β -barrel membrane proteins: local solvation environment modulates spin label configuration†

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Abstract

In the present work, EPR spectroscopy and X-ray crystallography were used to examine the origins of EPR lineshapes from spin labels at the protein-lipid interface on the β -barrel membrane protein BtuB. Two atomic-resolution structures were obtained for the methanethiosulfonate spin label derivatized to cysteines on the membrane-facing surface of BtuB. At one of these sites, position 156, the label side-chain resides in a pocket formed by neighboring side chains; however, it extends from the protein surface and yields a single-component EPR spectrum in the crystal that results primarily from fast rotation about the fourth and fifth bonds linking the spin label to the protein backbone. In lipid bilayers, site 156 yields a multicomponent spectrum resulting from different rotameric states of the labeled side chain. Moreover, changes in the lipid environment, such as variations in bilayer thickness, modulate the EPR spectrum by modulating label rotamer populations. At a second site, position 371, the labeled side chain interacts with a pocket on the protein surface leading to a highly immobilized single component EPR spectrum that is not sensitive to hydrocarbon thickness. This spectrum is similar to that seen at other sites that are deep in the hydrocarbon, such as position 170. This work indicates that the rotameric states of spin labels on exposed hydrocarbon sites are sensitive to the environment at the protein-hydrocarbon interface, and that this environment may modulate weak interactions between the labeled side chain and the protein surface. In the case of BtuB, lipid acyl chain packing is not symmetric around the β -barrel, and EPR spectra from labeled hydrocarbon facing sites in BtuB may reflect this asymmetry. In addition to facilitating the interpretation of EPR spectra from membrane proteins, these results have important implications for the use of long-range distance restraints in protein structure refinement that are obtained from spin labels.

Spin labels have proven to be powerful tools to probe protein structure and dynamics. In the EPR-based technique site-directed spin labeling (SDSL), a spin-labeled side chain is used to probe the local structure and dynamics at the labeled site, and to provide distance restraints between pairs of labeled side chains (1-5). This approach is particularly valuable in the case of large protein complexes or membrane proteins, where other approaches may have limited utility. Spin labels have also been used extensively to refine structures using high-resolution NMR, where paramagnetic enhancements of nuclear relaxation provide long-range distance restraints between nuclei and spin-labeled side chains (6, 7). Although there are several

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approaches that can be used to covalently attach spin labels to proteins, the ease of attachment of labels based upon cysteine chemistry has made the methanthiosulfonate-derivitized cysteine side chain, R1, the most popular spin-labeled side chain for protein labeling (Figure 1a).

An important aspect of interpreting EPR spectra and long-range distances from spin-labeled sites is knowledge of the configuration of the label side chain. The configuration of the R1 side chain at labeled sites has been determined experimentally by examining the modes of motion that modulate the EPR spectrum (8, 9), and by crystallography on model proteins such as T4 lysozyme (10-13). These studies have largely examined aqueous-exposed helical sites, where the internal motion of the label is dominated by dynamics about the fourth and fifth dihedral angles that link the label to the protein backbone (see Figure 1a). Label motion at these sites is not strongly influenced by neighboring residues, and differences in EPR spectra at such sites largely reflect differences in protein backbone dynamics on the ns time-scale (8, 14). Moreover, the preferred rotameric states of the side chain are strongly influenced by a weak interaction between the distal sulfur atom and the Ca proton.

Information on the motion and configuration of the spin label at hydrocarbon-exposed sites in membrane proteins is more limited. Two structures were recently reported for the R1 side chain at helix surface sites in LeuT (15), and this study suggests that unlike soluble proteins, the label R1 at hydrocarbon sites tends to make interactions with the protein surface. Until now, no structures of the R1 label at the surfaces of β -sheets in either membrane proteins or soluble proteins have been reported; however, the R1 environment should be more sterically restricted on the surface of a β -sheet than the surface of a helix. Work on the soluble β -sheet cellular retinol-binding protein (16) using mutagenesis coupled with SDSL demonstrated that R1 motion is strongly affected by the identity of nearest-neighbor residues and that highly ordered states result from β -branched residues such as valine and isoleucine at the non-hydrogen bonded neighbor position. In the case of the β -barrel membrane protein BtuB (Figure 1b), the spectra are also modulated by the neighboring residues (17); however, there is no consistent pattern of label motion and EPR lineshape that can be correlated with the local steric environment. For example, the EPR spectrum for BtuB W371R1 is near the rigid limit on the X-band timescale, yet its nearest-neighbor residues consist of two threonines, one alanine, and one glycine. In contrast, the EPR lineshape from BtuB Y275R1 results from R1 having an intermediate rate of motion, yet its nearest-neighbor residues consist of two lysines, one tyrosine, and one leucine. Moreover, the EPR spectra at some sites in BtuB are strongly influenced by lipid acyl chain length, an observation which suggests that protein backbone dynamics or protein shape might be modulated by membrane thickness (17).

In the present work, we determine the crystal structures for two spin-labeled sites on the hydrocarbon-facing surface of BtuB, and examine the motion of the spin-labeled side chain, R1, as a function of the neighboring side chains and as a function of the local hydrocarbon environment. Labels at the β -barrel surface of BtuB do not assume the same rotameric states that are typically seen on solvent-exposed helical sites, and their motion is strongly influenced by interactions that are made with the protein surface. At position 156 in BtuB, which lies close to the solvent interface, two motional components are present in the EPR spectrum, which are a result of exchange between label rotameric states. In addition, the equilibrium between these states is strongly modulated by lipid environment. At position 170, which is in the bilayer interior, the EPR spectrum indicates that the spin label is immobilized, and this immobilization is independent of lipid environment and neighboring residue identity. The W371R1 crystal structure suggests that immobilization at sites in the bilayer interior is due to interactions of the label with the protein surface. Collectively, the results indicate that label motion and its interactions with the protein surface are highly dependent upon solvation at the labeled site. These results are important for correctly

interpreting lineshapes from β -barrel membrane proteins, probing the environment at the protein-lipid interface, and for the determination of structures and structural changes when spin labels are used as long-range probes of inter-spin distances.

MATERIALS AND METHODS

Mutagenesis, Expression, Purification, and Spin-Labeling of BtuB Mutants

All mutations in BtuB were introduced into a pAG1 vector (for EPR spectroscopy) or a pET22b vector (for crystallization) using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, California), and the mutations were subsequently verified by nucleotide sequencing. For EPR spectroscopy of BtuB mutants, the expression, purification, spin-labeling and reconstitution into lipid bilayers was performed following a procedure detailed elsewhere (18). All lipids were obtained from Avanti Polar Lipids (Alabaster, Alabama), and except where noted, all BtuB mutants were reconstituted into 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers for EPR spectroscopy. POPC forms a fluid-phase bilayer over a broad temperature range, and as discussed elsewhere (19) it has a hydrocarbon thickness that is similar to that of the native outer membrane of *E.coli*. For BtuB crystallization, the expression, purification and spin-labeling of BtuB was performed as described previously (20, 21).

Crystallization and Crystallographic Data Collection

Purified BtuB (11 mg/mL in 30 mM Tris pH 8.0, 20 mM C8E4) was crystallized by mixing 1 μ L of BtuB and 1 μ L of reservoir buffer in an EasyXtal hanging-drop tray (Qiagen, Germantown, MD), containing 200 μ L of total reservoir buffer for each crystallization condition, and followed by incubation at 290 K. The reservoir buffer consisted of 200–550 mM magnesium acetate, 5.0–7.5% PEG3350, and 20 mM Bis Tris at pH 6.6. Crystals were visible after 1–2 days, and grew to ~200 μ m in the longest dimension after 1–2 weeks. For x-ray diffraction, BtuB crystals were transferred to cryo-buffer (150 mM magnesium acetate, 2.5% PEG3350, 20 mM Bis Tris at pH 6.6, 10 mM C₈E₄, and 20% glycerol) for 1–2 min before loop mounting and cryo-cooling by insertion into liquid nitrogen. Diffraction data were taken at 90 K at the 22-ID and 22-BM beamline at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL).

Structure Determination

Indexing, integration, and scaling of the diffraction data was performed using HKL2000 (22). The structures were solved with Phaser (23) maximum likelihood molecular replacement method, using PDB deposition 1NQE (24) as a search model. To reduce model bias, the spin-labeled residues were deleted from the search model prior to molecular replacement. Model building was done in COOT (25), and unrestrained TLS (26) refinement was performed using Refmac (27) and PHENIX (28) was used to refine the occupancy of the spin label. The spin-labeled residues were manually built in COOT. Completed structures were evaluated and validated with MolProbity (29).

EPR Spectroscopy of Spin-Labeled BtuB Mutants

The room temperature (298 K) X-band EPR spectroscopy was performed on a Bruker EMX spectrometer equipped with a dielectric resonator (Bruker Biospin, Billerica, MA) or an E-line 102 Century series spectrometer from Varian outfitted with a loop gap resonator. Low temperature (200 K) X-band EPR spectroscopy was performed on an E-line 102 Century series spectrometer from Varian equipped with a loop gap resonator. For all X-band measurements, 5 μ L of approximately 100 μ M protein sample was loaded into Pyrex capillaries (0.60 i.d. $\times$ 0.84 o.d., Fiber Optic Center, Inc., New Bedford, MA) using a

syringe, and EPR spectroscopy on spin-labeled BtuB crystals was performed as described previously (21). Low temperature (200 K) Q-band EPR spectroscopy was performed on an ELEXSYS E-500 spectrometer equipped with an ER5106 QT-W resonator (Bruker Biospin, Billerica, MA) with 5 μ l of approximately 100 μ M protein samples loaded with a syringe into quartz capillaries (0.60 i.d. $\times$ 0.84 o.d., Fiber Optic Center, Inc., New Bedford, MA). All EPR spectra were recorded with either a 100 G (at T=298 K) or 150-200 G (at T=200 K) magnetic field sweep at 2.0 mW incident power. The phasing and normalization of EPR spectra was performed using LabVIEW software provided by Dr. Christian Altenbach (University of California, Los Angeles, California).

Saturation Recovery EPR

Saturation recovery was performed on an X-band ELEXSYS E-580 spectrometer equipped with an MS-2 split-ring resonator (Bruker Biospin, Billerica, MA). The spectrometer was fitted with a Stanford Research Instruments amplifier SR445A (Sunnyvale California) in place of the video amplifier originally supplied with the instrument. For these measurements, 5 μ l of approximately 100 μ M protein sample was loaded into Pyrex capillaries (0.60 i.d. $\times$ 0.84 o.d., Fiber Optic Center, Inc., New Bedford, MA) using a syringe. A 500 ns saturating pump pulse was applied to the center of the $m_I=0$ hyperfine line, and 2 mW CW observe power was applied at the same frequency. The field was stepped on- and off-resonance by 100 G at 5 Hz to subtract any background signal. Each measurement was independently repeated 3 times with good reproducibility (standard deviations within 75 ns), and the average T_1 relaxation times from the three measurements are reported.

Modeling of EPR Spectra

Low temperature (200 K) X- and Q-band spectra were fit using an effective Hamiltonian rigid limit model executed in LabVIEW software, courtesy of Dr. Christian Altenbach (University of California, Los Angeles, California). From these fits, the hyperfine A- and g-tensor values were determined and used as constraints for fitting the room temperature EPR spectra with the Microscopic Order Macroscopic Disorder (MOMD) model (30) implemented in the Multicomponent program developed by Dr. Christian Altenbach (University of California, Los Angeles, California). The tensor values used for spectral fitting are $g_{xx}=2.0085$, $g_{yy}=2.0059$, $g_{zz}=2.0021$; $A_{xx}=6.5$, $A_{yy}=5.6$, $A_{zz}=35.0$.

In the MOMD model, three coordinate frames are used to represent the microscopic motion of the nitroxide with respect to a fixed macroscopic director. In the molecule-fixed molecular frame (X_M , Y_M , Z_M), Z_M is taken to lie along the 2p orbital of the nitroxide, X_M coincident with the nitroxide bond axis, and Y_M is selected for a right-handed coordinate system. The molecular frame is the principal frame for the nitroxide hyperfine A- and g-tensors. The second coordinate frame is the rotational diffusion tensor frame (X_R , Y_R , Z_R), which is related to the molecular frame by a z-y-z Euler rotation using the angles γ_D , β_D , and α_D , where a positive angle produces a counterclockwise rotation when viewed from the positive side of the rotating axis. The rate of nitroxide motion about each of these axes and their orientation with respect to the molecular frame indicates which R1 dihedrals are undergoing torsional oscillations that are contributing to motional averaging in the EPR spectrum. A restoring potential can be implemented that constrains the amplitude of diffusion about Z_R within a cone defined by the instantaneous angle θ between Z_R and the symmetry axis of the conical potential, which defines the z-axis of the third coordinate frame, the director frame. The director frame is fixed with respect to the protein, and forms an angle ψ with respect to the external magnetic field. To get the final spectrum corresponding to an isotropic distribution of protein orientations, the spectra are summed over ψ .

The high degree of overlap between the fast and slow components in the 156R1 spectra introduces significant uncertainty into the fitting process. To address this issue, the single-component 156R1 spectrum in DLPC bilayers was initially fit, and the final parameters from this fit were used as initial input parameters for the fast component during the subsequent two-component fits. Additionally, due to the correlation between rotational diffusion and local order, the spectra were fit without a restoring potential to reduce fitting time and the number of parameters. In fact, better fits were obtained for the spectra presented here assuming anisotropic diffusion without a restoring potential, especially with respect to the high-field manifold.

During fitting, the R tensor (diffusion tensor in Cartesian representation) was allowed to vary for each component independently. Once a good fit to the central line was established, the Euler angles were adjusted for each component, paying special attention to the quality of fit at the high field manifold. If necessary, the R tensors were varied again; this process was iteratively repeated until a satisfactory fit was obtained. In cases of simple z-axis anisotropic motion, only γ_D and β_D angles were varied to constrain the number of fitting parameters. Likewise, for simple x-axis anisotropic motion, only γ_D was varied. Subsequently, the Lorentzian (and if necessary, Gaussian) linewidths, initially set to 0 Gauss, were allowed to vary in order to obtain the final fit. In some cases, the A-tensor values were allowed to vary by 0.6 Gauss if necessary to obtain the best fit. The quality of fit was assessed using the reduced χ^2 between the experimental and theoretical spectra, as well as visually evaluating the match between prominent spectral features.

RESULTS

In the present work, the *Escherichia coli* outer membrane protein BtuB was used to examine the molecular basis of R1 spin label motion at hydrocarbon-exposed sites on β -barrel membrane proteins. Shown in Figure 1 are EPR spectra obtained for several sites in BtuB that have been labeled with the spin label side chain R1 (17, 19) and reconstituted into POPC bilayers. The EPR spectra from the outer surface of BtuB are quite variable but can be divided into two general types: spectra that are clearly multicomponent indicating that R1 exhibits at least two types of motion (Figure 1c), and spectra which are dominated by a component due to a slowly moving nitroxide, where the hyperfine anisotropy is not averaged (Figure 1d). Although there are exceptions, the multicomponent spectra arise more frequently from residues in the aqueous phase or near the aqueous solvent interface, while the strongly immobilized spectra originate almost exclusively from spin-labeled sites buried in the membrane hydrocarbon.

Structural model of T156R1 from X-ray crystallography

T156R1 is at a site near the aqueous solvent interface that exhibits a multicomponent EPR spectrum (Figure 1c). To investigate the origin of these dynamic modes, the structure of T156R1 was determined at 90 K (Figure 2). The crystals of BtuB T156R1 diffracted to a resolution of 2.6 Å and the resulting structure was refined to an R_{free} of 25.39%; the complete data collection and refinement statistics are shown in Table 1.

Clear electron density is observed for the entire R1 side chain, allowing for determination of the χ_1 - χ_5 dihedral angles (Table 2). The first two dihedral angles, χ_1 and χ_2 , are in a {t, m} rotamer using the conventions of Lovell et al. (31). This rotamer has been observed previously for R1 at solvent exposed helical sites, but it is the least frequent of the three rotamers that are seen (32). Due to the large absolute value of χ_2 (-83°), the S_δ -HC $_\alpha$ stabilizing interaction that is frequently seen in spin-labeled α -helical structures is absent ($d = 4.2$ Å). Instead, the R1 side chain appears to be stabilized by other interactions with the protein backbone including S_δ -N $_{i+1}$ and S_δ -O=C interactions ($d = 3.5$ Å). These

interatomic distances are consistent with the other {t, m} rotamers reported in the literature (11, 12), and may be important R1-backbone interactions that stabilize the spin label in this configuration. In two recently published structures of R1 at hydrocarbon-exposed helical sites (15), R1 assumes the {m,m} rotamer (the most common rotamer at helical sites), and is found to fold back onto the protein surface. In contrast, the 156R1 side-chain projects away from the protein backbone where the nitroxide ring is localized in a hydrophobic pocket formed by the side chains of Q158, L160, V166 and L168.

The substitution of R1 at position 156 did not perturb BtuB structure; the all-atom pair-wise RMSD compared to the wild-type apo structure (24) is 0.21 Å; however, small changes in the local side chain rotamer distribution are evident in neighboring residues. The largest change occurs for the i+2 residue, Q158, where the first rotatable bond isomerizes moving the ϵ nitrogen approximately 3 Å from its position in the wild-type protein (Figure 3). Compared to the wild-type structure, the L160 conformer is also altered in the spin-labeled structure as is the L168 side chain (Figure 3).

EPR spectra and the crystal structure suggest a mode of motion for T156R1

Shown in Figure 4 are EPR spectra of BtuB T156R1 reconstituted into POPC bilayers and in the protein crystal at room temperature. The POPC spectrum can be simulated using the MOMD model (see methods) assuming that the spin label undergoes two modes of motion: a relatively fast (1.7 ns) anisotropic motion about the z-axis of the diffusion tensor, and a slower anisotropic x-axis motion (see Table 3). The room temperature EPR spectrum obtained from *in surfo* crystals of BtuB T156R1 is different, and can be simulated by a single component undergoing relatively fast z-axis motion (1.7 ns), similar to the fast component seen for the POPC sample. The EPR spectrum of BtuB T156R1 in crystallization buffer is identical to the crystalline spectrum, and lowering the temperature of this sample did not induce the appearance of a second slow component in the EPR spectrum, such as that seen in the POPC spectrum (data not shown). As a result, it is likely that the label conformer observed in the crystal structure (the {t,m} rotamer) gives rise to the EPR spectrum of the crystal at room temperature (and the fast component seen in the POPC spectrum). This assignment is consistent with the MOMD fit. From this fit, the Euler angles for the fast component can be used to plot the rotational diffusion tensor frame onto the T156R1 crystal structure (Figure 5). In the context of this model, the orientation of the z-axis is roughly in a direction that would be the average of the direction for the fourth and fifth bonds linking the spin label to the protein backbone, indicating that 156R1 is executing rapid rotation about the χ_4 and χ_5 bonds. Rotameric conversion about about χ_1 - χ_3 is unlikely to be rapid on the EPR time-scale, and motional averaging of the nitroxide magnetic interactions about χ_4 and χ_5 is consistent with previous studies of R1 in T4 lysozyme (9).

Mutagenesis of neighboring residues is consistent with the motional model for T156R1

For β -sheet structures, a residue may interact with hydrogen-bonded (HB) and non-hydrogen bonded (NHB) neighbors and with residues in the $i\pm 2$ positions. In general, these interactions are dependent on the side chain configuration and on the local strand twist and tilt (33). To test the model presented in Figure 5, EPR spectra were obtained from mutagenesis of the side chains surrounding T156R1. The EPR spectra are shown in Figure 6 and the fits to the spectra using the MOMD model are listed in Table 3.

All the spectra shown in Figure 6 result from multiple motional components and require two modes of motion to be reasonably simulated by the MOMD model. In some cases, the lineshapes of T156R1 are not strongly affected by mutation of the surrounding residues, although in many cases, these mutations shift the relative populations of the fast and slow components in the EPR spectrum of T156R1. Substitutions at the HB neighbor, L168, have

the largest effects and increased steric size at this site increases the fraction of slow component in the EPR spectrum. As seen in Figure 6 and Table 3, the L168W substitution produces a large change and inverts the populations to strongly favor the slow component. The larger side-chain at position 168 is expected to sterically interfere with the position of T156R1 in the {t, m} rotamer, and may force R1 to favor an alternate rotamer of χ_1 and/or χ_2 that yields the slow spectral component.

The i+2 residue (Q158) lies close to the label disulfide, and as discussed above, Q158 is also the only residue that assumes a significantly different rotamer in the T156R1 structure compared with the wild-type BtuB structures. Mutations at residue 158 affect the axes and rates of rotation for the fast component and the rates of motion for the slow component. Smaller side chains at position 158 result in faster motional rates for the slow component, and slower diffusion about the z-axis for the fast component. Additionally, the fraction of label giving rise to a slow component increases for Q158N and Q158A as compared to the wild-type residue.

As discussed below, the likely source of the slow component is an alternate label rotamer, which differs from that found in the crystal structure. We do not know the rotameric state that gives rise to the slow component in these EPR spectra, but the {t,t} rotamer is both energetically reasonable (32) and would be allowed within the local side chain environment around T156R1. Moreover, in this configuration, R1 would interact more closely with T138 and Q158, which could account for effects of these mutations on the EPR lineshapes of T156R1. The interaction between R1 and the non-hydrogen bonded neighbor T138 is not strong in the {t,m} state, and while two mutations do not change the motion of the label, the mutation T138Q increases the population of the slow component. This is consistent with modeling of the {t,t} rotamer indicating that this rotamer might be favored with glutamine rather than threonine at position 138. Another possible alternate configuration is the commonly observed {m,m} rotamer, although modeling of this rotamer into the T156R1 crystal structure requires adjustment of T156R1 dihedrals other than just χ_1 , and this rotamer would sterically clash with T138.

Bilayer thickness shifts dynamic modes of the BtuB T156R1 spin label

Previous work demonstrated that the 156R1 EPR lineshape was sensitive to hydrocarbon thickness (17). Specifically, it was found that thinner membranes resulted in increased motional averaging of 156R1, but that R1 motion was not strongly affected by the phase state of the surrounding lipid. It was proposed that R1 does not interact strongly with annular lipids but that it might report changes in the structure or dynamics of the β -barrel in response to a hydrophobic mismatch. Shown in Figure 7 are EPR spectra of T156R1 in lipid bilayers of four different thicknesses, all in the liquid crystalline state, as well as simulations of these spectra using the MOMD model (see Table 3 for parameters).

In DLPC bilayers, 156R1 is a single-component spectrum; the MOMD fits suggest that the spin label is undergoing rapid oscillations about the χ_4 and χ_5 bonds (1.7 ns). This spectrum is identical to the crystalline 156R1 EPR spectrum, where BtuB was crystallized from a C₈E₄ micellar solution. For the DMPC spectrum, this fast component is essentially unchanged, but an additional component is present having an intermediate motion (4.3 ns) about the x-axis of the molecular frame ($\gamma_D = 11$, $\beta_D = 0$). With increasing bilayer thickness, the population the slow component increases and reaches 59% of the total spin population in DiErPC bilayers. Although no pattern can be seen in the rotational rates of the slow component as a function of bilayer thickness, the axes of rotation change slightly with thicker membranes. These results suggest that changes in membrane thickness primarily affect an equilibrium between two dynamic modes of T156R1, which appear to represent different rotameric states (see below). The fast correlation time $\tau(z)$ that makes the most

significant contribution to the motional averaging of T156R1 remains essentially constant with membrane thickness, suggesting that membrane thickness does not affect lineshape directly through changes in ns backbone fluctuations as previously proposed (17).

Structural origins of the slow dynamic mode of T156R1

Some R1 rotameric exchange processes, such as isomerization about the disulfide, are slow on the X-band EPR timescale ($\tau > \sim 50$ ns, 9). Slow rotameric exchange can produce multiple components in the continuous wave EPR spectrum, which are indistinguishable from protein conformational equilibria that are also slow on the X-band timescale. However, since protein conformational exchange is typically at least an order of magnitude slower than rotameric exchange, and is also slow on the nitroxide T_1 -relaxation time-scale, T_1 measurements can be used to differentiate rotameric exchange from conformational exchange (34).

In order to determine the origin of the multicomponent lineshape, BtuB T156R1 was reconstituted into DLPC, POPC, and DiErPC bilayers and saturation recovery EPR was used to measure the T_1 relaxation time of each sample. As seen in Figure 8, each of the signals recovered to equilibrium with a single-exponential time course. The curves could be fit to

the expression $M_z(t) = M_0 \left(1 - e^{-\frac{t}{T_1}}\right)$, yielding a single-exponential fit and one apparent T_1 value. Since the T156R1 spectra in POPC and DiErPC have two motional components, the single exponential recovery indicates that the exchange rate between dynamic modes is fast

on the T_1 timescale: $k_{exAB} \gg \frac{1}{2} \left(\frac{1}{T_{1A}} - \frac{1}{T_{1B}} \right)$, where T_{1A} and T_{1B} represent the intrinsic spin-lattice relaxation times of the individual motional modes.

The lower limit on the exchange rate between the two motional modes of T156R1 could be estimated from the individual values of T_{1A} and T_{1B} . The MOMD fits (Table 2) indicate that the fast dynamic mode remains relatively unchanged with membrane thickness. If it is assumed that the T_1 relaxation time for the fast component remains constant, then the value of T_{1A} from the DLPC saturation recovery data can be taken to be that of the fast component in each spectrum, and T_{1B} can be estimated using the MOMD populations of each component (the measured saturation recovery signal should be a linear combination of T_{1A} and T_{1B} , e.g. for POPC, $(0.74)(723 \text{ ns}) + (0.26)(T_{1B})$ 1.90 μ s). Using this approach, the saturation recovery data indicate that in POPC bilayers the exchange rate is faster than 596 kHz and in DiErPC bilayers the exchange rate is faster than 557 kHz. These correspond to interconversion times of 1.68 and 1.80 μ s, respectively. Since most conformational exchange occurs no faster than tens of μ s (35), the two spectral components in T156R1 are likely the result of label rotameric exchange.

As indicated above, a likely alternate configuration for T156R1 is the {t,t} rotamer. Moreover, it is likely that the slow component in the EPR spectrum results from the interaction of the label in this rotamer with the hydrophobic surface of the protein. Dioxane has been used to probe weak interactions between R1 and protein surfaces (12), and it might be expected to alter T156R1 lineshapes by modulating the interaction of the label with the protein surface. Shown in Figure 9 are EPR spectra that result from titration of dioxane into BtuB T156R1 reconstituted into bilayers. For the POPC sample, dioxane increases the population of the 156R1 fast component as well as the correlation time of the slow component (MOMD fits not shown), suggesting that dioxane competes with the spin label for the surface of the protein and weakens the interaction between R1 and the protein surface. Interestingly, in DiErPC bilayers, dioxane has no effect at concentrations up to 10% v/v, but solubilizes the liposomes above this threshold, as reported previously (36). It is not clear why the behavior in DiErPC is different, but this result could indicate that the side

chain R1 interacts more strongly with the protein surface in these membranes and is not effectively displaced by dioxane.

Structural model of W371R1 from X-ray crystallography

W371R1 is located deep in the membrane bilayer and exhibits an EPR spectrum near the rigid limit (Figure 1d); however, its nearest-neighbor residues consist of a glycine, an alanine, and two threonines. To determine why R1 is highly immobilized at this site, the structure of 371R1 was determined at 90 K. The crystals diffracted to 2.3 Å and the resulting structure was refined to an R_{free} of 24.98%; the complete data collection and refinement statistics are shown in Table 1. As seen for 156R1, electron density for the entire R1 side-chain is resolved (Figure 10), and χ_1 and χ_2 are in a {p, p} rotamer (Table 2). This R1 rotamer has been reported only once in the literature (21), for a label in a sterically constrained environment in tertiary contact.

The W371R1 mutation does not affect BtuB structure; the all-atom pairwise RMSD compared to the wild-type apo structure (24) is 0.22 Å, and the neighboring side-chain rotamers are all unperturbed. It should be noted that residue 371 is near a BtuB crystal contact site on a neighboring β -strand; however, W371R1 does not contact the BtuB symmetry partner, and the rotameric state of R1 does not appear to be affected by this proximity. As seen for T156R1, the S_{δ} — HC_{α} stabilizing interaction found at many spin-labeled α -helical sites is absent ($d = 4.5$ Å). Instead, 371R1 may be stabilized internally by S_{δ} — N_{i+1} ($d = 3.6$ Å) and S_{δ} — $O=C$ (3.0 Å) interactions similar to that of the 156R1 side chain. The spin label sits in a pocket formed by Y389 and T373; a methyl group on the nitroxide ring interacts with the Y389 C ϵ 2-H ($d_{\text{H-H}} = 2.2$ Å), the R1 S_{δ} interacts with Y389 C δ 2-H and C ϵ 2-H ($d_{\text{S-H}} = 3.2$ Å) and the R1 C ϵ -H may interact with O γ of T373 ($d_{\text{H-O}} = 3.0$ Å). In other BtuB structures, W371 sits in this same pocket on the protein surface. Due to increased strand twist, there is limited interstrand hydrogen-bonding between the N-terminal end of strand 12 and the C-terminal end of strand 13. Nevertheless, this does not result in any measurable increase in backbone dynamics since the 371R1 lineshape is near the rigid limit. Although a considerable amount of strand tilt on β -strand 13 points the side-chains towards 371R1, the increased strand twist places the C α of the HB neighbor T391 about 2 Å below the C α of 371R1, decreasing the chances of a strong interaction between 371R1 and its hydrogen-bonded neighbor regardless of residue identity.

The configuration of W371R1 in this structural model is consistent with the broad spectrum that is obtained at room temperature. This model suggests that R1 at this deeply buried hydrophobic site interacts with a pocket on the protein surface formed by neighboring side chains. This site does not present a sterically restrictive environment to the R1 side-chain, and the interaction of R1 with this pocket must be sufficiently strong to immobilize the nitroxide on the ns-time scale. Unfortunately, we were unable to obtain a room temperature crystalline EPR spectrum, because there were not enough crystals to produce a spectrum with reasonable signal-to-noise. Unlike the label at site 156, W371R1 is not sensitive to hydrocarbon thickness, perhaps because it is more deeply buried in the bilayer (data not shown).

EPR spectra of G170R1 are insensitive to local side-chain substitutions

G170R1 is located at a site buried in the membrane interior that also yields a rigid-limit EPR spectrum, however it is surrounded by a more sterically restrained environment than W371R1. At this site, we mutated surrounding residues to alanine to determine whether specific side-chain interactions with G170R1 might be important for immobilization of the spin label. The resulting EPR lineshapes are shown in Figure 11. The results indicate that the motion of G170R1 is only very weakly dependent of the identity of nearest-neighbor

residues. Tyrosine, a residue that has been shown to interact with and stabilize the nitroxide ring (13), is the i+2 neighbor of G170. However, exchanging the tyrosine at residue 172 with an alanine did not significantly affect EPR lineshape; in fact, there was a minor decrease in the peak-to-peak amplitude, indicating additional immobilization of R1. The peak-to-peak amplitude of the central line also slightly decreases when the i-2 residue is mutated to alanine, indicating a further immobilization of the spin label when the local steric environment decreases on the same β -strand as R1. For all the mutants, small changes in g-anisotropy can be seen in the central line, which may reflect changes in local side chain polarity. Furthermore, in contrast to the spectra from 156R1, the EPR lineshape from G170R1 is not dependent upon lipid composition. These results suggest that G170R1 is not being immobilized by specific interactions made with neighboring residues; in other words, at this deeply buried site R1 can make strong interactions with the protein surface irrespective of neighboring side chain identity.

DISCUSSION

At aqueous solvent-exposed helical sites, the spin-labeled side chain R1 does not make strong interactions with neighboring residues (10, 14); however, the disulfide linkage interacts with the protein backbone and differences in EPR spectra are dominated by differences in backbone dynamics (37). On β -sheet proteins facing an aqueous environment, EPR spectroscopy indicates that the side chain R1 interacts strongly with neighboring residues and the motion of the label is strongly modulated by the identity of the HB and NHB neighbors (16). For labels in a hydrophobic environment, such as the surface of a membrane protein, the work presented here indicates that the R1 nitroxide ring makes interactions with hydrophobic pockets on the protein surface, and that the label-protein interaction is sensitive to the solvation environment.

Recent work on the structures of R1 at helix surface sites in LeuT indicates that R1 is more likely to interact with the protein surface at protein-hydrocarbon interfaces than at protein-aqueous interfaces (15). The work presented here is consistent with this result, and with the general finding that side-chain rotamers that face the hydrocarbon in transmembrane proteins (either α -helical or β -barrel) are different relative to those in soluble proteins (38). The data presented here indicate that T156R1, W371R1 and G170R1 interact with hydrophobic pockets formed by side chains on the β -barrel surface of BtuB. In the case of W371R1, the room temperature EPR spectrum is highly immobilized, even though the site is not sterically restrictive. The crystal structure of BtuB W371R1 indicates that this immobilization is due to the interaction of the nitroxide ring with a pocket formed by Y389 and T373. A common configuration for R1 at helical surface sites is the {m,m} or {t,p} rotamer for χ_1, χ_2 ; however, at position 371 the R1 side chain is in a {p,p} rotamer, which has only been observed once at a sterically hindered site (21). It is likely that this rotamer is being dictated by interactions of the R1 side chain with the pocket formed by Y389 and T373. The G170R1 mutagenesis data further indicates that R1 labels buried in the membrane interior are immobilized by interactions with the protein surface, and not by interactions with specific neighboring side chains. In the case of T156R1, the spin label conformer observed in the crystal structure projects away from the protein backbone and towards the solvent interface. The interaction of the spin label with the protein surface at this site appears to be weaker and modulated by the solvation environment.

Several other observations support the idea that a hydrocarbon environment promotes interactions of the R1 side chain with the protein surface. As indicated above, strongly immobilized EPR spectra on the surface of BtuB arise almost exclusively from labels positioned in the membrane hydrocarbon, while multicomponent spectra appear to be more prevalent at interfacial sites. Although sites on the positively curved surface of BtuB are less

sterically constrained than sites on cellular retinol binding protein (CRBP), a soluble β -sheet protein, the EPR spectra from CRBP (16) exhibit more motional averaging than those found on the hydrocarbon surface of BtuB. Interactions of R1 with the protein surface will become more important if interactions with the surrounding solvent are less favorable and this may be the case at the protein-hydrocarbon interface. Spin labels on BtuB were previously seen to be insensitive to the phase state of the bulk lipid (17), which is consistent with the finding here that R1 tends to interact with the protein surface at the protein-hydrocarbon interface.

The work presented here provides an explanation for the dramatic sensitivity of the EPR spectrum at position 156 upon lipid chain length. Membrane thickness modulates two rotameric states of R1 at this site, which gives rise to fast and slow components in the EPR spectrum. Why should the EPR spectrum of site 156 be sensitive to membrane hydrocarbon thickness? Conceivably, lipid thickness modulates the local acyl chain packing at this site or alters the exposure of this site to water. Altering the polarity gradient along the protein surface has, in fact, been shown to affect side-chain rotamer conformations (38). Similar to that seen in the thinnest bilayer formed from DLPC, BtuB T156R1 in the C_8E_4 detergent system only produces a single fast component in the EPR spectrum (see Figure 4). We speculate that local polarity or water penetration at this site are much greater in the detergent or short chain lipid.

Previously, it was noted that three other outward facing sites studied on β -strand 2 are sensitive to lipid thickness (17). The spectra at these sites (150, 152 and 154) also result from two motional components and lipid composition appears to modulate the populations of these components. As a result, the effects seen at 156 are not limited to sites near the solvent interface. These lipid effects have not been observed elsewhere on the BtuB barrel at deeply buried hydrocarbon sites (strands 3, 7, 12 and 17), which suggests that this end of the BtuB barrel may be different than other regions of the protein.

The length of the β -strands in the barrel of BtuB, and other TonB-dependent transporters, is highly asymmetric around the circumference of the protein. Recent work using nitroxide depth measurements indicates that the protein-bilayer interface is also asymmetric around the circumference of BtuB, and that the region near strand 1 is highly mismatched to the hydrocarbon thickness (19). Conceivably, this hydrophobic mismatch might alter the optimal lipid chain packing with the protein surface, create more local defects in the bilayer and/or alter water penetration around this surface of the protein, thereby modulating the interactions that labels make with the protein surface at this site. This region of BtuB (β -strands 22, 1 and 2) also has higher crystallographic B-factors in both the *in meso* and *in surfo* structures than other regions around the protein β -barrel (24, 39). Although the MOMD fits of spectra from T156R1 do not suggest that ns backbone dynamics are directly contributing to motional averaging of the label, it is possible that slow backbone fluctuations around strand 2 are altering label interactions with the protein surface.

In summary, the R1 spin-labeled side chain tends to interact with the protein surface at hydrocarbon facing sites in BtuB. In contrast to aqueous facing sites, the mobility of R1 on the BtuB barrel and the resulting EPR spectra at these sites is influenced by the availability of binding pockets for R1 on the surface of the protein and not necessarily by steric constraints due to neighboring residues. Moreover, R1 is highly sensitive to the solvation environment, and at some sites interactions of the label with the protein can be modulated by lipid chain length.

The R1 side chain is widely used as a probe to obtain long-range distance restraints for molecular modeling, either through measurements of paramagnetic enhancements of nuclear relaxation or through measurements of electron dipole-dipole interactions. In these cases,

knowledge of the likely side-chain configurations for R1 becomes an important factor in modeling the structure with desirable resolution. The work presented here indicates that the rotamers found for R1 at aqueous-exposed sites in β -barrel membrane proteins will likely be different than those found for sites facing the membrane hydrocarbon. Even in cases where R1 is not restrained by interactions with neighboring residues, it is likely to be restrained due to interactions with the protein surface at sites located within the membrane interior.

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Abbreviations used

EPR	electron paramagnetic resonance
MTSL	methanethiosulfonate spin label
C₈E₄	<i>n</i> -octyl tetraoxyethylene
PC	phosphatidylcholine
DLPC	dilauroylphosphatidylcholine
DMPC	dimyristoylphosphatidylcholine
MTSL	methanthiosulfonate spin label
POPC	palmitoyloleoylphosphatidylcholine
R1	spin-labeled side chain produced by derivatization of a cysteine with MTSL
SDSL	site-directed spin labeling

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Figure 1.

a) Model for the spin-labeled side chain R1 obtained by derivatization with an MTSL spin label. Five rotatable bonds link the R1 spin label to the protein backbone, but motions that average the nitroxide magnetic interactions are often dominated by motion about χ_4 and χ_5 (see text). **b)** Model of BtuB (PDB ID: 1NQH (24)) showing the position of 10 C α carbons that have been spin-labeled with the side chain R1. Previous work (19) indicates that when reconstituted into POPC bilayers, sites near the aqueous solvent interface **c)** tend to yield EPR spectra that are multicomponent (yellow spheres), whereas at sites in the membrane interior **d)** yield EPR spectra that are near the rigid-limit (red spheres). All spectra are 100 Gauss scans, and normalized to equivalent spin numbers, except amplitudes of the spectra in d) are scaled by a factor of 1.5. The arrows in d) indicate the positions of the hyperfine extrema in the EPR spectrum, which are not averaged in rigid-limit EPR spectra.

Figure 2.

a) The 90 K x-ray crystal structure of BtuB T156R1 (PDB ID: 3RGM) determined at 2.6 Å showing the R1 side chain (in a stick representation) and the positions of the nearest neighbor residues in a Corey, Pauling Koltun rendering. In **b)** and **c)** are shown alternate views of the site around T156R1, with the van der Waals surface shown in grey. In **b)** the $2F_o - F_c$ electron density is shown as blue mesh contoured at 1σ . Data collection and refinement statistics are given in Table 1.

Figure 3.

The BtuB T156R1 crystal structure superposed on the wild-type coordinates (PDB ID: 1NQE). T156R1 nearest-neighbor residues from the spin-labeled structure are rendered as sticks and shown in gray, whereas the same residues from the wild-type structure are shown in green. The Q158, L160 and L168 rotamers are altered upon introduction of R1 at residue 156.

Figure 4.

EPR spectra obtained from BtuB T156R1 reconstituted into POPC bilayers (top) and from a sample of ~20-30 in-tact protein crystals suspended in crystallization buffer (bottom). The dashed line represents the fit to the POPC spectrum (see Table 3 for the fit parameters). The room temperature crystalline EPR spectrum consists of a single component undergoing fast rotational diffusion on the EPR timescale; the rate and anisotropy of the motion is similar to that for T156R1 in DLPC bilayers. All spectra are 100 Gauss scans.

Figure 5. Rotational diffusion tensor frame for the fast component plotted onto the 156R1 crystal structure. From the correlation times determined from the MOMD fit (see Table 3), it is apparent that the label is executing rapid rotation about the χ_4 and χ_5 dihedrals.

Figure 6.

EPR spectra of BtuB T156R1 reconstituted into POPC vesicles, and spectra from 11 mutants of T156R1, reconstituted into POPC bilayers, where the nearest neighboring residues have been mutated. These spectra include those from mutations to the $i\pm 2$ residues (V154, Q158), the hydrogen-bonded (L168) and non-hydrogen bonded (T138) neighbors, and residue L160, which is on a periplasmic turn and forms the apex of a hydrophobic pocket near 156R1. The dashed lines below each spectrum represent the MOMD simulations for each spectrum (see Table 3 for the parameters used to generate each spectrum). All spectra are 100 Gauss scans.

Figure 7.

A comparison of T156R1 EPR spectra at 23°C that are reconstituted into lipid bilayers of increasing hydrocarbon thickness. The hydrocarbon thicknesses, determined previously (40, 41), are approximately 19.5, 25.0, 27.1 and 43.4 for DLPC, DMPC, POPC and DiErPC, respectively. The EPR lineshapes are shown in the solid traces and the MOMD fits are shown as the dashed lines below each spectrum. The DLPC spectrum could be fit with a single component, and the population of a second slow component increases with bilayer thickness (see Table 3 for the MOMD parameters). These spectra were reported elsewhere, but did not include the MOMD simulations (17).

Figure 8.

Saturation recovery data (black traces) for 156R1 reconstituted into DLPC (top), POPC (middle), and DiErPC (bottom) bilayers. Each signal could be fit with a single exponential recovery function (white trace), and the T_1 s are indicated in the figure. The residual to the fit is shown in grey. The first ~100 data points representing the microwave defense pulse were omitted from the fits and figures.

Figure 9.

Effect of dioxane on the EPR spectrum from BtuB 156R1. Spectra are shown in the absence of dioxane (black trace) and in the presence of 5% v/v (blue trace) and 10% (red trace) dioxane. In POPC bilayers, dioxane increases the population of the fast component, consistent with the proposal that the slow label conformer results from an interaction of the label with a hydrophobic pocket on the protein surface. In DiErPC bilayers, dioxane does not affect the rotameric equilibrium, presumably because this interaction is much stronger in lipid bilayers of greater thickness.

Figure 10.

a) The 90 K x-ray crystal structure of BtuB W371R (PDB ID: 3RGN) determined at 2.3 Å showing the R1 side chain (in a stick representation) and the positions of the nearest neighbor residues in a Corey, Pauling Koltun rendering. In **b)** and **c)** are shown alternate views of the site around W371R1, with the van der Waals surface shown in grey. W371R1 sits in a pocket formed by residues T373 and Y389. In **b)** the $2F_o - F_c$ electron density is shown as blue mesh contoured at 1σ . Data collection and refinement statistics are given in Table 1.

Figure 11.

EPR spectra of BtuB G170R1 reconstituted into POPC and DMPC bilayers, and spectra from 4 mutations surrounding G170R1, reconstituted into POPC bilayers, where single alanine mutations were made to the nearest neighboring residues. These spectra include those from mutations to the $i\pm 2$ residues (Y172, L168), the hydrogen-bonded (V154) and non-hydrogen bonded (L202) neighbors. All spectra are 100 Gauss scans.

Table 1

X-ray data collection and refinement statistics for BtuB T156R1 and W371R1.

Structure:	BtuB T156R1	BtuB W371R1
Data Collection		
Beamline	APS-22ID	APS-22BM
Wavelength (Å)	1.000	1.000
Temperature (K)	90	90
Reflections observed	224,348	266,642
Unique reflections	27,492	37,950
Resolution range (Å) ^a	50-2.60 (2.64-2.60)	50-2.30 (2.38-2.30)
Space group	P3 ₁ 21	P3 ₁ 21
Cell dimensions	a = b = 81.6 Å, c = 227.7 Å $\alpha = \beta = 90^\circ, \gamma = 120^\circ$	a = b = 81.7 Å, c = 227.1 Å $\alpha = \beta = 90^\circ, \gamma = 120^\circ$
R _{sym} (%)	6.2 (48.1)	10.5 (32.5)
Redundancy	8.2	7.0
Refinement		
Resolution range (Å)	44.3-2.60 (2.67-2.60)	33.4-2.30 (2.36-2.30)
Reflections used	26,070	35,973
Completeness (%)	98.3 (82.7)	95.2 (74.0)
R _{cryst} (%) ^b	21.32	22.16
R _{free} (%) ^c	25.39	24.98
RMS Deviations		
Bond lengths (Å)	0.020	0.023
Bond angles (°)	1.817	1.953
Number of Atoms		
Protein	4535	4612
Water	86	130
Other	C8E4 (6), Mg (11)	C8E4 (7), Mg (3)
PDB Accession Code	3RGM	3RGN

^aHighest resolution shell data shown in parenthesis.^b $R_{\text{cryst}} = \Sigma ||F_{\text{obs}}| - |F_{\text{calc}}|| / \Sigma |F_{\text{obs}}|$, where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively.^c R_{free} is R_{cryst} calculated using 5% of the data which is randomly chosen and omitted from the refinement.

Table 2

Rotamer designations and summary of R1 dihedral angles.

Mutant	Rotamer	χ_1	χ_2	χ_3	χ_4	χ_5
T156R1	{t, m}	176	-83	-94	-82	-30
W371R1	{p, p}	47	78	64	113	130

Table 3

Dynamic parameters from the MOMD fits[†]

Mutant	Comp.	%	$\tau(x)$ (ns)	$\tau(y)$ (ns)	$\tau(z)$ (ns)	α	β	γ
T156R1 DLPC	2	100	-	11	1.7	-23	65	-26
T156R1 DMPC	1	22	4.3	-	-	0	0	11
	2	78	-	12	2.1	-23	63	-26
T156R1 POPC	1	26	14	-	-	0	0	17
	2	74	-	20	1.7	-24	58	-18
T156R1 DiErPC	1	59	7.6	-	-	0	0	31
	2	41	17	-	2.4	-36	50	-13
T156R1/T138A	1	27	6.9	-	-	0	0	20
	2	73	-	13	2.3	-28	63	-17
T156R1/T138Q	1	49	11	-	-	0	0	22
	2	51	-	18	1.7	-34	54	-11
T156R1/T138W	1	31	4.6	-	-	0	0	13
	2	69	-	-	1.1	0	65	-31
T156R1/L168A	1	21	6.1	-	-	0	0	28
	2	79	-	13	2.7	-23	65	-27
T156R1/L168V	1	33	9.0	-	-	0	0	23
	2	67	-	13	2.4	-28	62	-22
T156R1/L168W	1	65	7.8	-	-	0	0	34
	2	35	-	-	0.96	0	55	0
T156R1/V154A	1	52	3.1	-	-	0	0	20
	2	48	-	12	3.0	-29	65	-31
T156R1/V154Q	1	64	5.4	-	-	0	39	11
	2	36	-	-	0.56	0	54	-40
T156R1/Q158A	1	39	4.6	-	-	0	0	21
	2	61	4.7	-	16	52	37	-10
T156R1/Q158N	1	50	6.2	-	-	0	0	25
	2	50	8.4	-	5.4	45	45	-2
T156R1/L160A	1	35	8.0	-	-	0	0	24

Mutant	Comp.	%	$\tau(x)$ (ns)	$\tau(y)$ (ns)	$\tau(z)$ (ns)	α	β	γ
	2	65	-	13	3.2	-31	62	-19

[†]The slow component is described as component 1, whereas the fast component is labeled as component 2. The slow component was fit with intermediate to slow x-axis anisotropic motion. In most cases, the majority of motional averaging in the fast spectral component was due to fast z-axis anisotropic motion. The dashes represent anisotropic correlation times from the fits that were slower than the timescale for averaging at X-band ($\tau > \sim 50$ ns). In these cases, to reduce the number of parameters, the correlation times were arbitrarily set to 167 ns ($R = 6.0$) for the remainder of the fit and thus are not reported.