Photoreceptor rhodopsin: structural and conformational study of its chromophore 11-*cis* retinal in oriented membranes by deuterium solid state NMR

Gerhard Gröbner[a], Gregory Choi[a], Ian J. Burnett[a], Clemens Glaubitz[a], Peter J.E. Verdegem[b], Johan Lugtenburg[b], Anthony Watts[a,*]

[a] *Biomembrane Structure Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK*
[b] *Leiden Institute of Chemistry, University of Leiden, 2300 RA Leiden, Netherlands*

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Abstract Rhodopsin is the retinal photoreceptor responsible for visual signal transduction. To determine the orientation and conformation of retinal within the binding pocket of this membrane bound receptor, an ab initio solid state ^{2}H NMR approach was used. Bovine rhodopsin containing 11-*cis* retinal, specifically deuterated at its methyl groups at the C_{19} or C_{20} position, was uniaxially oriented in DMPC bilayers. Integrity of the membranes and quality of alignment were monitored by ^{31}P NMR. Analysis of the obtained ^{2}H NMR spectra provided angles for the individual labelled chemical bond vectors leading to an overall picture for the three dimensional structure of the polyene chain of the chromophore in the protein binding pocket around the Schiff base attachment site.

Key words: Membrane protein; Rhodopsin; NMR, solid state ^{2}H-; 7 TMD GTP coupled receptor; Retinal structure; Ligand-receptor interaction

1. Introduction

Rhodopsin (39 kDa) is a photoreceptor, which converts an incident photon of visible light into an optic nerve impulse [1]. The protein belongs to the family of G-protein coupled receptors, all of them transducing chemical or optical signals across the cellular membrane [2]. Since all of these receptors share a common spatial organization in the membrane [3–5], rhodopsin can be considered a model system for this class of receptors. The activation of rhodopsin is initiated by photoisomerization of its chromophore 11-*cis* retinal which is tightly bound in a pocket but gets slowly released as all-*trans* retinal after activation [1]. Many similarities exist between photoreception in rhodopsin, where light acts as a 'hormone', and chemical hormone action in other 7 TMD, G-coupled receptors. Thus a precise knowledge of the structural organization of the retinal binding site in rhodopsin is essential if an understanding of the pharmacologically important signal transduction mechanism for a wide range of similar receptors is to be gained, which are not readily amenable for high resolution structural studies [6,7].

For rhodopsin a 7-helix bundle arrangement in membranes was suggested by Baldwin and others using topographic mapping, or biophysical and theoretical approaches [3–5]. The proposed 7-helix structure has recently been confirmed at 6 Å resolution using electron cryomicroscopy (EM) on vertebrate rhodopsin [8,9]. However, the resolution obtained in these EM studies is still too low to reveal any molecular details of the retinal binding site, although some indirect evidence exists for the retinal environment [10,11].

One direct approach to obtain high resolution, non-crystallographic structural information on membrane proteins ($M_r > 30$ kDa) is solid state NMR spectroscopy on macroscopically oriented membranes [12–18]. In such macroscopically oriented membrane systems, lipids and proteins are arranged uniaxially around the membrane normal. In combination with isotopic labelling (e.g. ^{2}H, ^{13}C, ^{15}N) well resolved NMR spectra can be obtained to generate orientational information for individual molecular bond orientations. In addition, solid state ^{2}H NMR on aligned membranes has widely been used to study the organization and dynamics of various membrane components [15,17–22].

In the present work, an ab initio ^{2}H NMR approach originally developed by us to probe the structure of the chromophore in the binding pocket of bacteriorhodopsin [18,22] has been used to obtain structural details of retinal in bovine rhodopsin, and hence an insight into the molecular mechanism of a G-protein coupled receptor at an atomic level. For this purpose we developed an alignment procedure [23], based on the ISDU technique [24], allowing us to obtain oriented phospholipid membranes containing rhodopsin which contained 11-*cis* retinal specifically deuterated at its methyl groups (as C-CD_3) at either the C_{19} or C_{20} position. ^{31}P NMR allowed an independent assessment of the quality of alignment and structural integrity of the membrane by using the phospholipids as an internal reporter [25]. Computer analysis of the ^{2}H NMR lineshapes obtained from samples at different inclinations relative to the magnetic field provided the orientational constraints for the various C-CD_3 bond vectors in the polyene chain of retinal in the ground state.

2. Materials and methods

2.1. Bovine rhodopsin in native and reconstituted membranes

Bovine rod outer segments (ROS) were isolated in dim red light, as described before [26,27]. After purification by sucrose density centrifugation, intact photoreceptor discs without peripheral proteins were obtained. Rhodopsin was then regenerated [28] with 11-*cis* retinal, which was specifically deuterated on the methyl group at either the C_{20} or C_{19} position following the procedure of Pardoen [29]. Reconstituted membranes were prepared by purification of the protein from photoreceptor disc membranes, followed by incorporation into

*Corresponding author. Fax: (44) (1865) 275-234 or -259.
E-mail: awatts@bioch.ox.ac.uk

Abbreviations: ISDU, isopotential spin-dry ultracentrifugation; ROS, rod outer segments; NMR, nuclear magnetic resonance; DMPC, dimyristoylphosphatidylcholine; CSA, chemical shift anisotropy

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DMPC (Sigma, UK) bilayers at a lipid/protein molar ratio of 50:1 [26,27]. Finally, 900 μl ROS membrane suspension in deuterium depleted water (Aldrich, UK) containing 20 nM of rhodopsin was filled into a SW28 ISDU cell and centrifuged (80 000×*g*; 4°C; 12 h) under simultaneous evaporation on 73 μm thin glass plates (Marienfeld Glassware, Bad Mergentheim, Germany). Pelleted membrane samples were kept at controlled humidity (52%) at 4°C. For NMR experiments, all samples were placed in a 10 mm NMR sample tube, either round or square, and finally sealed after adding 30 μl of $NaHSO_4$ solution (52% humidity).

2.2. NMR measurements

^{31}P NMR measurements were performed on a Bruker MSL 400 spectrometer at 161 MHz using an Hahn-echo pulse sequence and broadband ^{1}H decoupling (25 kHz) with appropriate phase cycling. The pulse spacing was 50 μs and the width of the 90° pulse was 6 μs. Typically 500–2000 scans were accumulated with a 4 s repetition time. The temperature of the samples was regulated to within ±1° by a control unit (Bruker VT 1000). Orientation dependent ^{2}H NMR spectra of aligned membranes were acquired at 61 MHz using a quadrupolar echo pulse sequence (90°-τ-90°) with a 5.5 μs long 90° pulse, an interpulse delay of 30 μs, a recycle time of 400 μs and appropriate standard quadrature phase cycling.

2.3. Analysis of ^{31}P and ^{2}H NMR measurements

To obtain the value of the mosaic spread for the phospholipids in the aligned membranes, ^{31}P NMR lineshapes at different orientations with respect to the applied magnetic field were analysed by using a modified lineshape simulation algorithm [18,22,23]. The computer program was written in C++ using GAMMA [30] and simulations required between 1 and 10 min CPU time on an INDY R5600 workstation. Computer simulations of the ^{2}H NMR lineshapes obtained at various angles were performed using a distribution of C-CD_3 bond vectors along a rim of a cone due to the fact that retinal has a 2D symmetrical distribution around the membrane normal. In this way the orientation of the specifically deuterated molecular segment in the retinal relative to the symmetry axis of the aligned membrane was determined.

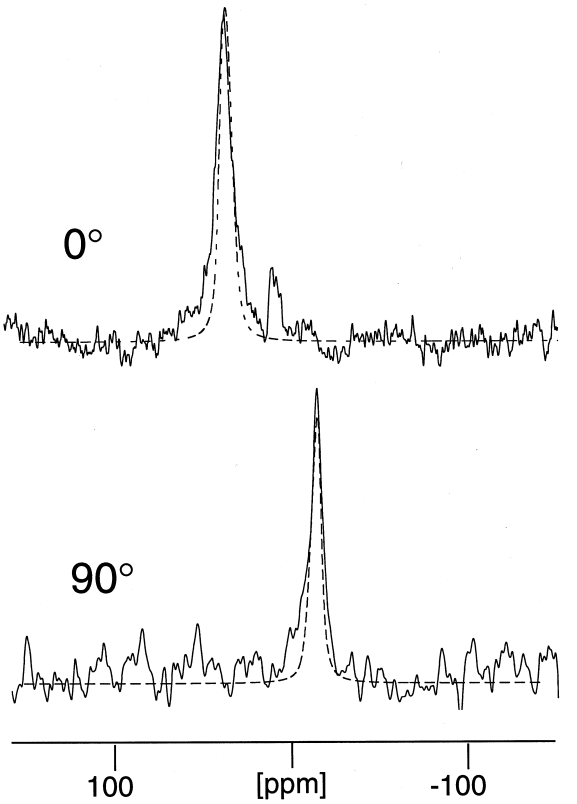

Fig. 1. ^{31}P NMR spectra (1000 scans) at RT of aligned DMPC/rhodopsin membranes at a 50:1 lipid/protein molar ratio. The sample was measured with the field direction either parallel (top) or perpendicular (bottom) to the membrane normal. Best fit simulations for determining the mosaic spread of the membrane directors (^{31}P NMR spectra) are superimposed in all spectra. See text for experimental details.

3. Results and discussion

Fig. 1 shows ^{31}P NMR spectra for DMPC bilayers, aligned by the ISDU method, containing bovine rhodopsin in its dark adapted ground state at a 50:1 lipid to protein molar ratio. Measurements were performed at two orthogonal inclinations with respect to the applied magnetic field. Using ^{31}P NMR spectroscopy the orientational sample quality and the integrity of the bilayer structure can be investigated [25], and an upper limit to the mosaic spread of the incorporated rhodopsin can be determined. The recorded spectrum consists of a single line which varies as a function of the sample inclination from 31 ppm ($\sigma_{\parallel}$) at 0° to −15 ppm ($\sigma_{\perp}$) at 90° orientation with respect to external 85% H_3PO_4 as a reference. This angular dependence of the chemical shift is typical for oriented bilayers where the phospholipid molecules undergo fast motions around the membrane normal [25]. Only a low amount (<5%) of unoriented material was detected from the spectrum at 0°. The angular dependence and shape of the NMR spectra clearly indicate that rhodopsin is incorporated into an intact uniaxially oriented phospholipid bilayer in the liquid crystalline phase [25] by the ISDU technique, which was not possible for native ROS disc membranes, where lipids formed major non-bilayer structures [31]. The measured 46 ppm for $\Delta\sigma$ ($\Delta\sigma = |\sigma_{\parallel} - \sigma_{\perp}|$) was slightly higher than for pure DMPC bilayers ($\Delta\sigma = 42$ ppm) [26,32] due to the lower hydration of the aligned membranes.

The observed ^{31}P NMR spectral linewidths at 0° orientation of 9 ppm, and at 90° orientation of about 8 ppm, compared to 2 ppm for pure DMPC membranes (spectra not shown), were caused mainly by a distribution of membrane directors reflecting the mosaic spread, which was determined to be 20±2°. The best fit simulation of the homogeneous line-broadening is superimposed in both ^{31}P NMR spectra recorded at each orientation (Fig. 1). The use of reconstituted phospholipid/rhodopsin bilayers instead of native ROS membranes provides a technical advantage since the lipid to protein ratio could be decreased from 75:1 in natural ROS membranes to 50:1, thereby improving the signal to noise ratio for the ^{2}H NMR measurements.

Fig. 2 displays the experimental ^{2}H NMR spectra of oriented membranes containing rhodopsin in which 11-*cis* retinal was incorporated as either [20,20,20-$^{2}H_3$]retinal or [19,19,19-$^{2}H_3$]retinal in the dark adapted ground state. The samples contained two stacks of 25 glass plates each. At the 0° inclination at 200 K, the observed lineshape for the C_{20} position exhibits a quadrupolar splitting of 41±2 kHz which is reduced to 22±2 kHz at 90° orientation (spectrum not shown). The obtained values, together with the quadrupolar splitting of 40 kHz obtained from an unoriented powder sample (spectrum not shown) at 200 K, indicates that the methyl groups rotate within the fast-motional regime, as already observed for retinal in bacteriorhodopsin at low temperatures [22]. With this assumption, the orientational dependence of the quadrupolar splitting and lineshape can then directly be analysed to obtain the bond angle between the C-C_{20} bond vector and the

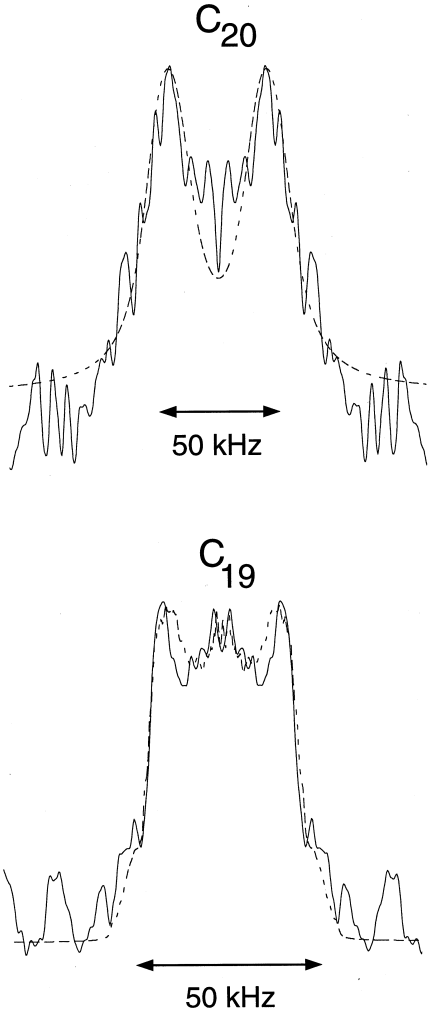

Fig. 2. ^{2}H NMR spectra (150 000 scans) of aligned DMPC/rhodopsin containing membranes (ca. 40 mg lipid) at a 50:1 lipid/protein molar ratio containing 11-*cis* retinal with deuterium-labeled methyl groups either at the C_{20} position (top) or C_{19} position (bottom). Spectra recorded at 200 K with the membrane normal parallel to the applied magnetic field. Simulation of the spectra are shown in dotted lines (see text for details).

membrane normal. By including into the lineshape simulation the values for mosaic spread obtained by ^{31}P NMR, finally a reduced value of $10 \pm 2°$ was deduced for the mosaic spread of the chromophore, indicating a better alignment of the protein compared to the lipid environment. This observation confirmed linear dichroism [24] and Fourier transform infra-red spectroscopy studies [33] on ROS segments aligned by ISDU, which also found for the chromophore a 10° mosaic spread despite the lipids forming mainly non-bilayer structures [31]. Performing the simulation using the reduced mosaic spread, a reduced quadrupolar splitting constant of 40 kHz and a line-broadening of 1500 kHz, the ^{2}H NMR lineshape and its orientational dependence could be analysed unambiguously (best fit simulations are superimposed in Fig. 2) and an angle of $30 \pm 5°$ for the C-C_{20} bond with respect to the membrane normal could be determined.

Similarly, the bond angle of the C_{19} segment, another methyl group along the polyene chain of the chromophore, was determined. In Fig. 2 is shown the ^{2}H NMR spectrum of aligned DMPC/rhodopsin membranes (50:1 molar ratio) containing 11-*cis* [19,19,19-$^2H_3$]retinal from uniaxially aligned membranes measured with the membrane normal parallel (at 0°) to the magnetic field at 200 K. The outer wings of the spectrum correspond to a quadrupolar splitting of 32 ± 2 kHz. Depending on the sign of quadrupolar splitting, which is not known for the observed value, two possible bond angles can correspond to this spectrum. Therefore a computer simulation was performed for a spectrum obtained at 90° orientation (spectrum not shown) to determine unambiguously the bond angle. In this way, the C-CD_3 bond vector of the C_{19} segment was measured to be an angle of $42 \pm 5°$ with respect to the membrane normal (best fit simulation for the 0° tilt spectrum is superimposed in Fig. 2).

The angles obtained for the C_{19} and C_{20} methyl group for 11-*cis* retinal in the binding pocket of rhodopsin provide sufficient information about the geometry and orientation of the polyene chain of the chromophore in the protein. In Fig. 3, retinal is shown in the binding pocket, based on the measured angles for the different methyl group orientations with respect to the membrane normal (identical to the protein long axis). Since the methyl groups in the structure were individually labelled, independent orientational constraints could be obtained, which are essential for an exact structural characterization. Focussing on the specific angles for the two methyl groups which are attached to the polyene chain containing conjugated double bonds, ideally lying in a plane, it can be concluded that the plane of the chromophore is distorted when bound to the protein as already seen for all-*trans* retinal in bacteriorhodopsin [34]. This distortion of the chromophore is suggested to be the main mechanism for light energy storage before rhodopsin activates the G-protein and all-*trans* retinal is released from the binding site [10,35]. The distortion of the 11-*cis* chain is then likely to consist of an in-plane curvature or, either with or without, an out-of-plane twist. However, the error of $\pm 5°$ in our measurements makes it difficult to quantify these deviations precisely from an ideal chromophore plane. Nevertheless, the data obtained here for the conformation and orientation of retinal in bovine rhodopsin are in good agreement with results obtained by Jäger [35] and Lewis et al. [11] using indirect approaches, namely linear dichroism and Fourier transform infrared spectroscopy on mutant forms of rhodopsin despite the completely different methods. Also included in their model derived from the optical methods is a twist in the chromophore around the C_6-C_7 and C_{12}-C_{13}

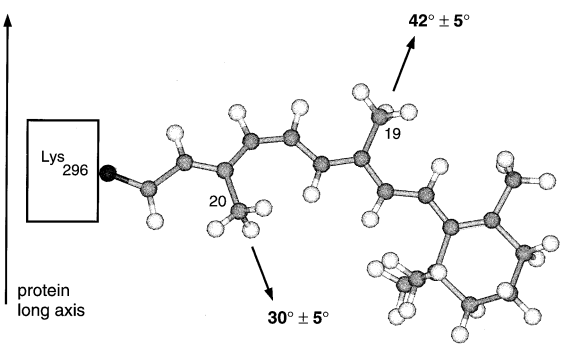

Fig. 3. Orientation and conformation of 11-*cis* retinal in the binding pocket of bovine rhodopsin in the ground state, constructed using the orientations of the methyl groups at the C_{19} and C_{20} position as determined by solid state ^{2}H NMR.

bonds. Recently, this model was refined by Tan [36] using exciton-coupled CD spectra of retinal analogues. In addition, a twisted chromophore has also been suggested using an indirect NMR approach, where constraints from ^{13}C chemical shift values [10,37] were combined with the structural model of Baldwin [3]. Since crystallographic data for the chromophore are not available the ^{2}H NMR studies shown here present the first direct approach to obtain an overall picture of the structure of 11-*cis* retinal in the binding pocket of rhodopsin which is essential for understanding of the general mechanism and function of this 7 TMD G-protein coupled receptor.

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