

X- AND W-BAND EPR SPECTROSCOPY COMBINED WITH MOLECULAR DYNAMICS SIMULATIONS UNRAVEL THE STRUCTURE AND STRUCTURAL CHANGES OF SPIN LABELED PROTEINS

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The investigation of protein motions and related conformational changes is a prerequisite for the understanding of enzymatic processes. Site directed spin labeling (SDSL) has emerged as a powerful technique to determine the required data, especially for problems where it is difficult to apply X-ray crystallography or solution NMR methods. In the present approach, X- and W- band electron paramagnetic resonance (EPR) spectroscopy is combined with molecular dynamics (MD) simulations to study the structure and conformational changes of site directed spin labeled proteins. The method is evaluated here with the light driven proton pump bacteriorhodopsin (BR) from *Halobacterium salinarum*.

Site directed mutagenesis has allowed to introduce nitroxide side chains at almost any desired site in a protein. Recent studies on a large number of spin labeled protein mutants have shown that the dynamic properties of the nitroxide side chain and thus the EPR spectral line shape contain direct information about the secondary and tertiary structure of the protein in the vicinity of the nitroxide binding site [1]. Supplementary data on the protein structure were extracted from the accessibility of the nitroxides for hydrophobic or hydrophilic quenchers and from the polarity of the nitroxide environment, which can be determined by high field-EPR spectroscopy [1-3](see also the contribution of Wegener et al.). Intramolecular distances were determined from the spin-spin interaction between two nitroxides attached to different sites of the protein molecule [4, 5] (see also the contribution of Radzwill et al.). The time resolved detection of these parameters for a set of spin labeled mutants of BR, e.g., allowed detailed characterization of conformational changes which occur during the photocycle [6].

The quantitative analysis of the experimental data in terms of motional mechanisms, fluctuation amplitudes and correlation times and their interpretation in terms of the protein structure and structural changes require the simulation of EPR spectra. A new method allows simulations of EPR spectra of nitroxides on the basis of molecular dynamics simulations of the spin labeled protein [7] [8]. We evaluated this method with EPR studies of the nitroxide side chains at positions 154 through 171 in the cytoplasmic moieties of helices E and F of BR and the interconnecting E-F loop. The nitroxide dynamics and their changes during the BR photocycle were characterized by combination of X- and W-band EPR spectroscopy and spectra simulations on the basis of MD simulations.

Nitroxide side chain mobility and BR structure

Sample preparation, spin labeling and EPR spectroscopy was performed as described [2, 6]. The orientation of the nitroxide side chains (R1) of the MTS spin label attached to the E-F loop and adjacent helical parts of BR, positions 154 through 171, (see fig.1) were deduced from the classification according to their mobility and accessibility for paramagnetic quenchers [2]. X-band spectra for nitroxides attached to positions 165 - 171 are shown in figure 2. The EPR results for the nitroxides attached to the cytoplasmic ends of helices E and F (positions 155 to 157 and 166 to 171) are in nice agreement with published structural data [9, 10]. The side chains attached to positions 155 and 156 face the protein interior, those attached to 157 and 158 are oriented towards the lipid phase. Position 159 is the first amino

acid side chain of the sequence which faces the aqueous phase. The high mobility is strong evidence for its position in a loop region. A similar high degree of mobility is found for the nitroxides attached to positions 161, 162, 164, 165 and 166. The reorientational motion of the nitroxides attached to 167 and 168 is strongly restricted by interaction with helices C and E, respectively. Position 168 is still accessible for water soluble quenchers. Positions 170 and 171 provide a location of the nitroxide already in the interior of the protein.

Due to high temperature factors, the structure of the E-F loop region appeared to be disordered in recent X-ray studies. This is in agreement with the EPR data, where the highest degree of mobility for certain nitroxides is found in the residue sequence from 158 to 165. Here, backbone flexibility and the residual motion of the side chain contribute to the observed nitroxide mobility. However, spectral components corresponding to restricted motion are also present to different extent and clearly dominate the spectra of A160R1 and M163R1. These components provide strong evidence for a side chain conformation with the nitroxide side chain oriented towards the protein. The arrangement of the loop residues 158 to 165 within a single turned and stretched loop agrees with the experimental EPR data and thus provides a reasonable model for the structure of the E-F loop in its physiological environment [2].

Molecular dynamics simulations of the nitroxide side chain motion allow to further evaluate this structural model. According to the method described in [7], MD trajectories were calculated for the complete sequence of spin labeled BR. Examples for a comparison of experimental with simulated spectra are shown in figure 2. The overall agreement between theory and experiment is strong evidence for the applicability of the method and for the validity of the structural model of the loop.

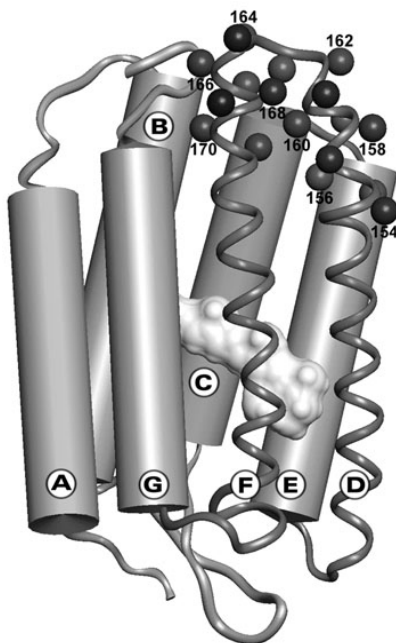


Fig. 1: Bacteriorhodopsin. Residues modified with the MTS spin label are indicated.

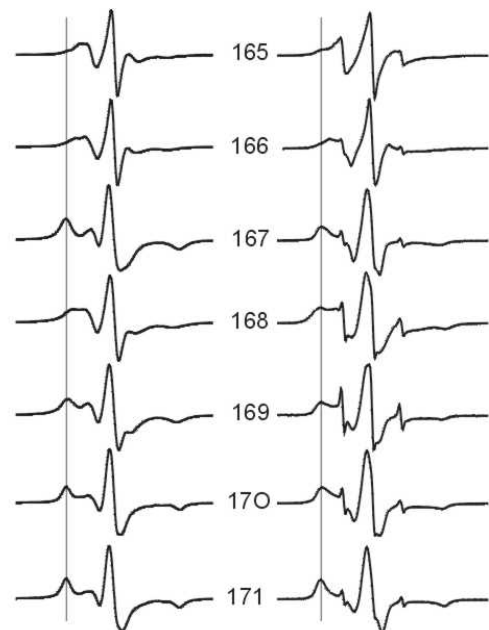


Fig. 2: Comparison of experiment (right) and simulation (left).

Conformational changes during the photocycle of BR

The sequence of intermediates in the photocycle of BR is associated with significant conformational changes as disclosed by diffraction experiments [11] and FTIR spectroscopy [12]. The diffraction experiments revealed prominent conformational changes in the vicinity of helices F and G. EPR experiments in combination with site-directed spin labeling provides real time resolution together with specific localization of structural changes. Applying this technique structural changes which influence all of the three cytoplasmic loops and of helix F could be uncovered [6].

The enhanced sensitivity of high-field EPR towards the anisotropy of the nitroxide dynamics provides a promising option to describe the influence of conformational changes on the nitroxide motion in even more detail. W-band EPR results on the double mutant V167R1/D96N are shown in figure 3 [3]. The lack of the proton donor D96 in this mutant leads to a prolonged photocycle which facilitates accumulation of the M intermediate with

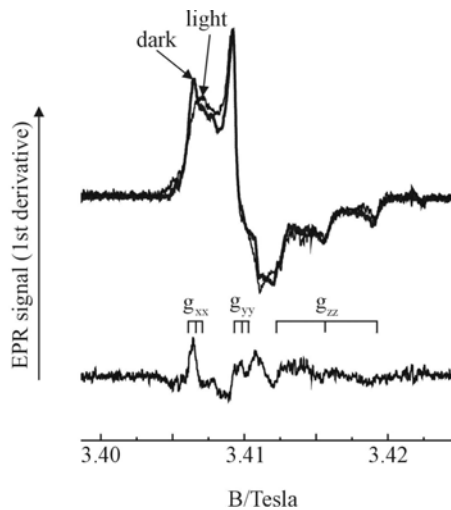


Fig. 3: W-band EPR spectrum (top) of the double mutant V167R1/D96N in the dark and in the light and the difference spectrum dark - light (bottom) [3]

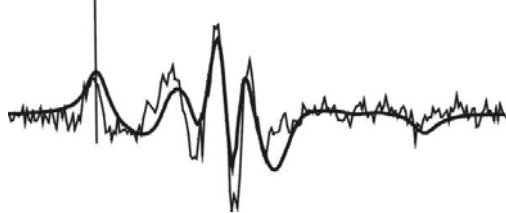


Fig.4: Experimental (noisy line) and simulated X-band EPR difference spectra (dark - light) determined for the nitroxide at residue 171. The vertical line indicates the position of the low field absorption extreme as shown in Fig.2.

deprotonated Schiff base at 293 K. Upon quasi-continuous light excitation major changes were revealed in the g_{xx} and g_{yy} regions of the spectrum compared to that of the BR ground state. The observed averaging of the hyperfine splitting in the g_{xx} spectral region and its shift towards the center line is strong evidence for an increased reorientational mobility, at least of the x-axis of the nitroxide in the M intermediate. The underlying structural change occurs at the cytoplasmic moiety of helix F. The nitroxide side chain of V167R1 is oriented towards helix C [2]. Thus, motion of helix F and/or helix C, which increases the inter-helical distance, would weaken the restrictions of the nitroxide mobility and, therefore, would account for the data.

This is further examined by EPR experiments and MD simulations of the nitroxide side chain motion at residue position 171, a single helix turn apart from position 167. An experimental difference X-band spectrum (dark - light) observed for F171R1 is shown in figure 4. The changes of the spectral amplitudes at the low field and high field extremes are evidence for a transient increase of the nitroxide mobility during the photocycle. We performed MD simulations of the reorientational dynamics of the nitroxide at position 171 for a modified BR structure with the F helix tilted outward by 0.5 nm and compared the results with those determined for the BR initial state. The mean square fluctuation amplitude of the spin label reorientational motion increased from 0.07 rad^2 for the BR initial state to 0.42 rad^2 for the modified structure. EPR spectra were calculated from the nitroxide reorientation MD trajectories for both conformations. The simulated difference spectrum shown in figure 4 reveals a remarkable agreement with the experimental difference spectrum. The difference

amplitudes in the low and high field region which are indicative for the change of the motional freedom are clearly reproduced by the simulation.

This result together with time resolved studies of the complete set of spin labeled BR mutants (positions 154 through 171) show that the EPR spectral changes occurring during the photocycle are consistent with a small movement of helix C and an outward tilt of helix F [6]. These helix movements are accompanied by a rearrangement of the E-F loop and of the C-terminal turn of helix E. The kinetic analysis of the transient EPR data and the absorbance changes in the visible spectrum reveals the conformational change to occur during the lifetime of the M intermediate. The most prominent transient rearrangements of nitroxide side chains are found in the vicinity of D96 and may indicate the preparation of the reprotonation of the Schiff base which occurs during the M to N transition. All structural changes reverse with the recovery of the BR initial state.

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