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Site-Directed Spin Labeling and Electron Paramagnetic Resonance (EPR) Spectroscopy: A Versatile Tool to Study Protein-Protein Interactions

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1. Introduction

The function of a living cell, independent of we are talking about a prokaryotic single-cellular organism or a cell in the context of an complex organism like a human, depends on intricate and balanced interaction between its components. Proteins are playing a central role in this complex cellular interaction network: Proteins interact with nucleic acids, with membranes of all cellular compartments, and, what will be in the focus of this article, with other proteins. Proteins interact to form functional units, to transmit signals for example perceived at the surface of the cell to cytoplasmic or nuclear components, or to target them to specific locations. Thus, the study of protein-protein interactions on the molecular level provides insights into the basic functional concepts of living cells and emerged as a wide field of intense research, steadily developing with the introduction of new and refined biochemical and biophysical methods.

Nowadays there is a vast of methods available to study the interaction between proteins. On the biochemical level mutational studies, crosslinking experiments and chromatographic techniques provide means to identify and characterize the interfaces on the protein surface where interaction takes place. Biophysical methods include calorimetric techniques, fluorescence spectroscopy and microscopy, and "structural techniques" like X-ray crystallography, (cryo-) electron microscopy, NMR spectroscopy, FRET spectroscopy, and EPR spectroscopy on spin labeled proteins.

Site-directed spin labeling (SDSL) (Altenbach et al., 1989a, 1990) in combination with electron paramagnetic resonance (EPR) spectroscopy has emerged as a powerful tool to investigate the structural and the dynamical aspects of biomolecules, under conditions close to physiological i.e. functional state of the system under exploration. The technique is applicable to soluble molecules and membrane bound proteins either solubilised in detergent or embedded in a lipid bilayer. Therein, the size and the complexity of the system under investigation is almost arbitrary (reviewed in Bordignon & Steinhoff, 2007; Hubbell et al., 1996; Hubbell et al., 1998; Klare & Steinhoff, 2009; Klug & Feix, 2007). Especially with respect to protein-protein interactions SDSL EPR can provide a vast amount of information

about almost all aspects of this interaction. Spin labeling approaches can provide detailed information about the binding interface not only on the structural level but also give insights into kinetic and thermodynamic aspects of the interaction. EPR also allows determination of distances between pairs of spin labels in the range from ~ 10-80 Å with accuracies down to less than 1 Å, thereby covering a range of sizes including also large multi-domain proteins and protein complexes.

This chapter will give an introduction into the technique of SDSL EPR spectroscopy exemplified with data from studies on the photoreceptor/transducer-complex *Np*SRII/*Np*HtrII, followed by a number of recent examples from the literature where protein-protein interactions have been studied using this technique.

2. Site-Directed Spin Labeling (SDSL)

2.1 Spin labeling of cysteines

For the modification proteins with spin labels, three different approaches have been established. The most commonly used method utilizes the reactivity of the sulfhydryl group of cysteine residues being engineered into the protein applying site-directed mutagenesis. This approach usually requires that the protein of interest possesses only cysteine residues at the desired sites, and that additional cysteine residues present can be replaced by serines or alanines without impairment of protein functionality. Among the various spin labels available the (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate spin label (MTSSL) (Berliner et al., 1982) is most often used due to its sulfhydryl specificity, and its small molecular volume comparable to that of a tryptophane side chain (Fig. 1A,B). The spin label is bound to the protein by formation of a disulfide bond with the cysteine, and the resulting spin label side chain is commonly abbreviated R1. The linker between the nitroxide ring and the protein backbone renders the R1 side chain flexible (Fig. 1B), thus minimizing disturbances of the native fold and the function of the protein it is attached to. In addition, the unique dynamic properties of this spin-label side chain provide detailed structural information from the shape of its room temperature EPR spectrum. Besides MTSSL, a variety of different nitroxide radical compounds are commercially available, for example the 1-oxyl-2,2,5,5-tetramethyl, 2,5-dihydro-1H-pyrrol-3-carboxylic acid (2-methanethiosulphonyl-ethyl) amide (MTS-4-oxyl) spin label (Fig. 1C), comprising different linkers and/or nitroxide moieties. Also pH sensitive spin probes have been used to label the thiol group, e.g. of a synthetic peptide fragment of the laminin B1 chain (Smirnov et al., 2004).

The widely used methanethiosulfonate spin labels suffer from a significant drawback which is the sensitivity of the disulfide bond towards reducing agents like DTT, leading to immediate release of spin label side chains. If reducing conditions are required for sample preparation and/or stability, acetamide or maleimide-functionalized spin label compounds (Steinhoff et al., 1991; Griffith and McConnell, 1966) (Fig. 1D) can be used alternatively. In this case, the spin label is bound via a CS bond, which is not affected by reducing conditions.

Isotopically labeled nitroxide compounds where ^{14}N is exchanged by ^{15}N are important for specialized applications. The corresponding EPR spectra are characterized by a two line spectrum instead of a three line spectrum of the ^{14}N, and the lines of a ^{15}N spectrum are well

separated from the ^{14}N lines so that both labels can be used simultaneously in a single experiment (Steinhoff et al., 1991).

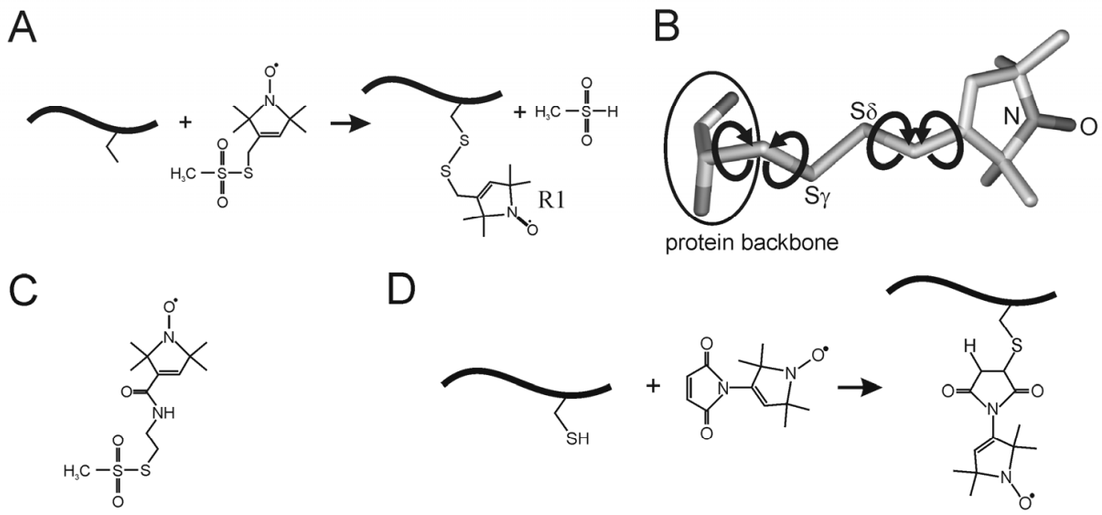

Fig. 1. Spin labeling of cysteines (A) Reaction of the methanethiosulfonate spin label (MTSSL) with a cysteine side chain, generating the spin label side chain R1. (B) Flexible bonds within the R1 side chain are indicated. (C) Chemical structure of the MTS-4-oxyl spin label. (D) Reaction of a maleimide spin label N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide with a cysteine side chain.

2.2 Spin labeling by peptide synthesis

A large variety of spin label building blocks for Boc- or Fmoc-based step-by-step peptide synthesis either on a solid support (SPPS) (Merrifield, 1963) or in solution have been synthesized (Barbosa et al., 1999; Elsässer et al., 2005). Being the most popular one, the paramagnetic α-amino acid TOAC (4-amino-1-oxyl-2,2,6,6,-tetramethyl-piperidine-4-carboxylic acid) (Rassat and Rey, 1967) is characterized by only one degree of freedom, the conformation of the six-membered ring (Fig. 2) The nitroxide is rigidly coupled to the peptide backbone, thereby providing the possibility to obtain direct information about the orientation of secondary structure elements, and has for example been used to study the secondary structure of small peptides in liquid solution (Anderson et al., 1999; Hanson et al., 1996; Marsh et al., 2007), and has also been successfully incorporated into the α-melanocyte stimulating hormone without loss of function (Barbosa et al., 1999).

The chemical synthesis of proteins with incorporated unnatural spin labeled amino acids relies on the ability to produce the constituent peptides, typically by SPPS. Although synthesis of polypeptides consisting of more than 160 amino acids (Becker et al., 2003) has become possible through improvements in peptide chemistry, aiming at the incorporation of spin labels into large proteins, esp. membrane proteins, SPPS has to be combined with recombinant techniques. The expressed protein ligation (EPL), also named intein mediated protein ligation (IPL) technique, can be used to semisynthesize proteins from recombinant and synthetic fragments, thereby extending the size and complexity of the protein targets.

The underlying chemical ligation of two polypeptide fragments requires an N-terminal cysteine on one and a C-terminal thioester moiety on the other fragment. After rearrangement through an S→N acyl shift, a native peptide bond is formed. The reaction can be performed also in the presence of other unprotected cysteine residues because of a reversible reaction preceding an irreversible step. Using this methodology, a spin-labeled Ras binding domain has been synthesized, showing a stable paramagnetic center detected by EPR (Becker et al., 2005).

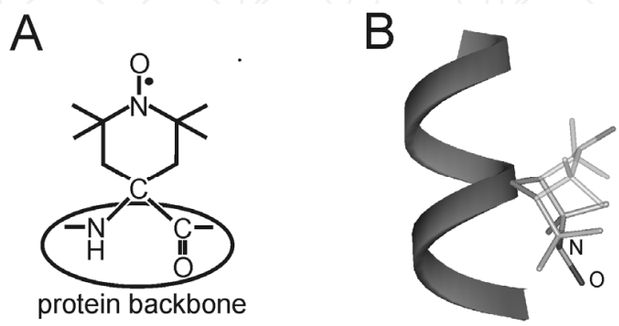

Fig. 2. The TOAC amino acid spin label. (A) chemical structure. (B) three-dimensional structure of the spin label incorporated into an a-helix. The flip of the six-membered ring as the only possible degree of freedom is shown in shaded representation.

2.3 Spin labeling using nonsense suppressor methodology

Spin label amino acids can been introduced into proteins by employing the nonsense suppressor methodology, for example by utilizing the amber suppressor tRNA chemically aminoacylated with the desired spin label amino acid (Cornish et al., 1994). Although this strategy might prove generally applicable in the future using unique transfer RNA(tRNA)/aminoacyl–tRNA-synthetase pairs (Chin et al., 2003), only few laboratories are currently equipped to apply this methodology successfully.

2.4 Spin labeling using nonsense suppressor methodology

As introduced by Kolb, Finn and Sharpless in 2001 (Kolb et al., 2001), the basic concept of "click chemistry" is the highly selective formation of a carbon-heteroatom bond under mild conditions with high yield. Its modular concept renders it a favorable tool for introducing labels into biomolecules. An example is the 1,3-dipolar cycloaddition of organic azides with alkynes in the presence of Cu which has been used to attach fluorescent probes to biomolecules (Deiters and Schultz, 2005). Recently, Tamas and coworkers (Tamas et al., 2009) described the synthesis of nitroxide moieties suitable for click chemistry, thereby opening this approach also for site-directed spin labeling.

3. EPR analysis of spin labeled proteins

In the following, the different experimental techniques of EPR spectroscopy on spin labeled proteins are introduced. Therein, the methods are exemplified with the sensory rhodopsin-transducer complex mediating the photophobic response of the halophilic archaeum

Natronomonas pharaonis. The photophobic response of this organism to green-blue light is mediated by sensory rhodopsin II, *Np*SRII, which is closely related to the light driven proton pump bacteriorhodopsin (for a recent review see (Klare et al., 2007)). *Np*SRII is a seven transmembrane helix (A–G) protein with a retinal chromophore covalently bound via a protonated Schiff base to a conserved lysine residue on helix G. Signal transduction to the intracellular two-component pathway modulating the swimming behavior of the cell takes place via the interaction of *Np*SRII with the tightly bound transducer protein, *Np*HtrII (halobacterial transducer), in a 2:2 complex. A transducer dimer comprising a four-helix transmembrane domain, a linker region consisting of two HAMP domains (Aravind and Ponting, 1999), and a cytoplasmic signaling domain, is flanked by the two SRII receptors.

3.1 Spin label dynamics

The shape of room temperature cw EPR spectra reflects the reorientational motion of the spin label side chain. The influence of spin label dynamics on the spectral shape has been reviewed in detail (Berliner, 1976; Berliner, 1979; Berliner & Reuben, 1989), and the relationship between the dynamics of the spin label side chain and protein structure has been extensively studied for T4 lysozyme (Columbus et al., 2001; Columbus & Hubbell, 2002; Fleissner et al., 2009, 2011; Mchaourab et al., 1996).

In general, the term "mobility" is used to characterize the effects on the EPR spectral features due to the motional rate, amplitude, and anisotropy of the overall reorientational motion of the spin label. Spin labeled sites exposed to the bulk water exhibit weak interaction with the rest of the protein as found for helix surface sites or loop regions and consequently display a high degree of mobility, that is characterized by a small apparent hyperfine splitting and narrow line widths (Fig. 3A & 3B, position 154). If the mobility of the spin label side chain is restricted by interaction with neighboring side chains or backbone atoms of the protein itself or an interaction partner, the line widths and the apparent hyperfine splittings are increased (Fig. 3A & 3B, position 159). Although the relation between the nitroxide dynamics and the EPR spectral line shape is complex, the line width of the centre line, ΔH^0, and the second moment of the spectra, $\langle H^2 \rangle$, have been found to be correlated with the structure of the binding site environment and can therefore be used as mobility parameters (Hubbell et al., 1996; Mchaourab et al., 1996).

The plot of these mobility parameters versus the residue number reveals secondary structure elements through the periodic variation of the mobility as the spin label sequentially samples surface, tertiary, or buried sites. This allows assignment of α-helices, β-strands, or random structures. A more general classification of regions exhibiting buried, surface-exposed, or loop residues can be obtained from the correlation between the inverse of the two mobility parameters, as shown in Figure 3C. Side chains from different topographical regions of a protein can be thereby classified on the basis of the x-ray structures of T4 lysozyme and annexin 12 (Hubbell et al., 1996; Isas et al., 2002; Mchaourab et al., 1996).

For a more quantitative interpretation of the experimental data in terms of dynamic mechanisms and local tertiary interaction, EPR spectra simulations have to be performed. Based on dynamic models developed by Freed and coworkers (Barnes et al., 1999; Borbat et al., 2001; Freed, 1976), excellent agreement of simulations with the corresponding

experimental spectra can be obtained. Furthermore, simulations of EPR spectra can be performed on the basis of molecular dynamics (MD) simulations (Beier and Steinhoff, 2006; Budil et al., 2006; Oganesyan, 2007; Sezer et al., 2008; Steinhoff et al., 2000a; Steinhoff and Hubbell, 1996). Thus, a direct link is provided between molecular structure and EPR spectral line shape, thus allowing verification, refinement, or even de-novo prediction (Alexander et al., 2008) of structural models of proteins or protein complexes.

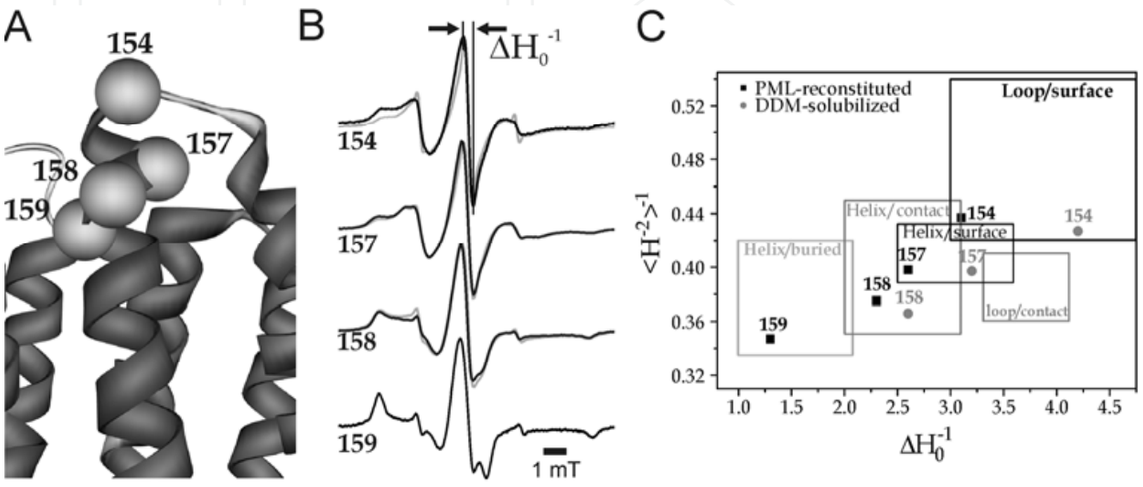

Fig. 3. Mobility analysis of spin labelled proteins. (A) Crystal structure of *Np*SRII (Luecke et al., 2001). The Cα atoms of spin labeled sites are shown as spheres. (B) X band EPR spectra of spin labeled *Np*SRII solubilised in detergent (gray) or reconstituted in purple membrane lipids (black). (C) Two-dimensional mobility plot of the inverse of the second moment *vs.* the inverse of the central linewidth (solubilized: gray circles, reconstituted: black squares), determined from the spectra in B. Boxes indicate the topological regions of proteins according to Isas et al. (2002) and Mchaourab et al. (1996).

The motion of a nitroxide spin label side chain is characterized by three correlation times, the rotational correlation time for the entire protein, the effective correlation time due to the rotational isomerization spin label linker, and the effective correlation time for the segmental motion of the protein backbone. These correlation times can significantly differ in the time scales they occur on. Thus, experimental data for all relevant time scales have to be available to set up an appropriate dynamical model. For this case, correlation times from μs (for the overall protein motion) down to ps (for the rotational isomerization) have to be covered by the experiment. EPR spectra at different microwave frequencies are sensitive to motions on different time scales. EPR at lower frequencies is sensitive to slower motions whereas faster motions are completely averaged out. On the other hand, high-frequency EPR can resolve such fast motions, but slower motions are "frozen" at the high-frequency time scale. Consequently, combining experiments at different microwave frequencies (multifrequency EPR) allows separation of various motional modes in a spin labelled protein according to their different time scales. Most of the work so far has been done using spin-labeled T4 lysozyme as a model system (Liang & Freed, 1999; Liang et al., 2004; Zhang et al., 2010).

Proteins and protein complexes are inherently dynamic structures that can exhibit a number of conformational substates often playing a key role for their function (Cooper, 1973,

Frauenfelder et al., 1988, 1991). A given state of a protein consists of a limited number of such substates with life times in the μs to ms range that can, for example, correspond to "bound" and "unbound" conformations of a protein binding interface. According to the life time of the substates they often can be recognized in room temperature cw spectra of spin labeled proteins if they are characterized by different spin label side chain mobilities due to structural changes in their vicinity. In the past years, Hubbell and co-workers established three experimental techniques to analyze conformational equilibria in proteins and to dissect them from spin label rotameric exchange, namely osmolyte perturbation (Lopez et al., 2009), saturation recovery (Bridges et al., 2010) and high-pressure EPR (McCoy & Hubbell, 2011).

3.2 Spin label solvent accessibilities

Supplementing the motional analysis, the accessibility of the spin label side chain toward paramagnetic probes (exchange reagents), which selectively partition in different environments of the system under investigation, can be used to define the location of spin label with respect to the protein/water/membrane boundaries. The accessibility of the nitroxide spin label side chain is defined by its Heisenberg exchange frequency, W_{ex}, with an exchange reagent diffusing in its environment. Water-soluble metal ion complexes like NiEDDA or chromium oxalate (CrOx) allow to quantify the accessibility from the bulk water phase, whereas molecular oxygen or hydrophobic organic radicals that mainly partition in the hydrophobic part of the lipid bilayer define the accessibility from the lipid phase. The concentration gradients of NiEDDA and molecular oxygen along the membrane normal can be used to characterize the immersion depth of the spin label side chain into the lipid bilayer (Altenbach et al., 1994; Marsh et al., 2006). Two experimental techniques can be used to determine the nitroxide's accessibility toward the paramagnetic probes: Cw power saturation, and saturation recovery.

Most commonly, Heisenberg exchange rates for nitroxide spin label side chains in proteins are measured using cw power saturation. Here, the EPR signal amplitude is monitored as a function of the incident microwave power in the absence and presence of the paramagnetic quencher. From the saturation behaviour of the nitroxide, an accessibility parameter, Π, can be extracted that is proportional to W_{ex} (Altenbach et al., 1989a; Altenbach et al., 2005; Farabakhsh et al., 1992). In Figure 4, the accessibility analysis performed on a 24 amino acid long segment starting at position 78 in the transmembrane region and extending to position 101 in the cytoplasm of the transducer protein *Np*HtrII in complex *Np*SRII is shown as an example for this technique (Bordignon et al., 2005). Figure 4A shows the crystal structure of the transmembrane region of the complex (Gordeliy et al., 2002). Power saturation experiments have been performed with air (21% O_2) and 50 mM CrOx, respectively. The Π values calculated from these experiments are shown in panel B versus residue number. The low Π values for both oxygen and CrOx for residues 78 to 86 indicate their location in a densely packed protein-protein interface. The clear periodicity of 3.6 residues (see inset in panel B) reflects the α-helical structure. For positions 87 to 94 a gradual increase in the Π_{CrOx} and Π_{oxygen} values is observed, indicating that this region is protruding away from the protein-protein interface into the cytoplasm. For positions 92 to 101 the Π_{CrOx} and Π_{oxygen} values observed are typical for water exposed residues. Also here a periodical pattern corresponding to an α-helical structure is observed.

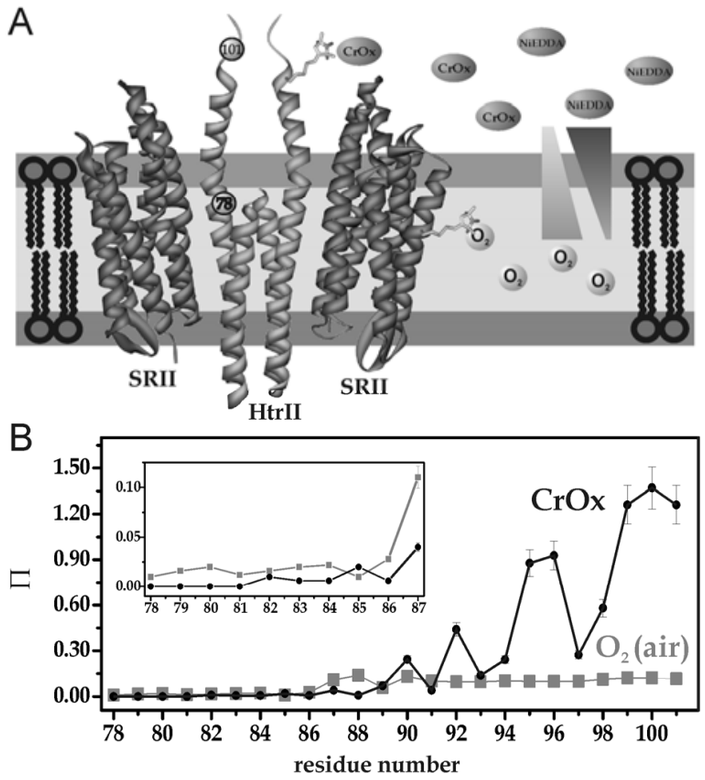

Fig. 4. Spin label accessibilities by power saturation. (A) Structure of the *Np*SRII/*Np*HtrII complex in a lipid bilayer (light gray: hydrophobic region, medium gray: headgroup region). The concentration gradients for water-soluble reagents (CrOx and NiEDDA) and lipid-soluble reagents (O_2) are indicated by shaded triangles. The first (78) and last residue (101) of the region investigated are indicated. (B) Accessibility parameters Π_{CrOx} (black circles) and Π_{Oxygen} (gray squares) vs. residue number. Π_{CroX} values have been obtained with 50 mM CrOx, Π_{Oxygen} values with air (21% O_2). The inset depicts magnified the region from residues 78 to 87 to show the periodicity of 3.6 for Π_{CroX} and Π_{Oxygen}.

Saturation recovery EPR (SR-EPR) allows measuring the spin–lattice relaxation time T_{1e} which is connected to the Heisenberg exchange frequency W_{ex} by

$$W_{ex} = \Delta\left(\frac{1}{T_{1e}}\right)_R \tag{5}$$

directly. In this type of experiment, saturating microwave pulses are applied to the sample in the absence and in the presence of exchange reagents, and the recovery of the *z*-magnetization is monitored as a function of time. Analyses of the recovery curves provide T_{1e} and thus the accessibility for the respective exchange reagent (Altenbach et al., 1989a, 1989b; Nielsen et al., 2004). One major advantage of SR-EPR compared to cw power saturation is that in the presence of multiple spin populations (see chapter 2.1) all corresponding T_{1e} values and accessibilities can be determined by SR-EPR. In contrast, cw power saturation can only provide an average accessibility for all components present in the cw EPR spectrum, and moreover, this average value will be biased towards the most mobile

component as it dominates the amplitude of the resonance lines. Moreover, saturation recovery can be used to distinguish between rotamer exchange for the spin label side chain (~ 0.1-1 μs range) and conformational exchange of the protein, which is at least one order of magnitude slower (Bridges et al., 2010).

3.3 Polarity and proticity of the spin label micro-environment

Polarity and proticity of the spin label microenvironment are reflected in the hyperfine component A_{zz} and the g tensor component g_{xx}. A polar environment shifts A_{zz} to higher values, whereas the tensor component g_{xx}, determined from the B-field of the canonical peak position ($g_{xx} = h\nu / \mu_B B$), is decreased. The A_{zz} component can be obtained from cw X-band EPR spectra of spin labeled proteins in frozen samples. The principal *g*-tensor components and their variation can be determined with high accuracy using high-field EPR techniques due to the enhanced Zeeman resolution (Steinhoff et al., 2000b). In regular secondary structure elements with anisotropic salvation (e.g., surface exposed α-helices), the water density and hence the tensor component values A_{zz} and g_{xx} are a periodic function of residue number. Therefore, similarly to accessibility measurements with water soluble exchange reagents (see 3.2), these data can be used to obtain structural and topological information and the polarity of the spin label environment can reveal detailed information on the protein fold.

The polarity parameter values for the sequence 88 to 94 in the first HAMP domain of *Np*HtrII in complex with *Np*SRII are shown in Figure 5A (Brutlach et al., 2006). It is evident that positions 90 and 93 in *Np*HtrII are located in a more polar, water accessible environment. The same holds for position 154 on the cytoplasmic surface of *Np*SRII. In contrast, positions 88, 89, 91, 92 and 94 reside in a more apolar environment that is less accessible to water. Also evident is a periodical pattern, characterizing the α-helical structure. The exceptional apolar character of position 78 indicates that this side chain is deeply buried in a protein-protein or protein-lipid interface. These results are reflected in a structural model, which has been based in

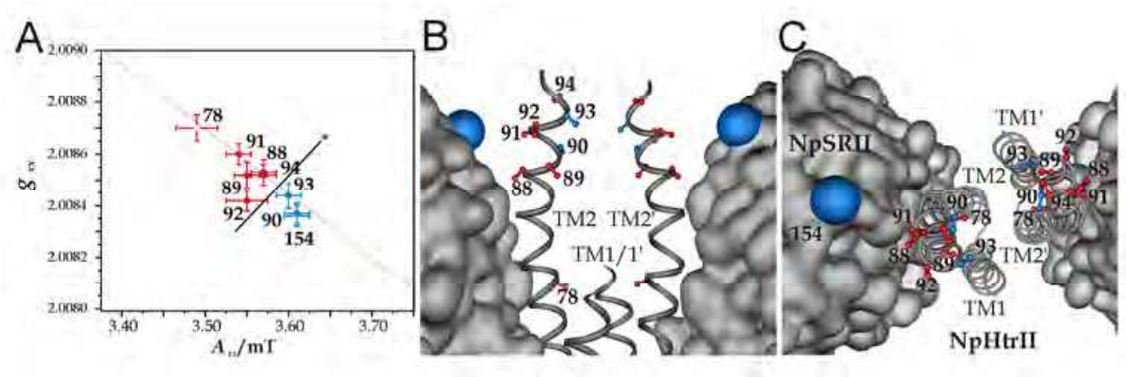

Fig. 5. (A) Plot of g_{xx} versus A_{zz} for positions 88 to 94 in *Np*HtrII according to Brutlach et al., 2006. The plot also includes values for position 78 on the second transmembrane helix (TM2) and for position 154 in *Np*SRII. An arbitrary threshold of g_{xx} / A_{zz} indicated by the diagonal line marked with * classifies the sites into more polar (blue) or more apolar sites (red).
(B) Side view onto *Np*SRII (surface representation) and the four-helix bundle of the transducer (ribbon representation) up to position 96 according to Bordignon et al. (2005).
(C) Cytoplasmic view of the structural model.

addition on mobility, accessibility and distance data (see 2.4) for this region (Figure 5B and C) (Bordignon et al., 2005). Residues 88, 91 and 92 are located in protein-protein interfaces, and positions 90 and 93 are positioned at the opposite side of the transducer helix. Position 78 is, in line with the exceptional low polarity of its micro-environment, buried in the densely packed four helix bundle of the *Np*HtrII dimer.

3.4 Inter-spin label distance measurements

If two spin label side chains are introduced into a biomolecule or two singly labeled molecules are in a stable macromolecular complex, the distance between the two labels can be determined through quantification of their spin–spin interaction, thus providing valuable structural information.

Spin–spin interaction is composed of static dipolar interaction, modulation of the dipolar interaction by the residual motion of the spin-label side chains, and exchange interaction. The static dipolar interaction in an unordered immobilized sample leads to considerable broadening of the cw EPR spectrum if the inter spin distance is less than 2 nm (Figure 6A, C). Distances can be quantified by a detailed line shape analysis of EPR spectra of frozen protein samples or proteins in solutions of high viscosity (Altenbach et al., 2001; Rabenstein and Shin, 1995; Steinhoff et al., 1991; Steinhoff et al., 1997). Pulse EPR techniques expand the accessible distance range up to 8 nm (Borbat and Freed, 1999; Pannier et al., 2000; Martin et al., 1998). Two major protocols have been successfully applied, the 4-pulse DEER or 4-pulse PELDOR (Figure 6D) and the Double Quantum Coherence approaches (for a recent review see (Schiemann and Prisner, 2007)).

The combination of cw and pulse EPR techniques, taking into account borderline effects in the region from 1.6-1.9 nm (Banham et al., 2008; Grote et al., 2008), provide means to determine interspin distances in the range from 1-8 nm, thereby covering the most important distance regime necessary for structural investigations on biomacromolecules. Remarkably, the DEER data can provide, besides the distance information, also information about the orientation of the spin label side chains, their conformational flexibility and the spin density distribution, thereby increasing the amount and quality of data for a setup or verification of structural models.

Based on inter spin distance measurements on 26 different pairs of spin labels introduced into the cytoplasmic regions of *N*pSRII and *Np*HtrII the arrangement of the transmembrane domains of this complex was modelled (Wegener et al., 2001) (Fig. 7A). Direct comparison of the EPR model with the later determined crystal structure (Gordeliy et al., 2002) (Fig. 7B) shows the consistency of the EPR model with the crystal structure concerning the general topology and the location and relative orientation of the transmembrane helices. Remarkably, also most of the side-chain orientations within the complex coincide quite well in the two models, although for the EPR based model the bacteriorhodopsin structure had to be used as a template for *Np*SRII, since its structure was not known at that time. In a later study it was shown that the structural properties of the HAMP domain as characterized by mobility, solvent accessibility, and intra-transducer-dimer distance data are in agreement with the NMR model of the HAMP domain from *Archaeoglobus fulgidus* (Döbber et al., 2008).

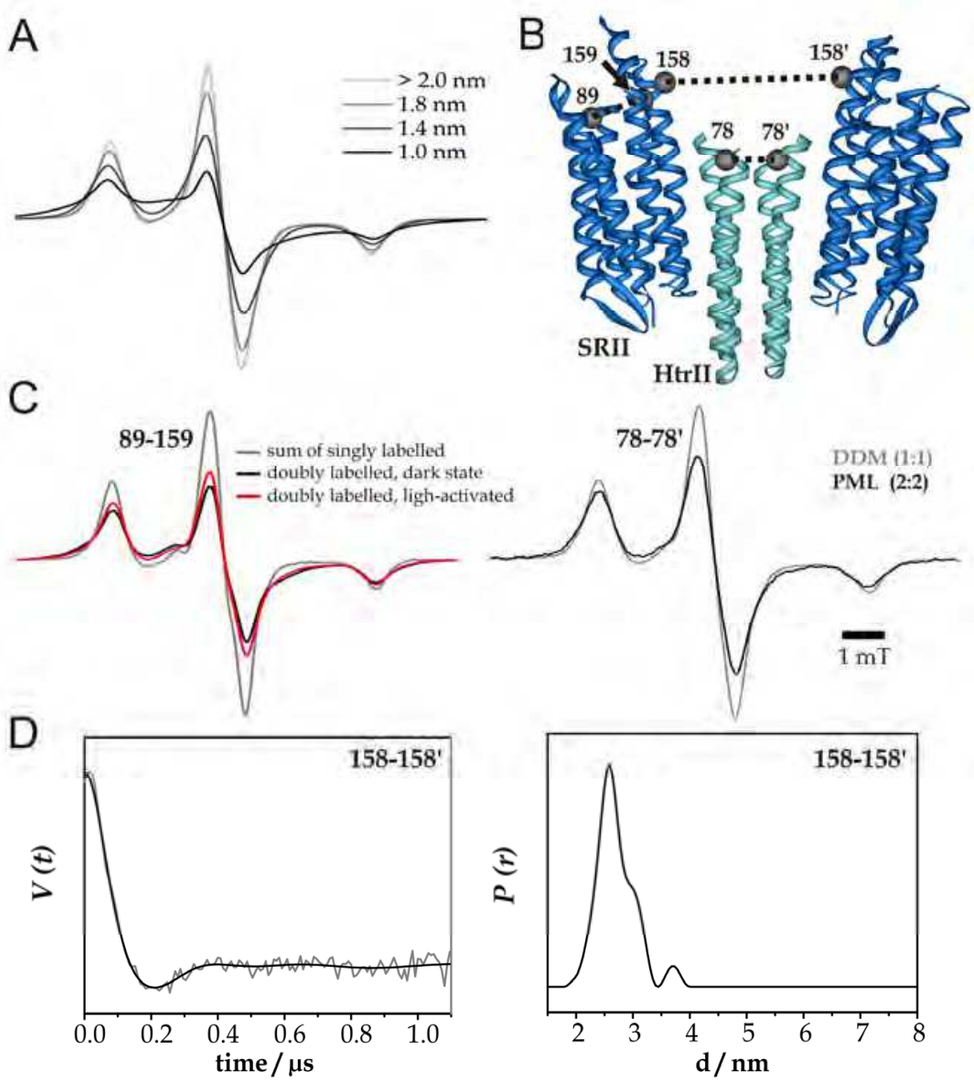

Fig. 6. Interspin distance measurements. (A) Simulated powder spectra (obtained by cw EPR on frozen samples) for different interspin distances. (B) Ribbon representation of the X-ray structure of the 2:2 *Np*SRII–*Np*HtrII complex (PDB 1H2S). Positions L89, S158 and L159 in *Np*SRII, and V78 in *Np*HtrII are shown as spheres. (C) left: cw EPR spectra (T = 160 K) of the double mutant L89R1/L159R1 in the receptor ground state (black) and in the trapped signaling state (M-state, red) compared to the sum of the spectra of the singly labeled samples (gray) reveals line broadening due to dipolar interaction. Interspin distances of 1.1 (±0.2) nm for the ground state and 1.3 (±0.2) nm for the M-trapped have been determined (Bordignon et al., 2007); right: *Np*HtrII–V78R1 solubilized in DDM (gray) or reconstituted in PML (black) in the absence of *Np*SRII. The interspin distance obtained in the reconstituted sample is 1.3 (±0.2) nm (Klare et al., 2006). (D) Left: background corrected DEER time domain for *Np*SRII-S158R1. The distance distribution shown in the right panel shows a mean distance of 2.6 nm between the two spin labels bound to positions 158 in the 2:2 complex that is in good agreement with the distance of 3 nm between the oxygen atoms of the respective serine residues as calculated from the crystal structure.

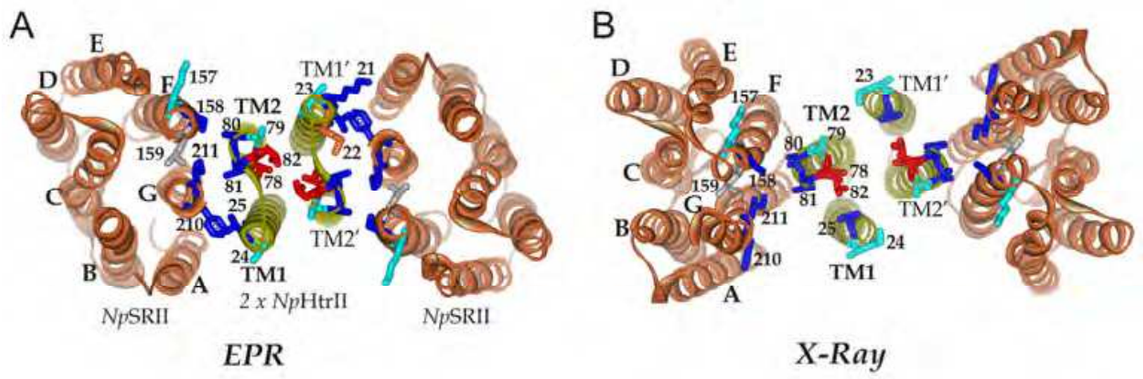

Fig. 7. (A) EPR based model of the transmembrane region of the *Np*SRII/*Np*HtrII complex (viewed from the cytoplasmic side). Side chains where spin labels have been attached are shown in stick representation. The color code represents the strength of the observed dipolar interaction (blue and red: strong; cyan and orange: weak). (B) Crystal structure of *Np*SRII/*Np*HtrII (PDB: 1H2S) (Klare et al., 2004). The color code for the side chains is the same as in (A).

4. SDSL EPR in protein interaction studies

4.1 Structure of the Na^+/H^+ antiporter dimer

An excellent example for the application of DEER spectroscopy to investigate interactions between proteins is the *E. coli* Na^+/H^+ Antiporter NhaA (Hilger et al., 2005, 2007). The protein is responsible for the specific exchange of Na^+ for H^+, and is known to be regulated by pH. From studies on two-dimensional crystals diffracting at 4 Å it had been revealed that NhaA forms a dimer in crystals, and *in vivo* studies as well as cross-linking data suggested that it also works as a dimer *in vivo*. Applying DEER on a NhaA variant labeled at position H225, it could be shown (Hilger et al., 2005) that a pH independent distance of 4.4 nm between spin labeled sites in neighboring molecules can be resolved, and that the degree of dimerization, as judged from the modulation depth of the DEER dipolar evolution data, strongly depends on pH. Thereby, experiments utilizing a singly spin labelled position yielded data strongly supporting the stoichiometry of the functional unit and providing evidences for a mechanistic picture of pH regulation, i.e. that the affinity between the protomers is modulated.

In a later study (Hilger et al., 2007), an extensive distance mapping of the NhaA dimer with nine different spin labeled amino acid positions was carried out. Based on these distance distribution data, explicit modeling of the spin label side chain conformations with a rotamer library approach, and combination with the available X-ray structures, a high-resolution structure of the presumably physiological dimer was determined.

4.2 Structure and function of the tRNA modifying MnmE/GidA complex

The GTP hydrolyzing protein MnmE is, together with the protein GidA, involved in the modification of the wobble position of certain tRNAs (Meyer et al, 2009). It belongs to the expanding class of G proteins activated by nucleotide-dependent dimerization (GADs). The

crystal structure shows that MnmE is a multidomain protein with a central helical part in which a Ras-like domain is inserted, and an N-terminal tetrahydrofolate-binding unit. MnmE was predicted to form a dimer in solution, in which the two G domains are separated with a distance of about 50Å between the two P-loops (Fig. 10A). A G domain dimerization had been proposed based on biochemical data and on the crystal structure of the isolated G domains in complex with the GTP hydrolysis transition state mimic GDP·AlF_x.

In a DEER study, distance measurements between spin labels positioned in the MnmE G domain and in the dimerization domain have been carried out (Meyer et al., 2009).The distance distributions for position E287 in the G domains (Fig. 8A) are shown in Figure 8B. In the apo state without any nucleotide bound, the two spin labels exhibit a distances of 55Å for E287R1 and a broad distribution of distances from 25Å to 50Å. GTP (GppNHp) binding induces the formation of additional distances at 27Å (S278R1) / 37Å (E287R1), contributing about 30% to the distance distributions. Upon GTP hydrolysis (GDP · AlF_x state), the distance distribution shows a single population maximum at 28Å (S278R1) / 36Å (E287R1). Thus, an equilibrium between an open conformation with distant G domains and a closed conformation with the G domains being in close proximity, exists when GTP is bound, and is shifted completely towards the closed state upon hydrolysis. A schematic representation of the proposed conformational changes of the MnmE G domains based on the EPR data is depicted in Figure 8C.

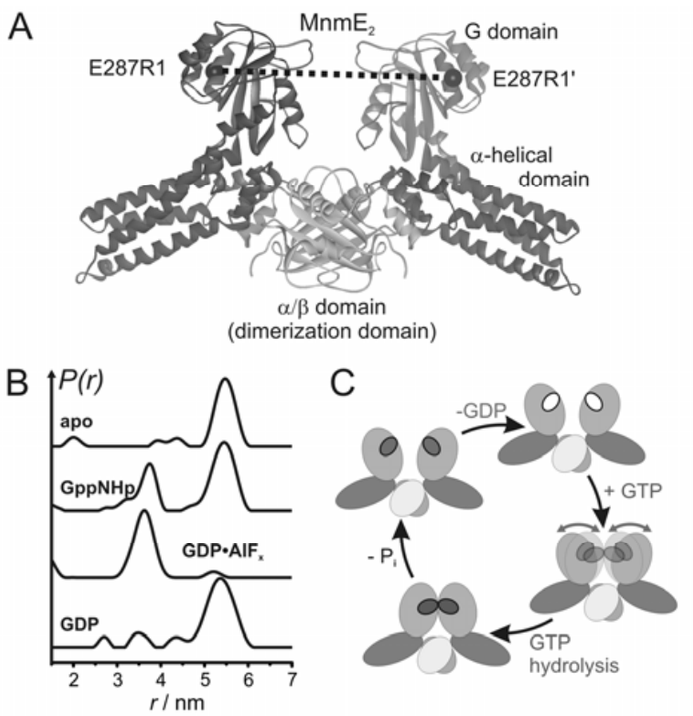

Fig. 8. G domain dimerization of MnmE monitored by DEER. (A) Structural model of the MnmE-dimer. Position 287 in the G domains, which was spin labeled is indicated by black spheres. (B) DEER distance distributions obtained by Tikhonov regularization using the program DeerAnalysis 2008. (C) Schematic representation of the G domain conformational states during the GTPase cycle.

In addition, spin labels attached at position 105 in the dimerization domain showed no significant distance changes during the GTPase cycle, indicating that the initial dimerization interface is largely preserved despite the large G domain movements. In addition, a dependency of the GTPase activity and consequently of the G domain motions on the presence of specific cations could be fully corroborated by the DEER analysis performed in this study. In a subsequent study, the influence of binding of GidA to MnmE in a 2:2 complex was investigated, showing that the interaction of GidA with MnmE partly abolishes the previously observed cation dependency.

4.3 Subunit binding in the chaperone/usher pathway of pilus biogenesis

Type 1 pili are adhesive multisubunit fibres in Gram-negative bacteria. During pilus assembly, subunits dock as chaperone-bound complexes to an usher, which catalyses their polymerization and pilus translocation across the membrane. In the background of the recently determined crystal structure of the full-length FimD usher bound to the FimC–FimH chaperone–adhesin complex, SDSL EPR was used to show that subsequent subunits bind to the usher c-terminal domains after undergoing so-called donor-strand exchange (Phan et al., 2011).

DEER spectroscopy was carried out on spin label pairs introduced into Fim proteins and the results ware compared to calculated distance distributions obtained from alternative models of the complex. The pair residue 74 of FimC and residue 756 of FimD in the complex revealed that the distance distribution obtained experimentally overlaps with that predicted when FimC–FimG (FimG is the adjacent subunit within the pilus) locates at the c-terminal domains. For the FimC-Q74R1/FimD-S774R1 the experimental distance distributions compared with those calculated for the crystal structure of FimD-C-H, assuming that the position of FimC-G, is similar to the previously bound chaperone-subunit complex FimC-H, are in good agreement, further supporting the assumption that FimC-G in the FimD-C-G-H complex locates at the c-terminal domains and is bound to c-terminal domain 2 (Phan et al., 2011).

4.4 Conformation of peptides bound to TAP

The ATP-binding cassette transporter associated with antigen processing (TAP) is involved in the adaptive immune defense against infected or malignantly transformed cells. It translocates proteasomal degradation products, i.e. peptides of 8 to 40 residues, into the lumen of the endoplasmic reticulum for loading onto MHC class I molecules. EPR spectroscopy and simulations based on a rotamer library were used to reveal conformational details about the bound peptides (Herget et al., 2011).

The authors used two different spin label side chains, namely the PROXYL spin label to monitor side-chain dynamics and environmental, and TOAC-labeled peptides (see chapter 2.2) to detect backbone properties. For different locations of the spin label on the peptide, striking differences in affinity, dynamics, and polarity were found. The mobility of the spin labels was found to be strongly restricted at the ends of the peptide. In contrast, the central region was flexible, suggesting a central peptide bulge. Furthermore, DEER spectroscopy was used for the determination of intrapeptide distances in doubly labeled peptides bound to TAP. Comparison with calculated distance distributions based on a rotamer library led

the authors to the conclusion that peptides bind to TAP in an extended kinked structure, analogous to those bound to MHC class I proteins (Herget et al., 2011).

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6. References

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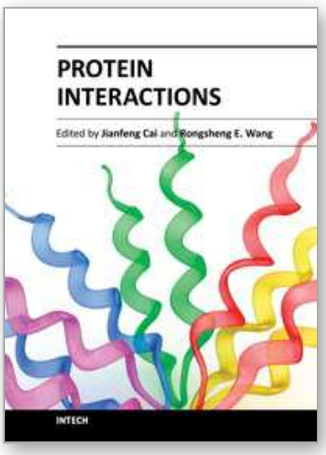

Protein Interactions

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Protein interactions, which include interactions between proteins and other biomolecules, are essential to all aspects of biological processes, such as cell growth, differentiation, and apoptosis. Therefore, investigation and modulation of protein interactions are of significance as it not only reveals the mechanism governing cellular activity, but also leads to potential agents for the treatment of various diseases. The objective of this book is to highlight some of the latest approaches in the study of protein interactions, including modulation of protein interactions, development of analytical techniques, etc. Collectively they demonstrate the importance and the possibility for the further investigation and modulation of protein interactions as technology is evolving.

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