Saturation Transfer Electron Paramagnetic Resonance on Membrane-Bound Proteins. I- Rotational Diffusion of Rhodopsin in the Visual Receptor Membrane

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SUMMARY : We have applied the technique of saturation transfer electron paramagnetic resonance to the study of spin labeled membrane-bound bovine rhodopsin. Based on the comparison with theoretical and experimental spectra corresponding to isotropic slow motion, the present data leads to a rotational correlation time for the membrane-bound rhodopsin molecule of 20 μsec at 20°C. Bleaching does not appear to influence the motion of the protein while addition of glutaraldehyde (5%) stops its rotation completely. These results are in good agreement with what is known about the motion of the membrane-bound rhodopsin, establishing the applicability of the saturation transfer technique to the study of slow anisotropic motions of membrane-bound proteins.

INTRODUCTION :

Conventional EPR spectroscopy of nitroxide radicals is limited to the measurement of correlation times shorter (faster) than 10^{-7} sec. However, a new EPR technique referred to as saturation transfer spectroscopy has recently been developed which has maximum sensitivity to rotational motions in the range of $10^{-7} < \tau_2 < 10^{-3}$ sec (1 to 7), where τ_2 is the rotational correlation time. Extensive theoretical work has been already published for the case of isotropic motion (3,4,5). The technique has proven applicable to the study of soluble proteins such as spin labeled hemoglobin and also spin labeled subfragment-1 in myosin (6,7). This latter system undergoes a partially anisotropic motion, but the reference spectra corresponding to isotropic motions were found to be useful

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in analyzing the data.

Since membrane-bound proteins are likely to experience even slower mo-
tions than soluble proteins, saturation transfer spectroscopy should be a
useful technique to investigate their properties. However the expected
high anisotropy of the movements involved makes it important to test the
method in a situation where the correlation time is already known. In that
instance rhodopsin is a unique case. In 1972 Cone (8) showed that the tran-
sient photodichroism of rhodopsin molecules in the visual receptor mem-
branes has a relaxation time of 20μ sec at room temperature. This value de-
termines the rotational time scale of the molecule.

In the present article rhodopsin is used as a model system to test the
applicability of saturation transfer to the study of the motion of a mem-
brane-bound protein. Since this technique enables us to study the protein
both in the dark and in the light, we eventually can test for any diffe-
rences in the motion of rhodopsin induced by bleaching.

MATERIALS AND METHODS :

Rod outer segment membranes were isolated from cattle retinas as des-
cribed by Osborne et al. (9). Membranes were used fresh or stored in li-
quid nitrogen before use. The rhodopsin concentration was determined by
the absorption at 500 nm, after solubilization of an aliquot in 3% Ammonyx
LO.

Spin label 3- maleimido-2,2,5,5 tetramethyl 1-pyrrolidinyloxyl was
purchased from Synvar (Palo-Alto). Infra-red spectroscopy showed no trace
of the iso-maleimide.
In a typical experiment 2 ml of 150μM rhodopsin was incubated overnight in
complete darkness with 2 to 5 spin labels per rhodopsin molecule at 4°C.
The unbound spin label was eliminated by washing 5 times (100 000 g for
30 min.). Labeled membranes were resuspended in minimum volume of 20mM
Tris-4Cl buffer pH 7.2 and transfered to a quartz cell for EPR spectrosco-
py. Final concentration of rhodopsin was up to 500μM.
EPR experiments. A Varian E-109, X band spectrometer was employed in the
absorption mode. A 50μl flat quartz cell was used together with a quartz
dewar for temperature control. For the saturation transfer experiments the
exact microwave field strength received by the sample in the 50μl quartz
cell was determined by calibration with Fremy's salt (3). It was found that
a microwave field amplitude of H_1 = 0.25 G corresponded to a display of
Po = 32.2 mw on the microwave power dial. Conventional EPR spectra were
recorded with 100 kHz field modulation (2 G amplitude). For the 2nd harmo-
nic out-of-phase the modulation frequency used is 50 KHz, with an amplitude
of 5 G. In the latter case the phase was adjusted to minimize the signal
of the second harmonic out-of-phase at a very low power (.5 mw).
The spectrometer was connected to a Tektronix 4051 computer (8 k me-
mory), allowing accumulation, subtraction and integration of the spectra.

RESULTS :

A conventional 1st harmonic absorption spectrum of the rod outer seg-
ment membranes labeled with maleimide spin label is shown on Figure 1a.
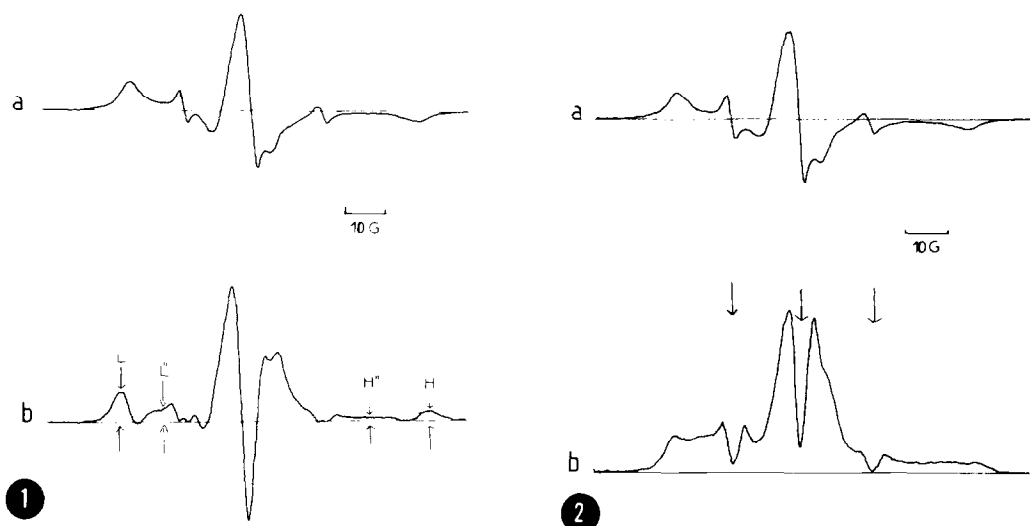
Figure 1 : Spin labeled rhodopsin membranes at 4°C. 1-a) conventional EPR
spectrum : 1st harmonic in phase, 10 mW, 2G, 100 KHz. The maximum splitting
is 65.9 ± .2G   1-b) saturation transfer spectrum : 2nd harmonic out-of-
phase, 32.2 mW, 5 G, 50 KHz. The parameters L, L", H" and H correspond to
the signal amplitude at the positions indicated by the arrows (for more
detailed theoretical explanation see ref. 3). Note that the weakly immobilized
signal appears as a small perturbation on the whole saturation transfer spectrum.

Figure 2 : Spin labeled rhodopsin membranes at 4°C after incubation with
5 % glutaraldehyde. 2-a) 1st harmonic in phase, same conditions as in
figure 1-a. The maximum splitting is 67.0 ± .2G 2-b) 2nd harmonic out-of-
phase, same conditions as in figure 1-b. The arrows indicate the regions
of overlap of the two types of signals, weakly and strongly immobilized.

The spectrum was recorded keeping the sample in the dark. Double integra-
tion of the ESR spectrum and comparison with the optical absorption indi-
cates that slightly less than one nitroxide was bound per rhodopsin mole-
cule. Delmelle and Virmaux (10) incubating with a larger excess of spin
label found more than one nitroxide bound per rhodopsin. In both cases,
a small fraction of the signal (5 to 10 %) corresponds to a weakly im-
mobilized probe. The ratio of the weakly immobilized to the strongly im-
mobilized signal was practically constant in all our experiments. The nar-
row lines superimposed on the broad spectrum can be generated with the
same spin label dissolved in a 50/50 mixture of glycerol-water at 4° C.
As a consequence computer subtraction of this contaminant is possible.

      Figure 1b is a 2 nd harmonic out-of-phase absorption display. The
gain is ten times greater than with the first harmonic. However, because
of the high concentration of membranes in our experiments, it is still pos-
sible to record the spectrum in a short period of time (16 minutes), with

TABLE 1 :

Rotational correlation time of rhodopsin as a function of temperature
deduced from the saturation transfer spectral parameters
according to Thomas et al (3) :

Temp. °C	– 4	+ 4	+ 20	+ 37	5 % Gluta. + 4° C
H''/H	.58	.37	.28	.16	1.05
$\tau_2 (\mu sec)$	80-100	50-80	20	7-10	$\geqslant$ 1 000

L''/L	.65	.48	.43	.42	~ 1.13
$\tau_2 (\mu sec)$	60	30	15-20	15-20	$\geq$ 1 000

The uncertainty in τ_2 is due   a) to the difficulty to measure L'' because
of the overlap with the weakly immobilized signal ; b) to the difficul-
ty to measure H'' because of its small value ; c) the discrepancy between
theoretical and experimental values of τ_2 in reference 3.

a high signal to noise ratio. 1st harmonic in-phase and 2 nd harmonic out-
of-phase spectra were recorded from -4° C to 37° C, in the dark. Increase
of temperature resulted only in a small decrease of the maximum splitting
for the first harmonic. In the same range of temperature the 2 nd harmo-
nic out-of-phase changes in a more dramatic way. These changes can be cha-
racterized by the measure of H''/H and L''/L (see fig.1) ; such parameters
appear in table I.

In order to see whether bleaching affects the spectra of the labeled
protein, a sample was labeled in the dark, then one half of the prepara-
tion was illuminated. The first and 2nd harmonic spectra of the two
samples (bleached and unbleached) in two different cells were then recor-
ded carefuly and repeatedly. No significant difference was detected. This
is a new finding, since the measurements by Cone were not possible with
bleached rhodopsin.

Finally, membranes after labelling were incubated with 5 % glutaral-

dehyde. Figures 2a and 2b show the resulting spectra in the two different modes.

DISCUSSION :

Figures 1 and 2 permit the modifications induced by glutaraldehyde on the classical EPR spectrum and on the saturation transfer spectrum to be compared. The advantage of the second technique is striking.

The fact that bleaching does not induce any conformation change detectable by saturation transfer does not question the validity of the method. Indeed, it is known by neutron diffraction (11) that the modification of the structure of the chromophore induce no gross change in the protein.

It is remarkable that the overall shapes of the second harmonic spectra shown on figures 1b and 2b appear very similar to some of the reference spectra generated with spin labeled hemoglobin by Thomas et al (3), i.e., with a protein tumbling in an isotropic environment. Furthermore , the decrease of temperature and the addition of glutaraldehyde both modified the spectra in the direction one would anticipate for isotropic motion.

Consequently it is reasonable to use those reference spectra to analyse our data on a quantitative level, in spite of the probable anisotropy of the actual motion. Peak ratios L''/L and H''/H have been indicated by Thomas et al as good reporters of τ_2. Table 1 shows the different values obtained with spin labeled rhodopsin from -4° C to 37° C.

Following these procedure, the correlation time estimated for the rotation of rhodopsin in the rod outer segment membrane is approximately 20μsec at 20°C (see table 1). Such numerical values agree with the values obtained by Cone (8). Hence a direct application of the rules established to interpret saturation transfer spectra for isotropic motion seems reasonable.

The type of motion most often proposed for membrane protein is cylindrical rotation about an axis normal to the membrane plane. Calculations of 2nd harmonic out-of-phase spectra in the case of slow cylindrical motion are in progress in our laboratory. In the case of a macroscopically oriented systems with all rotation axes parallel, these theoretical spectra are strongly affected by the cylindrical symmetry of the motion and are quite sensitive to the orientation of the spin label relative to the axis of rotation. Thus much more information could be gained from experiments if the membrane were oriented. Indeed, the present experimental results from a membrane suspension show no spectral features that indicate a deviation from isotropic motion.

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