ISSN 0021-3640, JETP Letters, 2007, Vol. 86, No. 2, pp. 149–152. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © S.B. Orlinskii, I.V. Borovykh, V. Zielke, H.-J. Steinhoff, 2007, published in Pis'ma v Zhurnal Éksperimental'noĭ i Teoreticheskoĭ Fiziki, 2007, Vol. 86, No. 2, pp. 158–161.

Use of Spin Labels to Study Membrane Proteins by High-Frequency Electron Nuclear Double Resonance Spectroscopy

S. B. Orlinskii[a, b], I. V. Borovykh[c, d], V. Zielke[c], and H.-J. Steinhoff[c]

[a] *Kazan State University, ul. Kremlevskaya 18, Kazan, 420008 Russia*
e-mail: Sergei.Orlinskii@ksu.ru
[b] *Huygens Laboratory, Department of Physics, Leiden University, 2300RA Leiden, the Netherlands*
[c] *Department of Physics, University of Osnabrueck, D-49076 Osnabrueck, Germany*
[d] *Laboratory of Biophysics, Wageningen University, 6703HA Wageningen, the Netherlands*

Received May 31, 2007

The applicability of spin labels to study membrane proteins by high-frequency electron nuclear double resonance spectroscopy is demonstrated. With the use of bacteriorhodopsin embedded in a lipid membrane as an example, the spectra of protons of neighboring amino acids are recorded, electric field gradients at the membrane surface are detected, and the constant of hyperfine interaction with the chlorine nucleus at the site of ion trapping is measured.

PACS numbers: 76.70.Dx, 87.16.Dg

DOI: 10.1134/S0021364007140172

In this work, we used spin-labeled bacteriorhodopsin from *Halobacterium salinarum* (*H. salinarum*) as an example in order to demonstrate the applicability of high-frequency (94.9 GHz) electron nuclear double resonance (ENDOR) spectroscopy to probe membrane proteins. The number of proteins containing intrinsic paramagnetic centers is about 1% of all the known proteins. In this connection, a procedure for inserting spin labels was developed in order to produce paramagnetic centers at specified positions in the protein structure. This procedure has been widely used for obtaining information on the dynamics of proteins [1, 2]. In this case, various versions of electron paramagnetic resonance (EPR) spectroscopy have been employed assuming significant localization of an unpaired electron at the N–O bond. Nitroxyl spin labels are stable organic free radicals with the electron spin $S = 1/2$. In this work, we used the (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate spin label (MTSSL) [3]. The mutations of cysteine at specified positions in the bacteriorhodopsin structure were performed in accordance with a published procedure [4]. The mutant proteins were isolated as purple membrane sheets in accordance with a protocol described by Oesterhelt and Stoeckenius [5]. The insertion of spin labels into bacteriorhodopsin was described by Radzwill et al. [6].

The EPR spectra of MTSSLs arranged at different positions in the bacteriorhodopsin structure have been studied [7, 8]. Typically, low-frequency ENDOR spectroscopy has been used previously to probe free spin labels unbound to proteins or bound to small molecules [9–12]. Recently, we found that, even at a low wavefunction density of an unpaired electron on surrounding nuclei, hyperfine interactions with them can be detected because of the high sensitivity and selectivity of high-frequency ENDOR spectroscopy [13–15]. All of the experiments were performed on a pulse EPR spectrometer, which was described elsewhere [16], at a test frequency of 94.9 GHz and a sample temperature of 1.6 K. The EPR spectra were obtained by recording the primary echo amplitude as the magnetic field was swept. The ENDOR spectra were measured using the Mims pulse sequence [17].

Figure 1a shows the ENDOR spectrum of the MTSSL at amino acid position 162 in bacteriorhodopsin. As found by Beier and Steinhoff [8], the label was oriented almost perpendicularly to the surface of a membrane into which bacteriorhodopsin was integrated. A characteristic EPR spectrum of a frozen solution with a resolved fine structure was observed. The singular points of this structure correspond to the canonical g-tensor orientations of the spin label; they are denoted by g_{xx}, g_{yy}, and g_{zz} in Fig. 1a. In addition, a resolved hyperfine structure was observed, which appeared due to the interaction of an unpaired electron with the ^{14}N nucleus with the nuclear spin $I = 1$ for labels whose g-tensor z axes were oriented along the external magnetic field.

Figure 1b shows the ENDOR spectra of protons measured in magnetic fields approximately corresponding to the canonical g-tensor orientations g_{xx}, g_{yy},

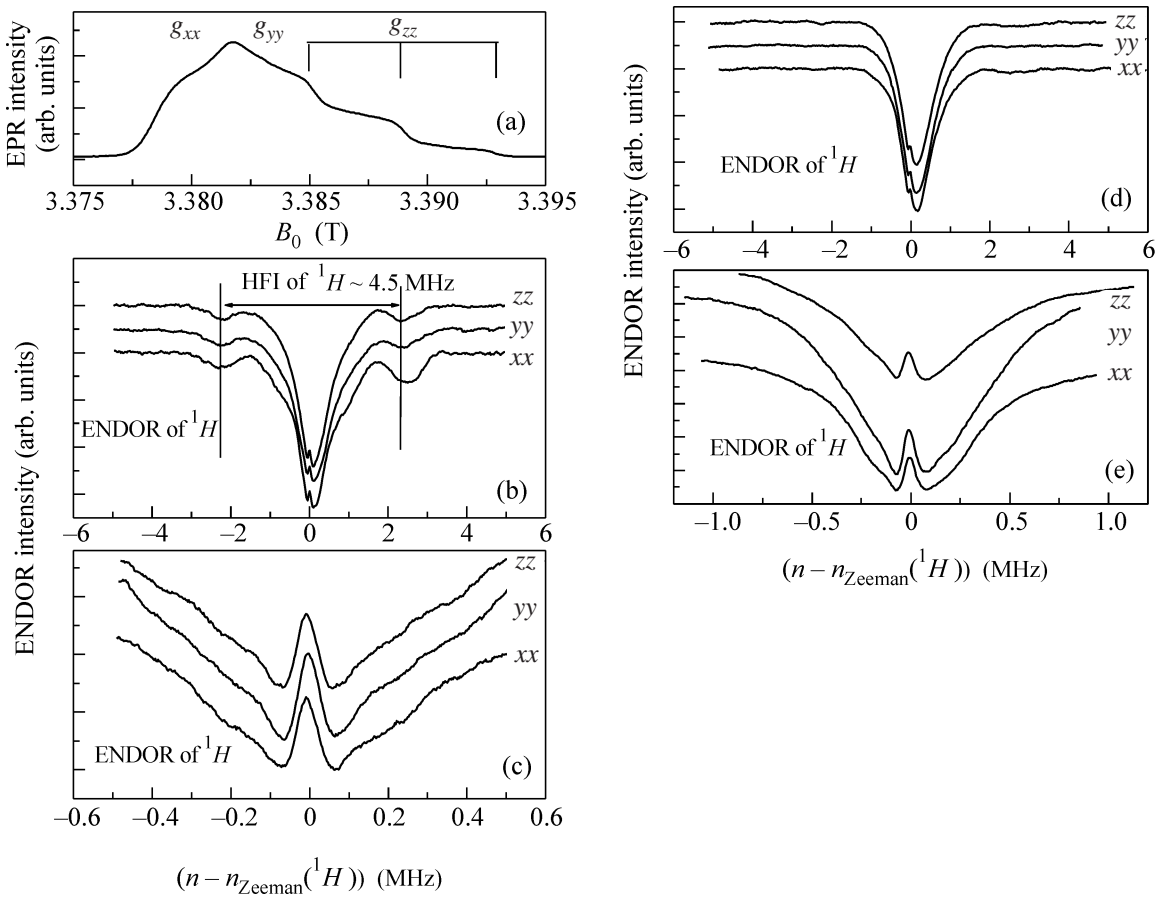

Fig. 1. (a) EPR spectrum of the protonated MTSSL at position 162 in bacteriorhodopsin; standard phosphate buffer solution (pH 7) at 100 mM NaCl concentration. (b) The ENDOR spectra of protons in the above sample are shown. The isotropic hyperfine interaction (HFI) of an unpaired electron with the nearest protons is marked. (c) The measurements in (b) performed at a higher resolution. (d) The ENDOR spectra of protons in a sample with the deuterated MTSSL at position 162 in bacteriorhodopsin are shown. (e) The measurements in (d) performed at a higher resolution.

and the first (low-field) line g_{zz}. The wavefunction density of an unpaired electron of the label is nonzero even at a sufficient distance from the nuclear core of the spin label. To demonstrate the possibility of measuring it at the magnetic nuclei of the protein, we performed the ENDOR measurements of protons in an analogous sample, in which the MTSSL occurred at the same position 162 but all of the protons of the label were replaced by deuterons. Figures 1d and 1e show these results. For better clarity, the central portions of the ENDOR spectra of protons were measured in more detail in both cases; Figs. 1c and 1e show these portions individually.

Figure 2a shows the EPR spectrum of the deuterated MTSSL at position 162 of bacteriorhodopsin. Figure 2b shows the ENDOR spectra of deuterons, which were measured in magnetic fields approximately corresponding to the canonical g-tensor orientations g_{xx}, g_{yy}, and the first (low-field) line g_{zz}. The distances to nuclei and the number of nuclei remain the same, and only their magnetic moment and spin are changed. In this case, the ratio between hyperfine interactions with protons and deuterons arranged at such structural positions is equal to the ratio of their magnetic moments 6.5. In Figs. 1b and 2b, hyperfine interactions for strongly interacting nuclei are marked; their ratio is 4.5/0.68 = 6.6. On this basis, we can conclude that their structural positions are identical. Therefore, the anisotropy observed in the ENDOR spectra of deuterons cannot be explained by hyperfine interaction anisotropy because it is absent from the ENDOR spectra of protons, which are shown in Fig. 1b. Because the deuteron spin is $I = 1$, this additional splitting can be explained by quadrupole interaction due to electric field gradients at the surface of a membrane into which bacteriorhodopsin was integrated. To test this hypothesis, the MTSSL can be removed from the membrane into a buffer liquid; under the same experimental conditions, the resolved anisotropic structure due to the quadrupole interaction disappeared. These conditions can be simulated by attaching the MTSSL to cysteine and placing it in the above buffer liquid (pH 7) at a 100 mM NaCl concentration. Figure 2c shows the ENDOR spectra of deuterons, which were measured for this spin label in magnetic

fields approximately corresponding to the canonical g-tensor orientations g_{xx}, g_{yy}, and the first (low-field) line g_{zz}.

The hyperfine interaction constant of an unpaired electron of the protonated MTSSL at position 158 in bacteriorhodopsin with the nucleus of the ^{35}Cl isotope at the site of ion trapping was measured. Its value was about 600 kHz, which is much greater than the expected value of 100 kHz based on the constant of hyperfine interaction with the nearest protons of the protein (about 1 MHz; see Fig. 1d) divided by the ratio of the magnetic moments of a proton and a ^{35}Cl nucleus (approximately 10). This relatively high wavefunction density of an unpaired electron of the spin label at the chlorine nucleus can be explained by the occurrence of a weak bond (such as a hydrogen bond) between the NO group and the chlorine atom.

The occurrence of quadrupole interaction and its anisotropy can be used to measure electric potentials and their changes in membranes under the action of external factors, such as pH and the presence of salts, or factors that appear in the course of the protein function. Moreover, analogous measurements can also be performed in proteins containing a large number of polar amino acids. The occurrence of a gradient of electric fields and its changes is very frequently associated with the functional activity of the protein. For example, bacteriorhodopsin is a proton pump activated by light, which induces retinol isomerization and, as a consequence, a change in local fields near this cofactor.

In nature, *H. salinarum* lives under conditions of a high sodium chloride concentration (about 4–4.5 mol/l, which is close to saturation). To explain the occurrence of a binding site for the chloride ion near position 158, we turn our attention to the bacteriorhodopsin structure. An analysis of this structure allows us to assume three possible binding sites, namely, the following amino acids having a positive charge: lysine 159, arginine 175, and lysine 172. In addition, we cannot exclude glycine 155, which also has a positive charge and occurs in a protein matrix. The most likely candidate is lysine 159 because, at a certain orientation of the spin label tail, the NO group can occur in the peptide pocket at a distance of 4.5–6 Å. At this orientation, the distance to other candidates was greater than 10 Å to make them less probable. Additional measurements with spin labels attached near amino acid 158, for example, at positions 157 and 159, should be performed in order to obtain more accurate information on the binding site.

This work opens up a new direction in the studies of proteins by high-frequency ENDOR spectroscopy. In this case, a wide range of currently available samples, which were synthesized for EPR-spectroscopic studies, can be used. Of course, ENDOR measurements in proteins with spin labels arranged near functionally important positions improve the information content of this technique. It also seems interesting to test other, both well-known and specially synthesized, spin labels with smaller unpaired electron localization in order to increase the sensitivity of ENDOR spectroscopy.

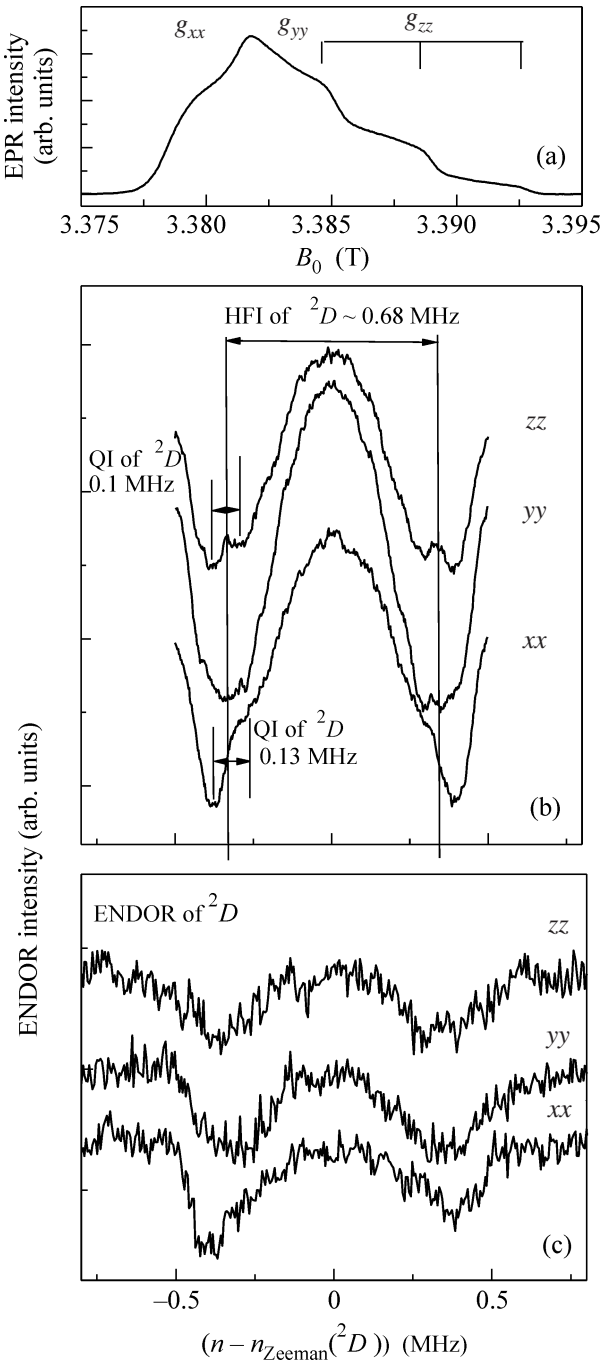

Fig. 2. (a) EPR spectrum of the deuterated MTSSL at position 162 in bacteriorhodopsin; standard phosphate buffer solution (pH 7) at 3 M NaCl concentration. (b) The isotropic hyperfine interaction (HFI) of an unpaired electron with deuterons and the quadrupole interaction (QI) of deuterons with electric field gradients for two orientations are marked. (c) The ENDOR spectra of deuterons in the sample where the deuterated MTSSL is bound to cysteine are shown.

In conclusion, note that standard spin labels were used in this work to probe proteins by high-frequency

ENDOR spectroscopy. We found that the high-frequency ENDOR of protons provides an opportunity to measure low wavefunction densities of an unpaired electron of the spin label at relatively large distances from its nuclear core. By now, the technique proposed for studying electric field gradients at the surfaces of both proteins and membranes that include these proteins is the only suitable technique for studying phenomena of this kind, which is based on the measurement of the anisotropy of the quadrupole interaction of MTSSL deuterons. The sensitivity of high-frequency ENDOR spectroscopy opens up new opportunities for the determination and characterization of ion binding sites; in this case, the absence of information on the protein structure is no obstacle. This fact is of paramount importance because it is well known that the functioning of proteins (particularly, transport and signal proteins) is directly related to the ion balance in the environment.

This work was supported by the Volkswagen Foundation (project no. I/78668). S.B.O. acknowledges the support of the Russian Foundation for Basic Research (project no. 07-04-00746-a). We are grateful to E.J.J. Groenen for helpful discussions.

REFERENCES

1. *Biological Magnetic Resonance*, Vol. 22: *Very High Frequency (VHF) ESR/EPR*, Ed. by O. Y. Grinberg and L. J. Berliner (Kluwer Academic/Plenum, New York, 2004).
2. *Spin Labeling: Theory and Applications*, Ed. by L. J. Berliner (Academic, New York, 1976).
3. H.-J. Steinhoff, EPR Newsl. **15**, 17 (2005).
4. E. Ferrando, U. Schweiger, and D. Oesterhelt, Gene **125**, 41 (1993).
5. D. Oesterhelt and W. Stoeckenius, Methods Enzymol. **31**, 667 (1974).
6. D. Radzwill, K. Gerwert, and H.-J. Steinhoff, Biophys. J. **80**, 2856 (2001).
7. H.-J. Steinhoff, A. Savitsky, C. Wegener, et al., Biochim. Biophys. Acta **1457**, 253 (2000); K. Mobius, A. Savitsky, Ch. Wegener, et al., Magn. Reson. Chem. **43**, S4 (2005); N. Radzwill, K. Gerwert, and H.-J. Steinhoff, Biophys. J. **80**, 2856 (2001).
8. C. Beier and H.-J. Steinhoff, Biophys. J. **91**, 2647 (2006).
9. D. Mustafi, G. B. Wells, H. Joela, et al., Free Radic Res. Commun. **10**, 95 (1990).
10. D. Mustafi and H. Joela, J. Phys. Chem. **99**, 11 370 (1995).
11. D. Mustafi and M. W. Makinen, J. Am. Chem. Soc. **117**, 6739 (1995).
12. D. Mustafi, A. Sosa-Peinado, and M. W. Makinen, Biochemistry, **40**, 2397 (2001).
13. S. B. Orlinskii, J. Schmidt, P. G. Baranov, et al., Phys. Rev. Lett. **92**, 047603 (2004).
14. S. B. Orlinskii, J. Schmidt, E. J. J. Groenen, et al., Phys. Rev. Lett. **94**, 097602 (2005).
15. S. B. Orlinskii, H. Blok, J. Schmidt, et al., Phys. Rev. B **74**, 045204 (2006).
16. O. G. Poluektov and J. Schmidt, Bruker Rep. **143**, 34 (1996).
17. W. B. Mims, in *Electron Paramagnetic Resonance*, Ed. by S. Geschwind (Plenum, New York, 1972).

Translated by V. Makhlyarchuk