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Review

Attaching a spin to a protein — site-directed spin labeling in structural biology

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Site-directed spin labeling and electron paramagnetic resonance spectroscopy have recently experienced an outburst of multiple applications in protein science. Numerous interesting strategies have been introduced for determining the structure of proteins and its conformational changes at the level of the backbone fold. Moreover, considerable technical development in the field makes the technique an attractive approach for the study of structure and dynamics of membrane proteins and large biological complexes at physiological conditions. This review focuses on a brief description of site-directed spin labeling-derived techniques in the context of their recent applications.

Keywords: site-directed spin labeling, electron paramagnetic resonance (EPR) spectroscopy, protein structure

INTRODUCTION

The rapid development of structural methods, such as NMR and X-ray crystallography, results in substantial increase of the speed of new protein structure determination. However, some issues remain generally unattainable to these powerful methods. The fact that the conformation found in the crystallized molecules may not represent the biologically active one, and difficulties in elucidation of the structure of high molecular mass proteins in solution and of membrane proteins are the major problems to solve. Moreover, methods that complement the static protein structures in crystals to offer a dynamic representation of proteins in solution and in large, functionally active supramolecular complexes are currently of exceptional interest.

Over the past decade, electron paramagnetic resonance spectroscopy (EPR) combined with site-directed spin labeling (SDSL) has emerged as a powerful method for investigating protein structure and its dynamics, allowing resolution at the level of the backbone fold (Hubbell & Altenbach, 1994). The absence of unpaired electrons in most biological materials would appear to impede the applicability of EPR methods, but in fact it is advantageous. Labeling of selected molecules on specified sites with a small spin label that has a simple EPR signal, together with the limited number of spins contributing to the EPR experiment enables a broad range of structural problems to be solved. Because the electron has a relatively high magnetic moment, EPR provides the necessary sensitivity to yield spectra with a good signal-to-noise ratio using less than 1 nanomole of a protein.

Protein EPR spectroscopy, based mainly on nitroxide spin labeling, yields information about the nitroxide mobility (Columbus & Hubbell, 2002), its

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Abbreviations: CW EPR, continuous wave electron paramagnetic resonance; DEER, double electron-electron resonance; DQC, double quantum coherence; DSDSL, double site-directed spin labeling; EM, electron microscopy; EPR, electron paramagnetic resonance; MOMD, microscopic order macroscopic disorder; MTSL, 3-methylthiosulfonyl-1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline; NMR, nuclear magnetic resonance; SDSL, site-directed spin labeling; TOAC, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid.

solvent accessibility (Oh *et al.*, 2000; Malmberg & Falke, 2005), the polarity of its microenvironment, and the distance between two nitroxides in double site-directed spin labeling (DSDSL) (Steinhoff, 2004). Thus, obtaining a set of EPR data of spin labeled variants of the examined protein is sufficient to determine its secondary structure elements, relative orientations of the latter and the overall topography of the protein. These methods are appropriate for studying even large membrane proteins or complexes with a great sensitivity to dynamic fluctuations of its structure. This paper reviews briefly the promising EPR methods for structural biology of proteins in the context of the major representative applications.

SPIN LABELING

Initially, biological EPR measurements were limited to metalloproteins possessing its own paramagnetic centres or to proteins with naturally occuring cysteine residues required for capturing spin labels. Since the application of molecular biology (which allowed engineering of cysteine residues at targeted sites in proteins) combined with EPR spectroscopy, SDSL has become a powerful new tool in structural biology (Altenbach *et al.*, 1990). The cysteine scanning method, where selected residues within the amino-acid sequence are substituted with cysteine residues, is easy to use and therefore quite common. Cysteine residues in the protein of interest can be subsequently modified with a variety of sulfhydryl-specific nitroxide spin labels (see Fig. 1). However, the methanethiosulfonate spin probe (MTSL) (Berliner *et al.*, 1982) is currently most often utilized. The resulting side chain, designated as R1, is comparable to phenylalanine or tryptophan side chains in molecular volume and exerts a negligible influence on the biological activity and stability as well as does not change the secondary structure (Mchaourab *et al.*, 1996; Bolin *et al.*, 1998). Moreover, the properties of R1 related to its enhanced sensitivity to protein backbone motion and secondary structure facilitate determination of subtle structural details from the shape of EPR spectra (Owenius *et al.*, 1999; Langen *et al.*, 2000). As indicated in Fig. 1, there is a rotational freedom of the χ_4 and χ_5 torsion angles in the R1 side chain, but the interaction between S_β and the C_α proton limits the χ_1, χ_2 and χ_3 rotamers.

Most recently, an interesting alternative for cysteine spin labeling has been developed. This is based on incorporation of unnatural amino acids into proteins. Tailor-made amino acids carrying a spin label, such as TOAC (McNulty & Millhauser, 2000), can be incorporated into the protein by solid-phase peptide synthesis, alternatively together with recombinant synthesis of proteins (Becker *et al.*, 2005) or by using synthesized amino acylated tRNA for spin-labeled protein expression in living cells (Shafer *et al.*, 2004). By those means, individually designed spin labeled side chains can be incorporated into specific sites of a protein. The reduced residual motion and the defined orientation of such side chains may improve measurements of inter-spin distances and orientations of the nitroxides relative to each other or with respect to the protein backbone.

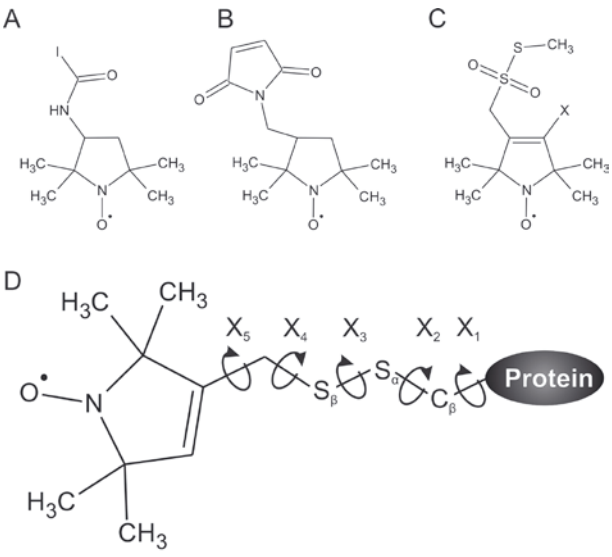

Figure 1. Nitroxide-based spin labels commonly used in SDSL experiments.
A. Proxyl-iodoacetamide (IAP); B. Proxyl-maleimide (MSL); C. Proxyl-methanethiosulfonate (MTSL). D. The structure of the R1 side chain produced by the reaction of MTSL with the sulfhydryl group of a cysteine residue. The dihedral angles associated with each bond are defined as χ_1–χ_5.

MOBILITY

The EPR line shape of the nitroxide side chain is sensitive to the local environment. The label mobility appears to be determined by both backbone motions and steric restraints of neighboring residues, depending on the length and flexibility of the linker between the nitroxide and the protein backbone (Berliner & Reuben, 1989; Hubbell *et al.*, 1996). The correlation between the side chain mobility, including effects due to the motional rate, amplitude and geometry, and the protein structure has been explored in most details in T4 lysozyme (Mchaourab *et al.*, 1996; 1999). Spin labels on the protein surface or in loops between secondary

structure elements display a high degree of mobility, which results in a small apparent hyperfine splitting and line width of the EPR spectrum. On the other hand, a strong interaction of the nitroxide with neighboring side chains or backbone atoms as found in the protein interior or at subunit interfaces is indicated by a broadened line and increased apparent hyperfine splitting (see Fig. 2). Despite the complicated nature of the nitroxide dynamics, reflected by the anisotropy of its motion and distribution of the motional states, EPR line shapes have been successfully analysed in terms of empirical parameters. The first of them is the inverse line width of the central resonance line (ΔH_0^{-1}) (see Fig. 2). Plotting of this parameter as a function of residue number may reveal the secondary structure, as the periodicity of the spin label mobility refers to a sequence of surface, contact or buried sites (Mchaourab *et al.*, 1996; Pfeiffer *et al.*, 1999; Mehboob *et al.*, 2005). The separation between the outer hyperfine extrema ($2A_{par}$) may also be taken as a measure of label mobility (see Fig. 2). A measure of nitroxide side chain mobility, which emphasizes contributions from the outer hyperfine extrema in the spectrum is the inverse second moment ($<H^2>^{-1}$) (see Fig. 2). This parameter provides a measure of the overall breadth of the spectrum (Mchaourab *et al.*, 1996). The reciprocal plot of $<H^2>^{-1}$ *versus* ΔH_0^{-1} has been found to group side chains from different topographical regions of the protein fold into defined sections on the plot demonstrating the correlation between these spectral parameters and regions of secondary structure and tertiary contact (Aihara *et al.*, 2006; Hubbell *et al.*, 1996; Isas *et al.*, 2002) (see Fig. 2). Mobility is not only a "sensor" of conformation, but may also serve as a sensitive monitor of conformational changes in both helices (Rink *et al.*, 1997; Columbus & Hubbel, 2004; Inanami *et al.*, 2005) and β structures (Jiang *et al.*, 1997; Jayasinghe & Langen, 2004) as well as in the folding processes (Morin *et al.*, 2006).

A more quantitative understanding of the motions that generate a given line shape needs a simulation of EPR spectra. Simulations based on dynamic models are in excellent agreement with the corresponding experimental spectra of spin labeled proteins or other biological molecules helping to get a more precise view of the dynamics of the analysed system (Barnes *et al.*, 1999; Borbat *et al.*, 2001). Moreover, molecular dynamics EPR spectra simulations facilitate the study of the influence of the structure adjacent to the spin labeled site on the spectral line shape (Steinhoff *et al.*, 2000; Beier & Steinhoff, 2006). Complications of samples may be described as microscopically ordered but macroscopically disordered (MOMD). The MOMD model has been developed to describe local side chain motion by an order parameter and an effective correlation time which are related to the amplitude of motion and the rate of movement, respectively (Budil *et al.*, 1996). Employing the model to spectra simulations it is possible to determine whether the line shape corresponds to isotropic or anisotropic motion and to detect multiple components of the spectra. The existence of the latter may refer either to several conformational states of the attached spin label or to the heterogenity of protein conformations. Several experimental approaches have been applied to unambiguously assign the components of the EPR spectrum to their respective molecular background, including experiments with spin labels of various structure (Columbus *et al.*, 2001) and studies as a function of temperature (Hanson *et al.*, 2006a). Mutating amino acids adjacent to the spin label helps to

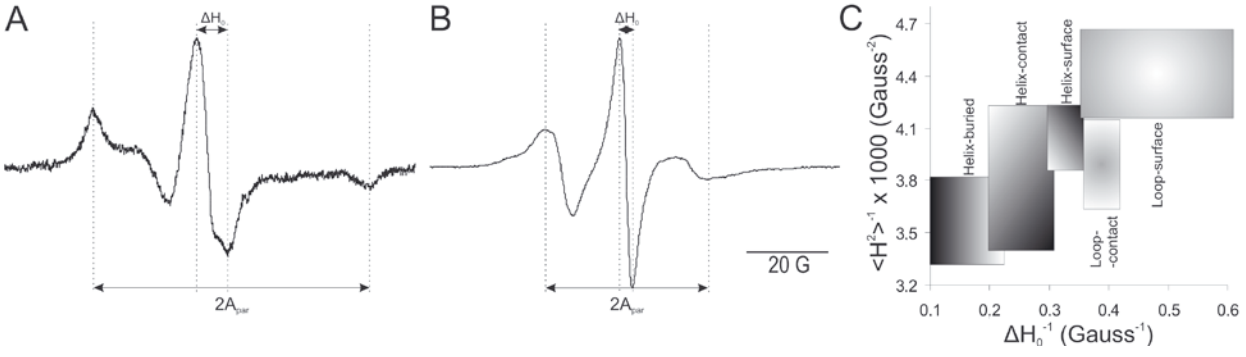

Figure 2. Spin label mobility, as revealed by EPR spectra line shape.
Examples of X-band EPR spectra of (A) motionally restricted spin label of a buried side chain of a helix and (B) increased mobility of an exposed side chain of the same helix forming β spectrin lipid-binding domain (Czogalla, unpublished). The separation between the outer hyperfine extrema ($2A_{par}$) and the peak-to-peak separation of the central line width (ΔH_0) provide a measure of label mobility. C. Mobility map constructed as a plot of the inverse second moment of the EPR spectrum ($<H^2>^{-1}$) *versus* inverse central line width (ΔH_0^{-1}), which indicates the correlation between the measured parameters and regions of protein topology (Isas *et al.*, 2002).

understand the effect of the local environment (Lietzow *et al.*, 2004). The extension of continuous wave (CW) EPR to multiple fields or frequencies helped to increase the signal-to-noise ratio and to improve spectral resolution. High field studies reveal previously unattainable details of spin label motion in its local environment (Mobius *et al.*, 2005; Budil *et al.*, 2006). W-band EPR measurements appeared to be particularly useful for the detection of anisotropy of nitroxide motion when bimodal dynamics of the spin label exists (White *et al.*, 2007).

ACCESIBILITY

The analysis of the collision frequency of a nitroxide side chain with freely diffusing paramagnetic reagents provides additional structural information. Expressed in terms of the accessibility parameter (Π) (Klug *et al.*, 1997), it provides information on protein structure, the tilt of membrane proteins, orientation and position of proteins at the membrane interface (Hubbell *et al.*, 1998). The accesibility of a particular spin-labeled site is determined from the increase of the label T_1 relaxation rate, which is proportional to the bimolecular collisional exchange with a secondary paramagnetic reagent and is measured by changes of the amplitude of the EPR spectrum with increasing microwave power in the presence or absence of a relaxing agent. Thus, the relative accessibility of the spin label to the paramagnetic probe is expressed by the equation: $\Pi(\text{probe}) = P'_{1/2}(\text{probe}) - P'_{1/2}(N_2)$, where $P'_{1/2}$ (probe) and $P'_{1/2}$ (N_2) are the half-saturating powers for samples containing probe or purged with dinitrogen to remove the paramagnetic species, respectively. Such an approach is the most widely utilized one and is known as the power saturation method (Altenbach *et al.*, 1994; Oh *et al.*, 1996; Qin & Cafiso, 1996; Ball *et al.*, 1999). As a rule, the EPR signal amplitude increases linearly with the square root of incident power, until it begins to saturate and decreases in intensity. The microwave power at which the signal amplitude is half-saturated is proportional to the spin-lattice relaxation rate of the label. During exposition of the spin labeled side chain to paramagnetic reagents, an increase in the saturating power can be observed. Apolar oxygen and highly polar metal chelates, such as Ni(II)EDDA or $Cr(C_2O_4)^{3-}$, in a water/phospholipid bilayer system are partitioned between the aqueous and hydrophobic phase according to their properties. Measurements using one or more reagents, with concentration gradients in opposite directions across the biological membranes, may be employed to determine the depth parameter (Malmberg & Falke, 2005). The latter, which is reflected by the equation $\Phi = \ln[\Pi(O2)/\Pi(\text{metal chelate})]$, becomes positive for spin label locations deep in the membrane. Most recently, a ruler for determining the membrane depth and orientation of proteins considering the effect of oxygen alone has been developed (Nielsen *et al.*, 2005).

The accessibility of a spin labeled side chain can also be determined *via* pulsed EPR methods in an approach called saturation recovery (Nielsen *et al.*, 2004). One of the major advantages of this technique is a more precise determination of the side chain conformation and conformational exchange (Pyka *et al.*, 2005). Another innovative approach is the multi quantum EPR (Klug *et al.*, 2005), which allows a better resolution of multiple component spectra and enhanced spectral sensitivity to both T_1 and T_2 (spin-lattice and spin-spin relaxation, respectively).

The depth parameter may become insensitive when applied to membrane-adsorbed peripheral proteins, as the latter are located at the membrane-water interface more than 5 Å away from the lipid phosphates. While the electrostatic potential of a biological membrane surface declines with the distance from that surface, the gradient of electrostatic potential is distributed across the extent of the protein molecule. Thus, the use of charged metal-ion complexes (Lin *et al.*, 1998; Ball *et al.*, 1999) or paramagnetic lipid Ni(II) chelates (Gross & Hubbell, 2002) appeared to be effective for exploring the orientation of proteins or protein domains with respect to the membrane surface. EPR accessibility experiments, used collectively with high-resolution protein structures and molecular dynamics simulations has emerged as an adequate approach for analyzing interactions between proteins and lipid bilayers (Jaud *et al.*, 2007).

SPIN-SPIN DISTANCE

Distance measurement between two site-directed probes in a protein is a powerful method to determine its structure and dynamics. EPR techniques for estimating distances between spins are based on the dipolar interactions between them in double site-directed spin labeling (DSDSL) experiments (Eaton & Eaton, 2000; 2004). Although exchange interactions may also contribute to the interaction between two spin labels, they are negligible for relatively long interspin distances without a short through-bond pathway. The electron-electron dipolar interaction results in considerable broadening or dipolar splitting of the CW EPR spectrum when compared with the noninteracting spins. When two nitroxides adopt a specific, rigid geometry, both the relative distance and orientation between the two spins can be determined (Hustedt & Beth, 1999). The

recently developed tether-in-a-cone model allows one to analyse EPR spectra in terms of both distance distribution and restricted distributions of the relative orientation between probes (Hustedt *et al.*, 2006). In other cases, distance determination is followed by the use of different sets of assumptions.

In a frozen state, broadening of a CW EPR spectrum due to nitroxide–nitroxide interactions changes the relative intensities of the three components of the spectrum. Considering that the dipolar spin-spin splitting is not resolved and that the relaxation rate (T_1) is slow relative to the splitting, the ratio of peak heights (d_1/d) (see Fig. 3B) can be used to estimate the distance between the two spins (Kokorin *et al.*, 1972; Sun *et al.*, 1999).

In the cases of relatively short inter-spin distances (up to 8 Å), where it is the most important to separate the dipolar interaction and exchange contributions, half-field transitions have been successfully applied (Eaton *et al.*, 1983; Persson *et al.*, 2001). However, the existence of a conformational heterogenity may shift the average distance values strongly toward shorter ones, when the half-field transition method is applied.

Most DSDSL experiments fall into the case when spins adopt a statistical distribution of distances and relative orientations. The deconvolution methods assume that there is sufficient flexibility in the linkage between the two nitroxides, which is indeed true for the R1 side chain, and thus the interspin vector orientation is randomly distributed relative to the magnetic axes of the paramagnetic centres (Steinhoff, 2004). In the crystal structures of spin-labeled T4 lysozyme, the electron density for the nitroxyl ring was poorly defined (Langen *et al.*, 2000), which is consistent with a distribution of

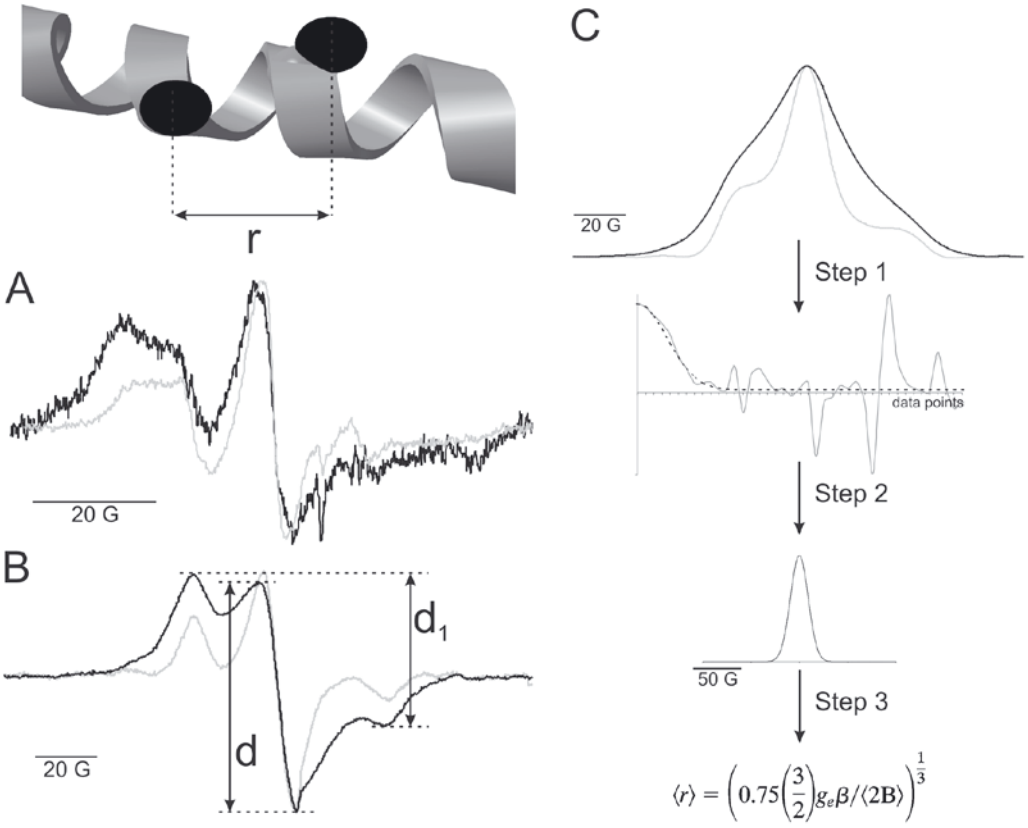

Figure 3. Distance measurements between two spin labels attached to the helical region of the lipid-binding domain of β spectrin (Czogalla *et al.*, 2007).
EPR spectra of a peptide labeled on the *i, i+3* positions measured in 40% sucrose (A) or at liquid nitrogen temperature (B) with the corresponding spectra of singly labeled peptides (grey). Peak heights d and d_1 of the spectrum of the doubly labeled peptide are indicated. C. The algorithm for distance analysis using Fourier deconvolution method (Rabenstein & Shin, 1995; Xiao & Shin, 2000). Step 1: Absorbance spectrum of two interacting labels (Π(B)) (black) and non-interacting reference spectrum (S(B)) (grey) are Fourier transformed to Π*(ω) and S*(ω), respectively. Dividing Π*(ω) by S*(ω) the broadening function in the Fourier space (M*(ω)) is obtained. A Gaussian curve (dotted line) is fit to the broadening function (M*(ω)) (grey) in order to suppress noise. Step 2: The fit function is reverse-Fourier transformed to provide the broadening function in real space (M(B)). Step 3: The latter is utilized to estimate average splitting value (<2B>) and the mean spin-spin distance (<r>) is determined according to the equation, where g_e is the isotropic g value for electrons, β is the electron Bohr magneton, and μ_0 is the permittivity of a vacuum. All EPR spectra are shown with the y-axis scale adjusted to match peak heights and the broadening of the doubly labeled spectra corresponds to the average distance between nitroxides about 9 Å.

conformations. The main idea of the deconvolution methods is to compare the spectra for the doubly spin-labeled proteins with the corresponding singly labeled samples with noninteracting nitroxides (see Fig. 3). Motional averaging of the dipolar interaction is restricted by freezing the samples (Rabenstein & Shin, 1995; Steinhoff *et al.*, 1997) or increasing the viscosity of solution (Altenbach *et al.*, 2001). An EPR "spectroscopic ruler" method is based on Fourier deconvolution of the dipolar broadening function for two interacting spins from the dipolar broadened CW EPR spectrum (Rabenstein & Shin, 1995; Xiao & Shin, 2000). That function is then used to obtain the mean interspin distance and the variance of its distribution. The method has proven to be effective in the range of 7.5–25 Å (Xiao & Shin, 2000; Zhang *et al.*, 2002) with a standard deviation of 0.9 Å for the entire range. An alternative approach consists in fitting of the simulated dipolar broadened spectrum to the experimental one by multiplication of the monoradical spectrum by a broadening function (Steinhoff *et al.*, 1997). By that means the average distance as well as the width of the Gaussian distance distribution between the attached spin labels is calculated from the fitted line width function (Radzwill *et al.*, 2001). The approaches originally developed for rigid conditions can be successfully used to estimate dipolar interactions between nitroxides even at physiological temperatures, provided the rotational correlation time of the interspin vector is long enough (Altenbach *et al.*, 2001). Applying the above methods it is necessary to take into account the variable fraction of incomplete spin-labeled protein, as it may be a major source of error in most DSDSL experiments.

The weaker dipole-dipole interactions at distances longer than 20 Å result in a gradual loss of line broadening. Combination of DSDSL with pulsed EPR techniques increases the measurable distance range up to 80 Å. However, such experiments require specialized EPR spectrometers that only recently became comercially available. One of these methods, double electron-electron resonance (DEER), also called pulsed electron-electron resonance (PELDOR), reveals distance information by producing a spin echo that is modulated with a periodicity related to the dipolar interaction and the resulting oscilations in the spin echo amplitude correlate with the distance and its distribution (Pannier *et al.*, 2000; Jeschke, 2002). The great sensitivity of the DEER technique allows studies of membrane proteins in their native environment (Jeschke *et al.*, 2004). Even more sensitive is another pulsed method, double quantum coherence (DQC) (Borbart & Freed, 1999; Chiang *et al.*, 2005) which is especially suitable for long distance measurements (Hara *et al.*, 2007). The major limitation of the DEER and DQC methods is the necessity to perform measurements at low temperatures at which the spin-spin relaxation time is long enough to allow echo detection.

By using multiple DSDSL techniques a more complete picture can be obtained, as the CW EPR and pulsed EPR methods perfectly complement each other and the obtained results are in reasonable agreement (Persson *et al.*, 2001). However, due to multiple rotameric states of spin labels an uncertainity of the backbone-spin distance and difficulties in the interpretation of the spin-spin distances in terms of the protein backbone may occur. Using the modeling strategy to find the lowest energy rotamers, it is possible to obtain a higher resolution in measurements of distances and distance distributions (Sale *et al.*, 2005).

ILLUSTRATIVE APPLICATIONS

SDSL combined with the described EPR methods have been successfully used to determine the structure of proteins and, more importantly, to follow the conformational changes related to their biological activity. The distance measurements between nitroxides has proven to be a valuable approach for detailed helical geometry analysis (Czogalla *et al.*, 2007) as well as for following structural transitions in peptides while unfolding (McNulty *et al.*, 2001). A combination of line shape, accessibility and distance EPR measurements makes possible a precise location of secondary structure elements and their arrangement into a tertiary fold of flexible proteins such as apolipoprotein A-I (Langerstedt *et al.*, 2007). Using a similar set of EPR techniques structural models are obtained which can be subsequently confronted with the corresponding crystallographic results. For example, the crystal structure of the cytoplasmic domain of erythrocyte membrane band 3 has been recently confirmed in solution (Zhou *et al.*, 2005). On the other hand, EPR methods appeared to be more reliable and sufficient to investigate the structure and structural changes of the ABC transporter MsbA in bilayers (Dong *et al.*, 2005). Similarly, dynamic models of ligand-induced conformational changes can be obtained (Hurth *et al.*, 2004). SDSL and EPR experiments provide high resolution structural information detailing protein–protein interactions. This enables identification of molecular arrangements between subunits of intermediate filaments at physiological conditions (Hess *et al.*, 2002), interactions between arrestin and rhodopsin (Hanson *et al.*, 2006b) and docking between the SecB and SecA chaperones of *E. coli* (Crane *et al.*, 2005).

Accessibility analysis has provided new capabilities for positioning and orienting spin-labeled proteins or their domains at the membrane-water interface. Peripheral proteins containing C2 do-

mains analyzed by this technique include cytosolic phospholipase A2 (Malmberg *et al.*, 2003; Nielsen *et al.*, 2005), protein kinase Cα (Kohout *et al.*, 2003) and synaptotagmin I (Rufener *et al.*, 2005; Herrick *et al.*, 2006). An analogous approach was used to determine membrane binding and penetration of annexin B12 (Isas *et al.*, 2005; Hedge *et al.*, 2006) and peptides derived from myelin basic protein (Musse *et al.*, 2006).

One attractive feature of the SDSL methods is the ability to follow the changes in the protein structure with millisecond timescale. Thanks to time-resolved EPR experiments on doubly labeled bacteriorhodopsin, it was possible to monitor the light-induced rotation of its transmembrane helices (Radzwill *et al.*, 2001; Xiao *et al.*, 2000). Similarly, structural changes upon protein–protein interaction such as rhodopsin–transducer interaction (Wegener *et al.*, 2000) and receptor-activated conformational cycle of Gα protein (Oldham *et al.*, 2006) can be observed. DSDSL studies also indicated the structural details responsible for the activation of rhodopsin and the effect of mutations which cause permanent activation of the visual transduction cascade (Kim *et al.*, 2004). The structure and function of rhodopsin appeared to be influenced by detergent-solubilization or lipid reconstitution, indicating applicability of the EPR structural methods as evaluation tools for non-native conditions imposed by a variety of biochemical techniques (Klare *et al.*, 2006; Kusnetzow *et al.*, 2006). Recent applications of SDSL also include examination of the structure and gating mechanisms of membrane ion channels such as mechanosensitive MscL ion channel (Perozo *et al.*, 2002), voltage dependent KvAP potassium channel (Cuello *et al.*, 2004), and proton-dependent potassium channel KcsA (Cordero-Morales *et al.*, 2006). Furthermore, the power of EPR spectroscopy of proteins has been demonstrated by determination of the structure and conformational changes of transporters, including the PutP proline-sodium symporter (Jeschke *et al.*, 2004), the multidrug-resistant transporter MsbA (Buchaklian & Klug, 2006), and the Ton box motif of the ButB (Fanucci *et al.*, 2003). In the case of the latter, a B_{12} vitamin transporter, it appeared that conformational changes may be modulated by solution osmolality, which reflects the differences between the crystal structure and the bilayer-reconstituted structure of the protein (Kim *et al.*, 2006). There are also a number of publications where SDSL was used to investigate the structure, conformation and assembly of the SNARE complexes (Chen *et al.*, 2004; Xu *et al.*, 2005; Zhang *et al.*, 2005), which enabled a more precise view of membrane fusion events and their regulation.

CONCLUDING REMARKS

Recent technical advances together with widespread applications have established SDSL as a well-developed structural biology technique. EPR measurements can be used not only to obtain the structure of a protein but also to elucidate the mechanisms of its biological activity, which is often unattainable from other biophysical techniques. Moreover, SDSL studies can complement other methodologies (e.g. X-ray crystallography, NMR, cryo-EM) yielding a new and powerful class of experimental approaches. The utility of the method is not limited to proteins, and a great progress has been made in applying SDSL to study nucleic acids and other biologically active molecules. Taken together, SDSL emerges as a promising technique for the study of structure and conformational dynamics in a wide range of biological systems.

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