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Communication

Analysis of side chain rotational restrictions of membrane-embedded proteins by spin-label ESR spectroscopy

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ABSTRACT

Site-directed spin-labeling electron spin resonance (SDSL-ESR) is a promising tool for membrane protein structure determination. Here we propose a novel way to translate the local structural constraints gained by SDSL-ESR data into a low-resolution structure of a protein by simulating the restrictions of the local conformational spaces of the spin label attached at different protein sites along the primary structure of the membrane-embedded protein. We test the sensitivity of this approach for membrane-embedded M13 major coat protein decorated with a limited number of strategically placed spin labels employing high-throughput site-directed mutagenesis. We find a reasonably good agreement of the simulated and the experimental data taking a protein conformation close to the one determined by fluorescence resonance energy transfer analysis [P.V. Nazarov, R.B.M. Koehorst, W.L. Vos, V.V. Apanasovich, M.A. Hemminga, FRET study of membrane proteins: determination of the tilt and orientation of the N-terminal domain of M13 major coat protein, Biophys. J. 92 (2007) 1296–1305].

1. Introduction

To identify the biological functions of proteins, it is imperative to know their three-dimensional structure. In this context, the least understood class of proteins are the integral membrane proteins [1,2]. Although they represent 30–40% of all expressed sequences, they amount to less than 1% of proteins of known structure [3]. Thus membrane proteins remain an enormous challenge in structural biology.

The progress of high-resolution structural studies of membrane proteins using the two common techniques, NMR and X-ray diffraction, has been limited because both approaches are restricted by technical and practical difficulties [4]. As a result, there is an urgent need for new biophysical methodologies that can provide detailed structural information. Among the more modern biophysical techniques, site-directed spin-labeling electron spin resonance (SDSL-ESR) appears to show a high potential to further advance the field [5–10].

The basis of this technique is a high-throughput site-directed mutagenesis to introduce unique cysteine residues at desired locations in the protein. As site-directed mutagenesis is becoming an increasingly powerful tool in protein preparation, the usefulness of SDSL-ESR in membrane protein studies grows tremendously [7]. An additional advantage is that the membrane proteins can be examined in their native membrane environment, such as reconstituted lipid bilayer systems under their physiological conditions.

Our objective is to present the basic ideas of a new method tailored to transfer the SDSL-ESR data into structural information. To demonstrate the power of our analysis, we use the M13 major coat protein, a small reference membrane protein, and we decorate it with a limited number of strategically placed spin labels. We extract the experimental free rotational space of the spin labels attached to the protein as published previously [11–13]. Here we develop a molecular model to describe local conformations of the protein in a lipid environment in terms of the available free rotational space for the spin label, showing that our method provides a new advance for spin-label ESR spectroscopy in the determination of protein structures.

2. Methodology

For a membrane-embedded protein, the conformational space of a spin-labeled side chain is determined by three main factors: (i) the local rotations of the spin-label side chain attached to the protein backbone, (ii) the restrictions of the rotamers by the backbone and side chains of the neighboring amino acid residues, and

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(iii) the restrictions imposed by the surrounding lipids. These effects are illustrated in Fig. 1. In our conformational modeling, it is assumed that at room temperature the backbone motion is slow on the ESR time scale and significantly slower than the motion of the side chains [14]. Thus the protein fold on a timescale beyond several nanoseconds is defined by series of pairs of dihedral angles φ and ψ. Possible dihedral angle pairs are restricted due to steric clashes of the backbone atoms by taking into account the minimal interatomic distances (van der Waals distances, contact distances) [15,16]. The bond lengths and angles are fixed to the values reported in the literature [17,18], as there is no need to resolve the individual conformation at the atomistic resolution. Instead, we want to detect the shape of the restricted conformational space that is experimentally measured by ESR. For each amino acid position including the spin-labeled cysteine side chain, the full conformational space of a side chain is generated by discrete rotations around the single bonds (Fig. 1A and B). The torsion potentials are modeled by a discrete set of equiprobable but not equidistant rotational states, such that their density increases with the depth of the torsion potential at a given angle.

The statistical weight p_i of a certain conformation of spin label i is given by:

$$p_i = \left\{ \begin{array}{l} 0, \text{backbone overlap} \\ 1, \text{no backbone overlap} \end{array} \right\} \times \left[\prod_{k \in \text{neighboring amino acids}} \left(1 - \frac{N^k_{k-i\,\text{overlap}}}{N^k_{all}} \right) \right] (1 - \sin \vartheta_i), \qquad (1)$$

where the product in the central factor runs over all neighboring amino acid residues that share the space with the conformations of the spin label.

The first factor in Eq. (1) indicates that the conformations of the spin-labeled cysteine side chain, which overlap with the backbone are completely rejected (Fig. 1C and D) as the motion of the backbone is much slower than the motion of the side chains. However, the overlap with the neighboring amino acid side chains (Fig. 1E and F) is assumed to be "soft" rather than "hard", as the wobbling of the side chains is fast on the ESR time scale. This is taken into account by the second factor in Eq. (1), which describes the reduction of the statistical weight by the ratio of the number of overlapping conformations and the number of all possible conformations of the neighboring side chains that are allowed by the backbone overlap check. Finally, the statistical weight of each conformation of a side chain of the spin label is also decreased by restrictions due to adjacent lipids. The aligning effect of the lipids is parameterized by the angle ϑ between the membrane normal and the direction of the side chain of a particular conformation (which is defined as the direction from the Cβ to the oxygen atom of the nitroxide) and in a first approximation described by $(1 - \sin\vartheta)$ (Fig. 1G and H). This is provided by the third factor in Eq. (1). This factor is a simplification, based on the following requirements: (1) there are no restrictions in case of a parallel orientation with respect to the lipids; (2) as soon as there is a non-zero angle, there should be a non-zero first derivative effect; (3) at a direction perpendicular to the lipids, the restriction should be strongest; (4) the derivative of this perpendicular effect should be zero again: there is not a very large difference whether lipid molecules are perfectly or nearly perpendicular to the side chains. The most simple and effective function that meets those criteria is the $(1 - \sin\vartheta)$ function. Since the side chain of the 3-maleimido proxyl spin label, which is used in the ESR experiments, is twice as large as compared to amino acid side chains, the aligning effect of the lipids on the other amino acid side chains can be ignored. Based on similar arguments we did not take into account the restrictive effects of amino acid side chains on one another.

Thus, the conformational space of a spin label at a specific site on a membrane-embedded protein will be sensitive to its local environment. For membrane-embedded M13 coat protein, the location of the protein relative to the lipid bilayer is defined by locking the positions of the amino acids that were experimentally determined to be at water–lipid interface [19,20]. Note that this kind of description is proposed to describe the time-averaged SDSL-ESR experimental data and cannot be compared to the much more time-consuming molecular dynamics approach, which on the other hand would actually resolve the time evolution of the conformations.

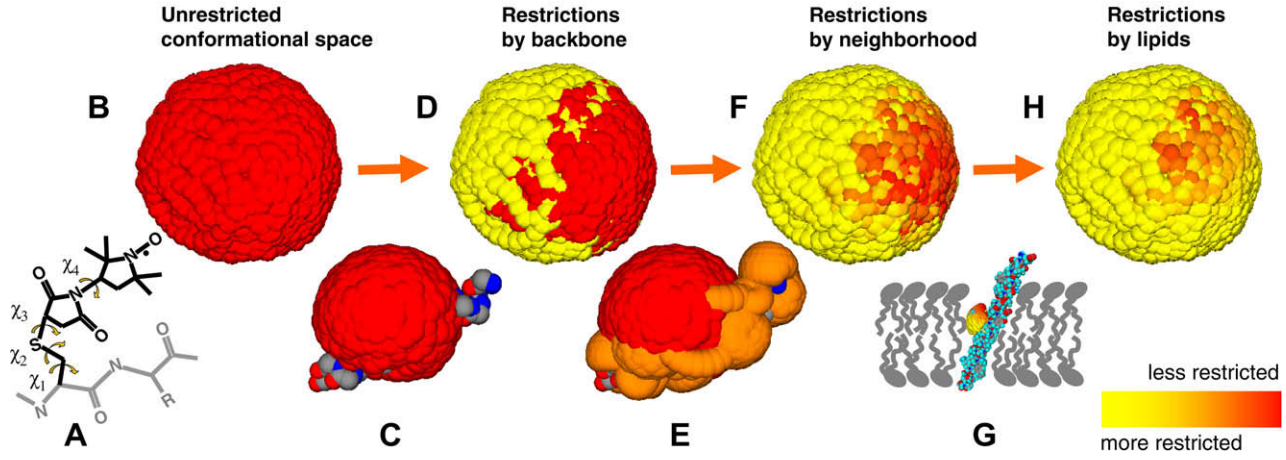

Fig. 1. Schematic illustration of the conformational space of the spin-label side chain for membrane-embedded 3-maleimido proxyl spin-labeled M13 coat protein. For simplicity, the protein is assumed to be in a perfect α-helical conformation and embedded in a bilayer of 1,2-dierucoyl-*sn*-glycero-3-phosphocholine between amino acid positions 9 and 46; the spin label is attached to a cysteine residue at position 25. (A) The spin label attached to a cysteine residue has four free rotations (χ_1, χ_2, χ_3, χ_4) around the four single bonds. (B) Unrestricted spin label conformational space (shown in red) resulting from the free rotations of the side chain around the four single bonds. (C) Steric overlap of the spin label with the protein backbone reduces the set of possible conformations. (D) The available spin label conformational space after steric overlap with the protein backbone (forbidden conformations are shown in yellow). (E) The wobbling spin label shares space with the wobbling side chains of the neighboring amino acid residues (indicated in orange). (F) The available spin label conformational space after steric overlap with both the backbone and the side chains of the neighboring amino acid residues. The soft interaction with the neighboring amino acids is indicated by a continuous yellow–orange–red color scale (see inset). (G) As the lipids tend to orient the amino acid side chains, conformations that are perpendicular to the membrane normal are highly restricted, which further reduces the set of allowed spin label conformations. (H) The final available spin label conformational space subject to all three types of restrictions.

Recently we have introduced a method of analysis of ESR spectra of site-directed labeled proteins, which provides information about the conformational space of the spin-labeled sites [11–13]. The conformational space of a spin label is quantified by the normalized free rotational space Ω, which measures the effective solid angle of the cone left for spin label wobbling. This parameter can also be deduced from molecular modeling of the restriction in the rotational space of the side chains (Fig. 1), by interpreting the results of the modeling in terms of a cone model [12,13]. For this, we calculate the average direction of the nitroxide N–O bonds using the statistical weights of the conformations. The averages are converted into two cone angles ϑ_0 and φ_0 that characterize the anisotropy of the rotational space. From the cone angles we finally compute the simulated normalized free rotational space Ω as follows:

$$\Omega = \frac{\vartheta_0 \varphi_0}{(\pi/2)}, \tag{2}$$

which can then be compared to the experimental values of Ω [12].

In summary, the free rotational space of a spin label is an attractive parameter to consider for protein structure analysis, as it will be affected by its local environment as given by the primary sequence, fold of the protein backbone, adjacent protein domains in a tertiary protein structure and, for membrane proteins, the phospholipids in which the protein is embedded. All computer models were realized as Delphi classes using the Borland Delphi 6.0 environment. The Pascal classes and the software are available from the authors upon request.

3. Results

The protein modeling was tested by comparing the simulated free rotational space of a membrane-bound M13 major coat protein to recently published experimental data [12] (Fig. 2, red triangles). For this protein, consisting of 50 amino acid residues, 27 single cysteine mutants were available. They span the whole primary sequence of the protein and they cover almost the complete range of values of the free rotational space Ω of the 3-maleimido proxyl spin label for the protein reconstituted in phospholipid bilayers consisting of 1,2-dierucoyl-*sn*-glycero-3-phosphocholine [11,20].

The experimental free rotational space Ω was compared with the value of Ω obtained from the simulation of the restrictions of the side chain rotational spaces (Fig. 2). For simplicity, we assumed a membrane-embedment of the protein based on a recently published model, using an α-helical protein with a tilt angle of 18° with respect to the membrane normal and with membrane crossing points at positions 9 and 47 [19,21,22]. To analyze the effect of protein conformation and membrane-embedment on the simulated free rotational space Ω, we generated a number of 5000 different helical structures of the protein with dihedral angles ϕ and ψ uniformly distributed around the values for an α-helix: $-57 \pm 30°$ and $-47 \pm 30°$, respectively. The Ω values related to the original α-helical protein model ($\varphi = -57°$ and $\psi = -47°$) are indicated with white triangles in Fig. 2. The observed variation in Ω values represents the effect of the various amino acid residues in the primary sequence of the protein. In one set of simulations, we left out the lipid effect in Eq. (1), showing the variation of Ω for a 'free' protein (Fig. 2A). At all spin label positions along the primary sequence of the protein the simulated Ω values were summarized into frequency histograms (see the cyan–blue histograms of the relative frequency of a given value of Ω in Fig. 2). As can be seen, the calculated restrictions from the simulated helical structures produce a wide range of Ω values that nicely cover the experimental data (red triangles). In a second simulation approach, the effect of the lipids was included. In this case, there is a reasonably good agreement between the SDSL-ESR experimental data and the simulated data for all spin label positions (Fig. 2B). The deviating positions 25–29 most likely indicate that the simulated structure did not produce locally a secondary structure motif that would sufficiently restrict the conformational space of the spin label. We will address this problem by introducing an optimization procedure in our calculation, which would tune the backbone dihedral angles and in fact eventually would produce an optimized ensemble of best-fitting structures.

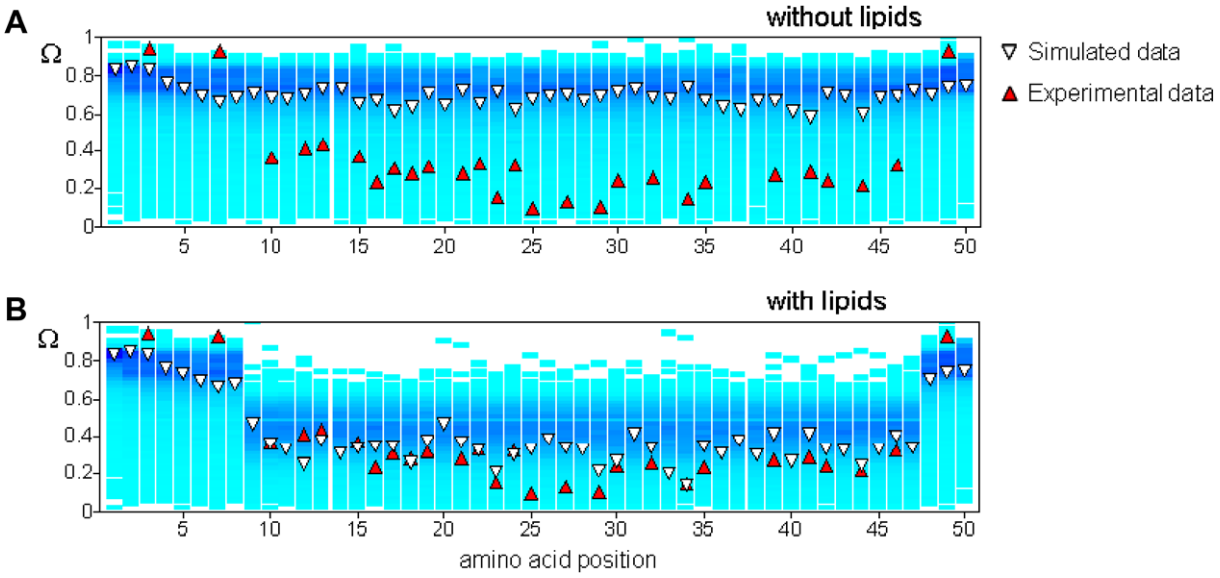

Fig. 2. Sensitivity of the free rotational space to the primary sequence, variations of the protein secondary structure and the effect of the lipids for membrane-embedded spin-labeled M13 coat protein. The histograms of the relative frequency of a given value of Ω (color-coded by continuous shades of blue, such that cyan is the lowest frequency and dark blue is the highest frequency within the set of 5000 modeled near helical structures; see text) at an amino acid position along the primary sequence are plotted both for the 'free' protein (A) and for the protein in a lipid environment (B). The red triangles correspond to the experimental values of Ω. The white triangles indicate the Ω values related to the original α-helical protein model ($\varphi = -57°$ and $\psi = -47°$) as defined in [19,21,22].

4. Conclusions

The key factor to the efficiency of our computational approach is the adjusted spatial and temporal resolution of the molecular modeling guided by the characteristic scales of the spin-label ESR experiments. The ESR experiment is insensitive to the exact atomic coordinates, but it enables us to track the rotational conformations of the amino acid side chains. With this in view, the simulation algorithm is designed to optimize its runtime without compromising the level of detail of the analysis. Since ESR spectroscopy is very sensitive to the available space of the fast rotational motion of the spin label attached to the protein, the rotational conformational space of the side chain can be taken as the most strategic unit in our protein modeling. The proposed search of the conformational space *for each spin-labeled protein mutant* requires a new approach in the modeling strategy as the standard modeling techniques and molecular dynamics simulations are not ideally suited for such an ESR data analysis and consequently are not realistically applicable within the computer time frames possible.

The next step will be to set up an optimization algorithm that will enable to find the best possible structures of the protein based on the Ω data. In this respect, the backbone dihedral angles will be continuously changed and the local restrictions will be recalculated, thereby optimizing the secondary structure of the protein. The goodness of fit to the experimental data will guide the optimization procedure through the search space towards more favorite structures. At the end of the optimization, more than one structure can produce equally good fits to the experimental Ω data, indicating a set of allowed global protein conformations. Such a method is comparable to the distance geometry approach employed in two-dimensional solution NMR spectroscopy that also results in a family of structures [23]. Based on our experience with evolutionary optimization methods [24,25], the estimated time frame for the structure optimization for a protein of size of 150 amino acid residues and 50 different single cysteine mutants will be about 4 weeks using twenty 4-GFLOP processors, which makes this approach highly competitive compared to other high-resolution methodologies.

As compare to well-established ESR tools of structure determination, such as accessibilities and distance constraints, our method provides an alternative approach. Our method has the advantage of providing direct information about the local secondary structure at physiological temperatures (i.e., room temperature) with singly labeled protein samples, without changing the sample conditions. In the case of accessibility experiments relaxation agents, such as Ni^{2+} ions or oxygen, need to be added to the sample. To determine distance constraints, two spin labels need to be engineered at the protein and for spin echo ESR experiments the sample has to be cooled to a low temperature (around 50 K) [7].

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References

[1] A. Arora, L.K. Tamm, Biophysical approaches to membrane protein structure determination, Curr. Opin. Struct. Biol. 11 (2001) 540–547.
[2] J.-J. Lacapère, E. Pebay-Peyroula, J.-M. Neumann, C. Etchebest, Determining membrane protein structures: still a challenge!, Trends Biochem Sci. 32 (2007) 259–270.
[3] S. White, Membrane proteins of known 3D structure, 2008, <http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html>.
[4] J. Torres, T.J. Stevens, M. Samsó, Membrane proteins: the 'Wild West' of structural biology, Trends Biochem. Sci. 28 (2003) 137–144.
[5] W.L. Hubbell, D.S. Cafiso, C. Altenbach, Identifying conformational changes with site-directed spin labeling, Nat. Struct. Biol. 7 (2000) 735–739.
[6] L. Columbus, W.L. Hubbell, A new spin on protein dynamics, Trends Biochem. Sci. 27 (2002) 288–295.
[7] M.A. Hemminga, L.J. Berliner (Eds.), ESR Spectroscopy in Membrane Biophysics, Springer, New York, USA, 2007.
[8] C. Beier, H.-J. Steinhoff, A structure-based simulation approach for electron paramagnetic resonance spectra using molecular and stochastic dynamics simulations, Biophys. J. 91 (2006) 2647–2664.
[9] D.E. Budil, K.L. Sale, K.A. Khairy, P.G. Fajer, Calculating slow-motional electron paramagnetic resonance spectra from molecular dynamics using a diffusion operator approach, J. Phys. Chem. A 110 (2006) 3703–3713.
[10] L.E.W. LaConte, V. Voelz, W. Nelson, M. Enz, D.D. Thomas, Molecular dynamics simulation of site-directed spin labeling: experimental validation in muscle fibers, Biophys. J. 83 (2002) 1854–1866.
[11] D. Stopar, J. Štrancar, R.B. Spruijt, M.A. Hemminga, Exploring the local conformational space of a membrane protein by site-directed spin labeling, J. Chem. Inf. Model. 45 (2005) 1621–1627.
[12] D. Stopar, J. Štrancar, R.B. Spruijt, M.A. Hemminga, Motional restrictions of membrane proteins: a site-directed spin labeling study, Biophys. J. 91 (2006) 3341–3348.
[13] J. Štrancar, T. Koklič, Z. Arsov, B. Filipič, D. Stopar, M.A. Hemminga, Spin label EPR-based characterization of biosystem complexity, J. Chem. Inf. Model. 45 (2005) 394–406.
[14] M. Karplus, J.A. McCammon, The internal dynamics of globular proteins, CRC Crit. Rev. Biochem. 9 (1981) 293–349.
[15] B. Ho, R. Brasseur, The Ramachandran plots of glycine and pre-proline, BMC Struct. Biol. 5 (2005) 14.
[16] B.K. Ho, A. Thomas, R. Brasseur, Revisiting the Ramachandran plot: hard-sphere repulsion, electrostatics, and H-bonding in the α-helix, Protein Sci. 12 (2003) 2508–2522.
[17] J.M. Word, S.C. Lovell, T.H. LaBean, H.C. Taylor, M.E. Zalis, B.K. Presley, J.S. Richardson, D.C. Richardson, Visualizing and quantifying molecular goodness-of-fit: small-probe contact dots with explicit hydrogen atoms, J. Mol. Biol. 285 (1999) 1711–1733.
[18] Z. Xiang, B. Honig, Extending the accuracy limits of prediction for side-chain conformations, J. Mol. Biol. 311 (2001) 421–430.
[19] R.B.M. Koehorst, R.B. Spruijt, F.J. Vergeldt, M.A. Hemminga, Lipid bilayer topology of the transmembrane α-helix of M13 major coat protein and bilayer polarity profile by site-directed fluorescence spectroscopy, Biophys. J. 87 (2004) 1445–1455.
[20] D. Stopar, R.B. Spruijt, M.A. Hemminga, Anchoring mechanisms of membrane-associated M13 major coat protein, Chem. Phys. Lipids 141 (2006) 83–93.
[21] P.V. Nazarov, R.B.M. Koehorst, W.L. Vos, V.V. Apanasovich, M.A. Hemminga, FRET study of membrane proteins: simulation-based fitting for analysis of protein structure, membrane embedment and association, Biophys. J. 91 (2006) 454–466.
[22] P.V. Nazarov, R.B.M. Koehorst, W.L. Vos, V.V. Apanasovich, M.A. Hemminga, FRET study of membrane proteins: determination of the tilt and orientation of the N-terminal domain of M13 major coat protein, Biophys. J. 92 (2007) 1296–1305.
[23] A. Bax, Two-dimensional NMR and protein structure, Annu. Rev. Biochem. 58 (1989) 223–256.
[24] B. Filipič, J. Štrancar, Evolutionary computational support for the characterization of biological systems, in: G.B. Fogel, D. Corne, (Eds.), Evolutionary Computation in Bioinformatics, Elsevier Science, San Francisco, USA 2003, pp. 279–294.
[25] A.A. Kavalenka, B. Filipič, M.A. Hemminga, J. Štrancar, Speeding up a genetic algorithm for EPR-based spin label characterization of biosystem complexity, J. Chem. Inf. Model. 45 (2005) 1628–1635.