High-resolution solid-state $^{13}$C-NMR study of carbons C-5 and C-12 of the chromophore of bovine rhodopsin

Evidence for a 6-S-cis conformation with negative-charge perturbation near C-12

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Solid-state $^{13}$C magic-angle spinning NMR spectroscopy has been employed to study the conformation of the 11-cis-retinylidene Schiff base chromophore in bovine rhodopsin. Spectra were obtained from lyophilized samples of bovine rhodopsin selectively $^{13}$C-labeled at position C-5 or C-12 of the retinyl moiety, and reconstituted in the fully saturated branched-chain phospholipid diphytanoyl glycerophosphocholine. Comparison of the NMR parameters for carbon C-5 presented in this paper with those published for retinyl Schiff base model compounds and bacteriorhodopsin by Harbison and coworkers [Harbison et al. (1985) Biochemistry 24, 6955 – 6962], indicate that in bovine rhodopsin the C-6 – C-7 single bond has the unperturbed cis conformation. This is in contrast to the 6-α-trans conformation found in bacteriorhodopsin. The NMR parameters for bovine [12-13C]rhodopsin present evidence for the presence of a negative charge interacting with the retinyl moiety near C-12, in agreement with the model for the opsin shift presented by Honig and Nakaniishi and coworkers [Kakitani et al. (1985) Photochem. Photobiol. 41, 471 – 479].

Rhodopsin is the photosensory pigment of the photo-receptor membrane in the rod cell of the vertebrate retina. It is an amphipathic intrinsic membrane protein comprised of the apoprotein opsin and a chromophoric group. Opsin is a single-chain polypeptide of 348 amino acids (40 kDa) [1 – 3]. Rhodopsin owes its visible absorption band peaking at 500 nm to the covalently bound chromophoric group: 11-cis-retinal (Fig. 1B) [4]. The primary event in vision is photon capture by rhodopsin which induces isomerisation of 11-cis-retinal into the all-trans configuration. This provokes conformational changes in the interior of the protein which propagate to the outside and create topological changes on the surface of rhodopsin. The latter are detected by elements of a signal transduction/amplification cascade, which finally leads to the closing of sodium channels in the plasma membrane of the rod cell within several hundred milliseconds after photon capture. For a review see [5, 6].

When 11-cis-retinal binds to opsin under formation of rhodopsin a large red shift occurs in its absorption maximum (378 nm to 500 nm). In rhodopsin the chromophore is bound via a Schiff base bond to the ε-amino group of lysine 296 [1, 2, 7]. Resonance Raman spectra indicate that this bond is protonated in native rhodopsin [8]. Model protonated Schiff bases of retinal absorb around 440 nm and apparently formation of a protonated retinal Schiff base per se cannot explain the absorption maximum of rhodopsin (500 nm) [9, 10]. The difference in wavenumber between rhodopsin and a model protonated Schiff base is called the 'opsin shift'. For rhodopsin it amounts to: $10^{7} \times (1/440 - 1/500) = 2730 \text{ cm}^{-1}$. This so-called 'opsin shift' is not unique for rhodopsin but is observed in all photosensory pigments based on retinal. Its magnitude, however, depends upon the actual pigment and nature has developed a mechanism to construct photosignals which cover the spectral range 430 – 560 nm [11, 12].

The mechanisms by which a protein moiety (opsin) might significantly influence the absorption maximum of a protonated Schiff base of retinal can be divided into two categories. (a) Conformational changes forced upon the retinal moiety (e.g. twisting around intramolecular bonds). For instance, Honig et al. [13] estimated that twisting around the 6 – 7 single bond in a protonated retinal Schiff base can produce a red shift up to 50 nm. (b) Electrostatic interaction with charged protein residues [9, 10, 13, 14]. This second mechanism was first proposed in 1958 by Kropf and Hubbard [14]. It was pointed out by Honig et al. [13] that this would require at least two point charges in order to explain the observed opsin shifts: one negative charge, as a counterion, near the protonated Schiff base and a second negative charge or strong dipole along the retinylidene polyene chain would satisfy this criterion. Based on the spectroscopic properties of several dehydroretinal rhodopsin analogs [15], Honig, Nakaniishi and coworkers [16] developed a model in which the second negative charge is placed near C-12 of the polyene chain (Fig. 1B) [10].

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Abbreviations. CP, cross polarization; MAS, magic angle spinning; Phy$_2$GroPCho, a phosphatidylcholine with acyl groups 3,7,11,15-tetramethylhexadecanoyl, or phytanoyl; FID, free induction decay.
Harbison et al. [17, 18] have already demonstrated for bacteriorhodopsin that high-resolution solid-state (MAS) $^{13}$C-NMR spectroscopy is a very powerful, non-invasive technique to probe the structure of the chromophore (configuration, conformation, charge distribution and interaction with the peptide chain) in the active site of photosensory pigments. In this paper we will demonstrate the first application of this technique to bovine rhodopsin. For this purpose we used rhodopsin which was $^{13}$C-enriched in its chromophore by incorporation of 11-cis-retinal selectively labeled at either carbon C-5 or C-12 of the polyene chain. Careful analysis of our data leads us to the conclusion that the C-6–C-7 single bond of retinal in bovine rhodopsin is in the S-cis conformation. Our data also suggest the presence of a negative charge in the proximity of the C-12 atom.

MATERIALS AND METHODS

$^{13}$C-labeled retinals

11-cis-Retinals were selectively enriched over 90% with $^{13}$C either at the C-5 or the C-12 position as described by Pardoen et al. [19] and Courtin et al. [20].

Preparation of rhodopsin containing $^{13}$C-labeled retinal

Most procedures have been described before in detail [21]. Bovine rod outer segment membranes were isolated in the illuminated form (opsin), using a continuous sucrose density gradient procedure, from fresh retinas homogenized in the light in the presence of NADPH. The resulting rod outer segment opsin membranes can be stored as a pellet for several weeks at −70°C.

For the formation of labeled pigments, an equimolar amount of the selectively $^{13}$C-labeled 11-cis-retinal was added as a concentrated ethanol solution to rod outer segment opsin membranes resuspended in aqueous buffer (20 mM Pipes, pH = 6.5). The formation of pigment was followed spectrophotometrically at 520 nm. In order to remove any specifically bound retinal as well as the highly unsaturated lipids, which both interfere with the $^{13}$C-NMR analysis, labeled rhodopsin was purified by affinity chromatography over ConA-Sepharose (Pharmacia, Uppsala, Sweden) [22]. The purified labeled rhodopsin was reconstituted in a lipid/rhodopsin ratio of 30:40:1 with diphytanoylglycerolphosphocholine (Phy2GroPCho, Avanti Polar Lipids Inc. Birmingham, AL, USA) by the detergent-dilution method [23].

The type spinners we used (see below) became highly unstable when filled with aqueous membrane suspensions. Therefore the membrane preparations were lyophilized before they were transferred to the spinner. Under the proper conditions lyophilization does not affect the spectral properties of membrane-bound rhodopsin [24]. Rod outer segment membranes that were rehydrated after lyophilization show a normal photolytic cascade. Furthermore Harbison et al. [17] did not detect significant differences between the high-resolution CP MAS $^{13}$C-NMR spectra taken of fully hydrated and of lyophilized bacteriorhodopsin-containing purple membranes.

Recording of solid-state CP-MAS $^{13}$C-NMR spectra

High-resolution solid-state CP-MAS $^{13}$C-NMR spectra were obtained on a Bruker CXP-300 NMR spectrometer working at 75 MHz for $^{13}$C nuclei. The rotors were of the Andrew-Beams design and were constructed from the industrial ceramic boronitride (BN). These do not show $^{13}$C signals which is a disturbing aspect of the more routinely used Delrin rotors. Cross polarisation from $^1$H to $^{13}$C spin systems was achieved with rotating fields of 1.5 mT and 6.0 mT respectively and a mixing time of 2 ms. A 2-s relaxation delay between pulses was used. Spectra were recorded at different spinning speeds between 2.1 kHz and 2.5 kHz. The $^1$H decoupling field was equivalent to 60 kHz. To prevent baseline distortions, phase cycling was used combined with spin temperature alternation (cyclops). All chemical shifts are relative to external tetramethylsilane (Me₄Si).

Calculation of chemical shift tensor elements

Values for the chemical shift tensor elements were calculated from the intensity distribution between the centre band and the rotational sidebands identified in the MAS-NMR spectra, according to the graphical approach developed by Herzfeld and Berger [25]. Calculations were performed on spectra obtained at two different spinning speeds (2.1 kHz and 2.5 kHz). The centre band and first- and second-order side bands in the spectra were fitted with a gaussian lineshape and their area was determined graphically. In principle, the two first-order sidebands are already sufficient to calculate the tensor elements, but additional sidebands were used to check or refine the values obtained. Thus, the final accuracy in the values calculated for the $^{13}$C-tensor elements was substantially better than the accuracy with which the intensity of each individual band could be determined. Analysis of the calculations and comparison of values obtained at different spinning speeds, indicated an upper level of ± 6 ppm for the error in the values of the tensor elements. It also showed that the values for $\sigma_{22}$ and $\sigma_{33}$ were less susceptible to variations in the intensity measurements than the value for $\sigma_{11}$.  

![Structure of all-trans retinal showing the numbering of atoms and the positions of the labels (*) in this study.](image-url)
the very high content of polyunsaturated fatty acids in rod outer segment membranes [28, 29] and indeed disappears following lipid removal (not shown). The resonances from the ethylenic $^{13}\text{C}$-labeled positions in retinal are also expected to occur between 125 ppm and 145 ppm [17, 18] and a relatively intense resonance at 128.5 ppm therefore will seriously hinder the analysis of the resonances in spectra of rhodopsin in which labeled retinal has been incorporated.

Hence it was necessary to remove the unsaturated lipids. However, lipid-free rhodopsin is a less suitable substrate for this kind of investigation, as it is not very stable and does not survive lyophilization very well [24]. The best solution therefore is to exchange the unsaturated lipids for saturated ones via a purification and reconstitution step. A complicating factor occurs here because reconstitution in fully saturated straight-chain phospholipids affects the photochemical behaviour of rhodopsin [30]. Instead we have therefore selected the fully saturated branched-chain phosphatidylcholine diphytanoylglycerophosphocholine (acyl chain: 3,7,11,15-tetramethylhexadecanoyl, abbreviated Phy), in which rhodopsin shows fairly normal photoysis (Fig. 3). The presence of the four bulky methyl groups probably prevents the close packing adopted by phospholipids with straight saturated acyl chains, and creates a more fluid environment comparable to lipids with unsaturated acyl chains [31].

Fig. 4 compares the CP-MAS $^{13}\text{C}$-NMR spectra of (a) pure Phy$_2$GroPCho, (b) unlabeled rhodopsin reconstituted in Phy$_2$GroPCho, and rhodopsin labeled with (c) 11-cis-[5-$^{13}\text{C}$]-retinal or (d) 11-cis-[12-$^{13}\text{C}$]-retinal which, following purification, was reconstituted in Phy$_2$GroPCho. Only the low-field sides of the spectra are shown. The intense peak at 128.5 ppm present in the spectra of rod outer segment membranes (Fig. 2) has now been very strongly reduced (asterisk). In the spectra of the labeled rhodopsins new resonances are apparent in the region 100–160 ppm (arrows), which derive from the single $^{13}\text{C}$-labeled retinal. This is very evident in the difference spectra shown in Fig. 5A and B obtained by subtracting the spectrum of unlabeled rhodopsin (Fig. 4B) from the spectra of the labeled rhodopsins (Fig. 4C, D). The centre and side bands of the new resonances were identified by analysis of spectra recorded at different spinning speeds. The isotropic chemical shifts of the carbons C-5 and C-12 are located at respectively 130.5 ppm and 133.5 ppm ($\pm$ 0.3 ppm). Several spinning side bands can be observed additional to the centre band, indicating a large $^{13}\text{C}$ chemical shift anisotropy for both nuclei. Bleaching of the samples (300-W tungsten lamp through a 530-nm cut-off filter) resulted in a mixed population of photoproducts. In the spectra this caused a large decrease in the sharp resonances assigned to C-5 and C-12, and a complete loss of the spinning side bands (Fig. 5C). The decrease of resonance intensity at $\sigma_1$ is probably due to line broadening as a result of chemical heterogeneity provoked by the bleaching.

Useful information on the molecular environment of a nucleus can be obtained by resolving its chemical shift tensor elements and comparing this to model systems [18]. Recently Herzfeld and Berger [25] developed a graphical approach to derive the values for the principal chemical shielding tensor elements from the intensity distribution between the centre band and the rotational sidebands in NMR spectra recorded at the magic angle. We used this approach to calculate the tensor elements $\sigma_{11}$, $\sigma_{22}$ and $\sigma_{33}$ of the $^{13}\text{C}$-5 and $^{13}\text{C}$-12 nuclei of retinal in rhodopsin, from spectra recorded at two spinning speeds (2.1 kHz and 2.5 kHz). This yields values of 33 ppm, 143 ppm and 215 ppm for carbon C-5 and 41 ppm,

Despite the fact that we concentrate on only one particular carbon atom out of a complex of 3500, the signal-to-noise ratio of the centre band and first rotational sidebands is reasonably good. The signal-to-noise ratio of our spectra is comparable to similar spectra published for bacteriorhodopsin (see [17]) and the latter authors arrive at a comparable accuracy in the values of the tensor elements.

RESULTS

Fig. 2 shows typical MAS $^{13}\text{C}$-NMR spectra of dark-adapted lyophilized rod outer segment membranes under various conditions. A conventional $^{13}\text{C}$-MAS spectrum, recorded with 90° pulses, is given in Fig. 2A. It shows an abundance of sharp resonances which all derive from the mobile lipid acyl chains, since they disappear following lipid extraction (not shown). The rather immobile protein resonances of carbonyl and aromatic residues probably relax too slowly and do not show up in these spectra. The intensity of less mobile groups can, however, be selectively enhanced by $^1\text{H}$-$^{13}\text{C}$ cross-polarization. With this approach (Fig. 2B), characteristic protein features like the resonances of the carbonyls at 172 ppm and of the aromatic residues at 128 ppm now appear clearly, together with strong rotational sidebands. Both give relatively broad resonances because of chemical heterogeneity of the carbonyl and aromatic groups in the protein and in the case of the carbonyls also as a consequence of dipolar coupling to $^{14}$N in the peptide bond [26]. Very apparent is the intense resonance peak at 128.5 ppm. This resonance has been assigned to the double-bond CH = CH carbon atoms in the fatty acyl chains of the phospholipids [27] in view of

\[ \text{A} \]

\[ \text{B} \]

Fig. 2. $^{13}\text{C}$-MAS NMR spectrum of lyophilized rod outer segment membranes measured with (A) 90° pulses and (B) $^1\text{H}$-$^{13}\text{C}$ cross-polarization. Spectra were taken using a sample spinning speed of 2.1 kHz and each spectrum is an accumulation of approximately 30000 FIDs. For spectrometer settings see the Methods. Samples contained about 450 nmol rhodopsin. Ar = aromatic centerband (c) and rotational sidebands; Ca = carbonyl centerband (c) and rotational sidebands.
Fig. 3. Photolysis of rhodopsin in PhyzGroPCho compared to native membranes. Absorbance spectra of a suspension of photoreceptor membrane (A) or rhodopsin reconstituted in either StezGroPCho (B) or PhyzGroPCho (C), all suspended in 20 mM PIPER buffer, pH = 6.5, 20°C (spectrum 1). After flash illumination (spectrum 2), subsequent spectra (3-7) were recorded every 3 min. (A) and (C) show the normal sequence: metarhodopsin I (380 nm) is generated immediately upon illumination and slowly decays under formation of metarhodopsin II (455 nm). For further details see [23].

149 ppm and 209 ppm for carbon C-12 respectively. The error in these values is in the order of 6 ppm. This error is largely due to the signal-to-noise ratio in the complicated spectra, which under the present experimental conditions cannot be substantially improved. Tables 1 and 2 compare the values we obtain for the tensor elements with values of model compounds and bacteriorhodopsin published by Harbison et al. [18]. This comparison strongly suggest the presence of a 6-S-cis linkage in the chromophore of rhodopsin and the presence of a negative charge in the vicinity of C-12.

DISCUSSION

The present results on two 13C-labeled rhodopsins prove that by means of high-resolution solid-state NMR spectroscopy very detailed information can be obtained about the structure of the chromophore and its interaction with the

Fig. 4. 13C MAS-NMR spectra of lyophilized samples of (A) PhyzGroPCho, (B) bovine rhodopsin in PhyzGroPCho, (C) [5-13C]retinylidene bovine rhodopsin reconstituted in PhyzGroPCho, (D) [12-13C]retinylidene bovine rhodopsin in PhyzGroPCho. Spectrometer settings as in the Methods. Each spectrum is an accumulation of 30000 FIDs. Samples contained 450 nmol labeled rhodopsin. (*) Resonance arising from residual unsaturated fatty acid (the amount slightly varies between preparations); (++) centerband of the labeled retinyl resonance; (1) rotational side bands of the labeled retinylidene resonance.

Fig. 5. Identification of the 13C-labeled retinylidene resonances in bovine rhodopsin, by subtraction of the spectra in Fig. 4. (A) Resonances of 13C-12 (Fig. 4 D minus Fig. 4B). (B) Resonances of 13C-5 (Fig. 4C minus Fig. 4B). (C) As (B), but after illumination of the sample.
Table 1. The isotropic chemical shift and chemical shielding tensors of bovine [5-13C]rhodopsin compared with those of several [5-13C]retinoids having the 6-S-cis or 6-S-trans conformation

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\sigma_1$</th>
<th>$\sigma_{11}$</th>
<th>$\sigma_{22}$</th>
<th>$\sigma_{33}$</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Retinoids with the 6-S-trans conformation</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoclinic all-trans retinoic acid</td>
<td>135.9</td>
<td>27</td>
<td>143</td>
<td>237</td>
<td>[17]</td>
</tr>
<tr>
<td>All-trans [5-13C]retinylidene bacteriorhodopsin</td>
<td>144.8</td>
<td>27</td>
<td>170</td>
<td>237</td>
<td>[17]</td>
</tr>
<tr>
<td>Retinoids with the 6-S-cis conformation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triclinic all-trans retinoic acid</td>
<td>128.8</td>
<td>28</td>
<td>141</td>
<td>217</td>
<td>[17]</td>
</tr>
<tr>
<td>11-cis-[5-13C]Retinylidene bovine rhodopsin</td>
<td>130.5</td>
<td>33</td>
<td>143</td>
<td>215</td>
<td>this paper</td>
</tr>
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</table>

Table 2. The isotropic chemical shift and chemical shielding tensors of bovine [12-13C]rhodopsin compared with those of several other retinoids

<table>
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<tr>
<th>Compound</th>
<th>$\sigma_1$</th>
<th>$\sigma_{11}$</th>
<th>$\sigma_{22}$</th>
<th>$\sigma_{33}$</th>
<th>Reference</th>
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<tr>
<td>All-trans-retinal</td>
<td>134</td>
<td>55</td>
<td>131</td>
<td>216</td>
<td>[18]</td>
</tr>
<tr>
<td>All-trans-retinylidene unprotonated Schiff base</td>
<td>136</td>
<td>67</td>
<td>130</td>
<td>210</td>
<td>[18]</td>
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<tr>
<td>All-trans[12-13C]retinylidene bacteriorhodopsin</td>
<td>134.3</td>
<td>56</td>
<td>130</td>
<td>211</td>
<td>[35]</td>
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<tr>
<td>11-cis[12-13C]Retinylidene bovine rhodopsin</td>
<td>133.5</td>
<td>41</td>
<td>149</td>
<td>209</td>
<td>this paper</td>
</tr>
<tr>
<td>11-cis-retinylidene protonated Schiff base</td>
<td>129</td>
<td></td>
<td></td>
<td></td>
<td>[36]</td>
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protein, as has been found before with bacteriorhodopsin. In addition to the isotropic chemical shift values, the three principal chemical shift tensor elements ($\sigma_{11}$, $\sigma_{22}$ and $\sigma_{33}$) can be obtained as well. Such data provide information that is otherwise inaccessible. The $\sigma_{11}$ value is an indicator for steric perturbation ($\gamma$-effect), the $\sigma_{22}$ value for charge density and $\sigma_{33}$ may reflect isomeric configuration [17, 18].

An unperturbed S-cis C-6 – C-7 single bond in rhodopsin

Valuable information on configuration and perturbation around C-5 of the chromophore in rhodopsin can be deduced from comparison with reported values for the isotropic chemical shift and tensor elements for several retinal derivatives and bacteriorhodopsin at this position (Table 1). For C-5 in rhodopsin the values for both the isotropic shift $\sigma_1$ and the tensor elements $\sigma_{11}$, $\sigma_{22}$ and $\sigma_{33}$ are in close agreement with those found for triclinic retinoic acid. In the latter the conformation around the C-6 – C-7 single bond is a non-planar 6 – 7 S-cis conformation, the thermodynamically more stable conformation, and the favored conformation of retinal in solution [18, 32]. This means that the conformation of the 11-cis chromophore in rhodopsin is in the non-planar unperturbed 6 – 7 S-cis form with no special interactions with the protein. This differs considerably from the situation in bacteriorhodopsin where the chromophore occurs in the planar 6 – 7 S-trans conformation (downfield shift of $\sigma_{22}$) and the C-5 carbon is perturbed by the close proximity of a negative charge from the peptide chain (downfield shift of $\sigma_{22}$).

Charge perturbation near C-12 in rhodopsin

Due to the lack of appropriate crystalline 11-cis-retinal derivatives not much detailed information about position C-12 in a model system is available. At this moment it is most appropriate to compare the values of the chemical shift tensor elements with those of C-12 in bacteriorhodopsin [35], since there is no evidence that at this position special interactions occur with the protein moiety. The isotropic chemical shift value is also compared with those found for a 11-cis protonated Schiff base in solution (Table 2). The most pronounced difference occurs in the $\sigma_{22}$ value. The unperturbed state has $\sigma_{22}$ values near 130 ppm irrespective of headgroup, but in rhodopsin it is shifted to 149 ± 6 ppm, i.e. a significant downfield shift of 19 ± 6 ppm. This downfield shift in $\sigma_{22}$ might also explain why the isotropic shift in rhodopsin is shifted down-field relative to the unperturbed 11-cis retinylidene protonated Schiff base in solution. Habrison et al. [17] present several arguments that such a specific downfield shift in $\sigma_{22}$ reflects accumulation of positive charge, which might be caused by interaction with a negative charge of the protein. Hence a downfield shift of the $\sigma_{22}$ value in rhodopsin reflects the presence of a much larger positive charge on the C-12 carbon than in bacteriorhodopsin. This may be due to the proximity of a negative charge in the protein near position C-12 in rhodopsin similarly as has been argued for C-5 in bacteriorhodopsin [17].

The $\sigma_{11}$ value of rhodopsin shows an upfield shift. As a matter of fact, the $\sigma_{11}$ tensor value reflects steric interactions, but it is not easy to find a satisfactory explanation in this case, since the value for $\sigma_{11}$ show considerable variance (Table 2) and reliable data for 11-cis retinylidene model compounds are still lacking.

Wavelength regulation in visual pigments

It is clear that the measurements on these two 13C-labelled rhodopsins show the power of contemporary solid-state NMR techniques for the study of the interaction of the chromophore in the active site in the protein. The present study proves that the conformation of the C-6 – C-7 single bond is unperturbed non-planar 6 – 7 S-cis. Although all details are not yet known, it also presents a clear indication for the presence of a negative charge in the neighbourhood of C-12 in the chromophore of bovine rhodopsin. These data so far agree with theoretical models for wavelength regulation in rhodopsin [10]. However a more complete interpretation of the presented data has to be based on 13C-NMR spectra of the other carbon atoms in the polyene chain of the chromophore. Studies on bovine rhodopsin labeled in other positions in retinal are in progress. Analysis by 13C-CP-MAS NMR at low temperatures will further allow to investigate retinal conformation and retinal-opsin interaction occurring after photon capture.

The difference in wavelength regulation in rhodopsin (6-S-cis, no charge near C-5, negative charge near C-12), and bacteriorhodopsin (6-S-trans, negative charge near C-5, no charge near C-12) [17], bivalent cations are implicated in wavelength regulation [33] might indicate that there is no close evolutionary relationship between these two classes of photosensory retinal-based pigments: the visual pigment and
REFERENCES