A HIGH-FIELD EPR STUDY OF THE POLARITY PROFILE OF THE BACTERIO-RHODOPSIN PROTON CHANNEL

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Introduction

Electron paramagnetic resonance (EPR) spectroscopy in combination with site-directed spin-labeling (SDSL) allows to obtain structure and dynamics even for proteins in solution or in the membrane. This information is obtained from the dynamical properties of the nitroxide side chain, its accessibility for paramagnetic quenchers and from the polarity in the vicinity of the nitroxide binding site [1].

The sensitivity of the magnetic tensors of nitroxide spin labels towards the polarity of the environment has been used in the past to characterize the behavior of the hydrophobic properties of membranes and proteins [2-4]. Whereas the spectral analysis of X-band spectra had to be restricted to the value of the nitrogen hyperfine tensor element A_{zz}, the enhanced resolution of high-field EPR spectra with respect to the g-tensor anisotropy allows additionally to follow the behavior of g_{xx} of nitroxides with very high accuracy. Due to this high resolution the g-tensor variations due to solute-solvent interactions could be followed [5-7]. The improved sensitivity to local structural influences on spin labels reveals significant changes in the g-tensor as a function of the polarity of the spin label environment [2, 8].

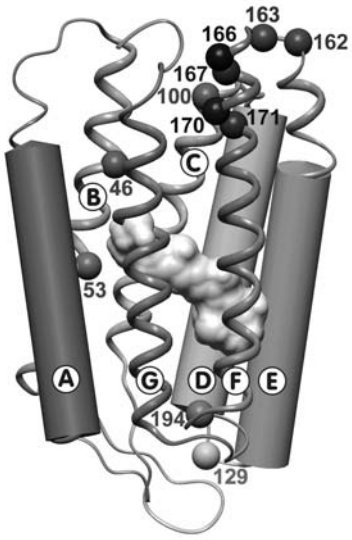

Fig. 1. Structural model of bacteriorhodopsin (BR) with the helices A to G and their interconnecting loops according to the data of Essen et al. [11]. Residues which were modified by the spin label are indicated, the chromophore retinal is shown space filled.

In the present report 95GHz (W-band) EPR spectroscopy is applied to study the polarity profile along the proton channel of spin-labeled bacteriorhodopsin (BR), an integral membran protein which acts as a light driven proton pump [9].

Results and discussion

Single cystein residues were introduced along the BR proton channel at the positions shown in Fig. 1 using site-directed mutagenesis techniques. The cysteins reacted with the sulfhydryl specific MTS spin label to yield the spin label side chain (R1). Inspection of the photocycle of the spin-labeled mutants did not give any evidence for protein unfolding and it was concluded that the overall structure and function of BR is retained [3]. EPR spectra were measured at 200 K to restrict molecular motion. In this temperature regime the dynamics of proteins exhibits glasslike behavior, and the reorientational correlation time of an otherwise unre-

stricted spin label side chain exceeds 100ns [10], i.e., the nitroxide may be considered as immobilized on the EPR time scale. Examples of spectra are shown in Fig. 2. The experimental spectra exhibit the typical nitroxide powder pattern line shape expected for a dilute distribution of nitroxides. The spectra are clearly resolved into three separate regions corresponding to the components g_{xx}, g_{yy} and g_{zz}. The variation of g_{xx} with the nitroxide binding site is revealed by the shift of the position of the low-field maximum, generally a polar environment shifts g_{xx} to smaller values. G- and A- tensor values were determined from the second derivative of the experimental spectra.

The g_{xx} values for the studied samples are plotted in Fig. 3 (left) as a function of the distance, r, between the nitroxide binding site and the cytoplasmic surface. The distances were measured from the projections of the C_β positions of the respective residue to a line connecting residues R164, the position of which is arbitrarily set to 0 nm, and G73 on the extracellular surface of the protein. An error bar of 0.7 nm indicates the uncertainty of the location of the N-O group (for positions of the respective side chains, cf. Fig. 1). The smallest values of g_{xx} are found for S162R1 and K129R1. These values are slightly smaller than those found for unbound MTS spin label in aqueous glycerol solution, which yields 2.00834. This difference is most likely due to different salt concentrations present in the spin label solution compared to the BR samples. The behavior of g_{xx} reveals a maximum near positions 46 and 53 in the vicinity of the retinal.

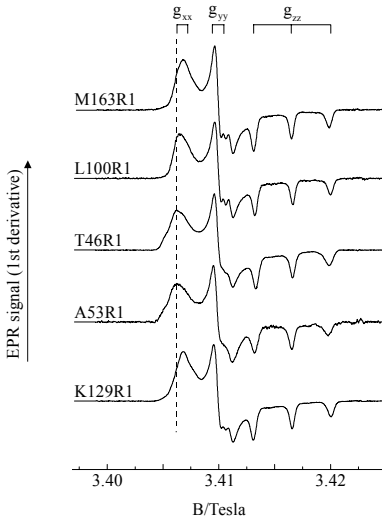

Fig. 2. Experimental high-field spectra (1st derivative represantation, T=200K, v=95GHz) for a set of spin labeled BR mutants. The vertical line marks the g_{xx} position of the T46R1. The variation of g_{xx} reflects the change of polarity when moving through the BR proton channel from the cytoplasmatic to the extracellular side.

The significant variation of g_{xx} with the nitroxide position in BR results from changes in the polarity of the nitroxide environment. According to the molecular model [11, 12] residues S162R1 and M163R1 are located in the E-F loop at the cytoplasmic surface, whereas residue K129R1 is positioned in the D-E loop on the extracellular surface. The high polarity in the environment of these residues is clear evidence that the nitroxides are accessible to water, which is in agreement with the structure [13]. The environmental polarity of the nitroxide at positions 100, 167 and 171 is significantly less and reaches its minimum at position 46 between the proton donor D96 and the retinal. The overall behavior of g_{xx} with the varying polarity of the nitroxide environment is consistent with previous results from nitroxides in organic solvents [8]. Earlier measurements of magnetic tensors for different label position in pure lipid dispersions (dipalmitoylphosphatidylcholine, DPPC) revealed a total variation of g_{xx}, Δg_{xx}, of 6×10^{-4} [2]. The values for g_{xx} found in the present study for the nitroxides located in the proton channel of BR are lower and Δg_{xx} amounts to approximately 3×10^{-4}. Hence, this is evidence for the presence of water, or of polar or charged residues in the vicinity of the nitroxides.

It is also very informative to study g_{xx} as a function of the hyperfine tensor component A_{zz} of the N-atom of the nitroxide group. This dependence is plotted in Fig. 3 (right) for the various spin label positions in BR. Obviously, the plot is suggestive of straight-line correlations.

Theoretically, both g_{xx} and A_{zz} are expected to be linearly dependent on the π spin density ρ_O at the oxygen atom of the nitroxide group [14-16]. For g_{xx} , however, apart from a direct proportionality to ρ_O, there is an additional dependence on specific electronic properties of the oxygen lone-pair orbitals [14-16]. The lone-pair orbital energy E_n affects g_{xx} via the excitation energy $\Delta E_{n->\pi^*} = E_{\pi^*} - E_n$ [17] and is known to be sensitive to a polar environment, e.g. water, and to be particularly sensitive to direct H-bonding of the lone pairs to water or to polar amino acid residues. Kawamura et al. [18] who studied the g_{iso} *vs* A_{iso} (X-band data) de-

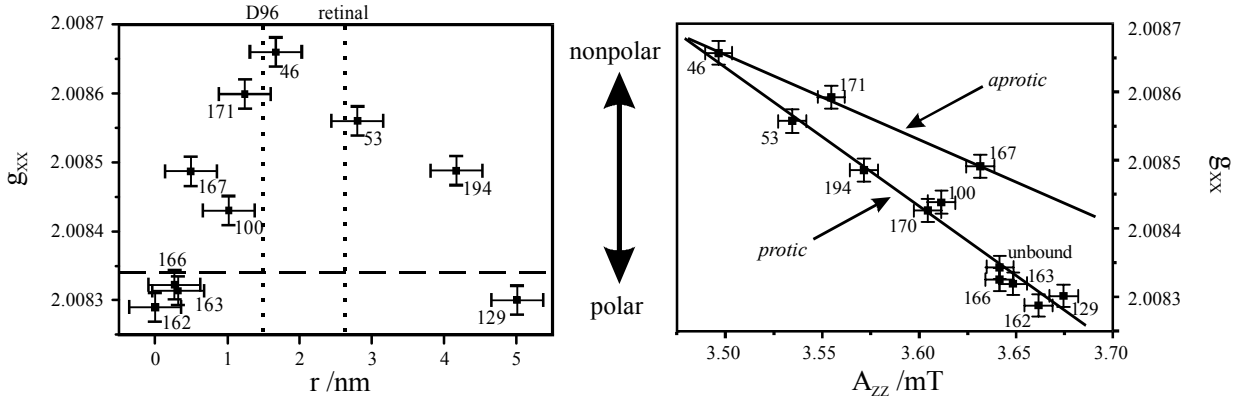

Fig. 3. Left: The tensor element g_{xx} of spin labels oriented towards the aqueous phase or into the proton channel of BR as a function of the nitroxide location with respect to position 164. The horizontal bars indicating the range of the possible locations of the N-O group. Vertical bars (2σ error $\pm 2x10^{-5}$) are determined by the S/N of the second derivative of the EPR spectra. The broken line shows the g_{xx} value of unbound spin label, the dotted lines indicate the positions of D96 and the Schiff base of retinal, respectively. The value of g_{xx} may be understood as a polarity index, thus the plotted data reflects the hydrophobic barrier in the BR proton channel. Right: Plot of g_{xx} versus A_{zz} of the nitrogen for various spin label positions in BR. The horizontal bars indicate 2σ errors of about $\pm 10^{-2}$mT for A_{zz}. The vertical bars are as in the left plot.

pendence of di-t-butyl nitric oxide (DTBNO) in various solvents found two different straight lines for aprotic solvents (e.g., n-hexane, acetonitrile) and for protic solvents (e.g. methanol, water).

We have attempted to establish similar straight-line relations for the various label positions in BR (see Fig. 3 (right)). The points belonging to label positions 46, 171 and 167 on the one hand and all the remaining points on the other hand are assignable to two different straight lines with slopes $-1.35T^{-1}$ and $-2.00T^{-1}$ for the „protic“ and „aprotic“ lines, respectively. It should be pointed out that the slope of $-1.35\ T^{-1}$ for the "aprotic" line is close to the slope found for aprotic n-PC/DPPC lipid dispersions by Earle et al. [2] for $n \leq 7$ (excluding sterically induced changes of A_{zz} for larger n in the phosphatidylcholine spin labels, n-PC, dispersed in the lipid dipalmitoylphosphatidylcholine, DPPC).

According to the structure of Lücke et al. [13] the residues T46R1, V167R1 and Y171R1 are located in the vicinity of the cytoplasmic proton pathway. Evidence for one or two water molecules could be found between D96 and the Schiff base. All other spin labeled residues are located in the extracellular pathway and Lücke et al. [13] found three well defined water molecules in this part of the channel. According to this structure five polar or charged residues are located in the vicinity of the cytoplasmatic proton pathway. On the extracellular pathway up to 10 residues may be involved in a hydrogen bound network. The influence of these residues on the different hydrophobic properties is clearly reflected in the behavior of g_{xx} *vs* A_{zz}. Additionally g_{xx} serves as a polarity index of the nitroxide environment (Fig.

3(left)). The present results strongly support and expand the polarity data which were extracted from X-band spectra on the basis of the behavior of A_{zz} [3].

Conclusion

Extensive theoretical work is done on the shape of the potential and the hydrophobic barrier in ion channels and ion pumps [19]. An important parameter for the understanding of the function of these proteins is the knowledge of the polarity profile within the ion channels. The experimental study done in the present report shows that the combination of site-directed spin labeling and high-field EPR spectroscopy is ideally suited for the collection of the necessary data.

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