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MAGIC ANGLE SPINNING NMR STUDIES ON THE METARHODOPSIN II INTERMEDIATE OF BOVINE RHODOPSIN: EVIDENCE FOR AN UNPROTONATED SCHIFF BASE

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Abstract—Magic angle spinning (MAS)^{13}C-NMR spectra of the metarhodopsin II intermediate have been obtained using bovine rhodopsin regenerated with retinal ^{13}C-labeled at the C-13 and C-15 positions to investigate the protonation state of the retinal Schiff base linkage. The ^{13}C-labeled rhodopsin was reconstituted into 1,2-dipalmitoleoylphosphatidylcholine bilayers to increase the amount of meta II trapped at low temperature. Both the ^{13}C-15 (159.2 ppm) and ^{13}C-13 (144.0 ppm) isotropic chemical shifts are characteristic of an unprotonated Schiff base, while the ^{13}C-15 shift is significantly different from that of retinal (191 ppm) or a tetrahedral carbinolamine group (70–90 ppm) previously proposed as an intermediate in the hydrolysis of the Schiff base at the meta II stage. This rules out the possibility that meta II non-covalently binds retinal or is a carbinolamine intermediate and provides convincing evidence that Schiff base deprotonation occurs in the meta I–meta II transition, an event that is likely to be important in triggering the activation of transducin.

INTRODUCTION

The retinylidene chromophore is the photoreactive group in the visual pigment rhodopsin responsible for light absorption and protein activation (for a recent review see Birge[1]). It is buried within the interior of the protein covalently attached to lysine 296 as a protonated Schiff base (PSB)†. Light absorption drives an 11-*cis* to all-*trans* isomerization of the chromophore to form bathorhodopsin which subsequently decays through a series of intermediates designated lumirhodopsin, metarhodopsin I and metarhodopsin II. Yoshizawa and Wald[2] proposed that rhodopsin undergoes a conformational change at the metarhodopsin stage based on the observation that low temperatures (<−50°C) block the ability to photoconvert metarhodopsin back to rhodopsin. Several subsequent studies have argued for both global changes in rhodopsin structure[3–6] and local changes in specific amino acid residues during the photoreaction[7,8]. More recently, it has been found that the GTP-binding protein, transducin, is activated at the meta II intermediate[9,10], most likely due to the protein conformational changes associated with meta II formation.

Meta II has a λ_{max} of 380 nm and is in a pH-dependent equilibrium with meta I (λ_{max} = 478 nm) where the equilibrium is shifted towards the meta II intermediate at lower pH[11] (Fig. 1). The pH dependence is opposite to that observed in squid rhodopsin[12] and is anomalous if meta II contains an unprotonated Schiff base as suggested by resonance Raman experiments[13]. Furthermore, bleaching of rhodopsin results in the uptake of at least one proton from solution[14,15]. In order to account for these various protonation changes, it was originally proposed that two protons are taken up by the protein at the meta II stage, one corresponding to the proton lost from deprotonation of the Schiff base and the second originating from solution[11]. Cooper and coworkers have subsequently proposed an alternative model for the meta I to meta II transition where only a single proton is taken up at meta II and is involved in hydrolysis of the Schiff base linkage[16,17]. They suggested that meta II is at least in part a carbinolamine, an intermediate in Schiff base hydrolysis.

Magic angle spinning (MAS) NMR yields high-resolution ^{13}C NMR spectra of membrane proteins (for a recent review see Smith and Griffin[18]). Specific sites on the retinal chromophore can be studied by regenerating rhodopsin with ^{13}C-labeled retinal[19]. The chemical shifts of the C-15 and C-13 retinal carbons are particularly sensitive to the protonation state of the Schiff base and the electrostatic polarization of the chromophore by the protein counterion[20,21]. In retinal PSB model compounds, the ^{13}C-15 (162–167 ppm) and ^{13}C-13 (162–163 ppm) isotropic chemical shifts vary depending on the size and nature of the counterion. MAS NMR has the

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†*Abbreviations*: DMPC, dimyristoylphosphatidylcholine; FTIR, Fourier transform infrared; GTP, guanosine triphosphate; MAS, magic angle spinning; MES, 2-[N-morpholineo]ethanesulfonic acid; meta I, metarhodopsin I; meta II, metarhodopsin II; NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; PSB, protonated Schiff base.

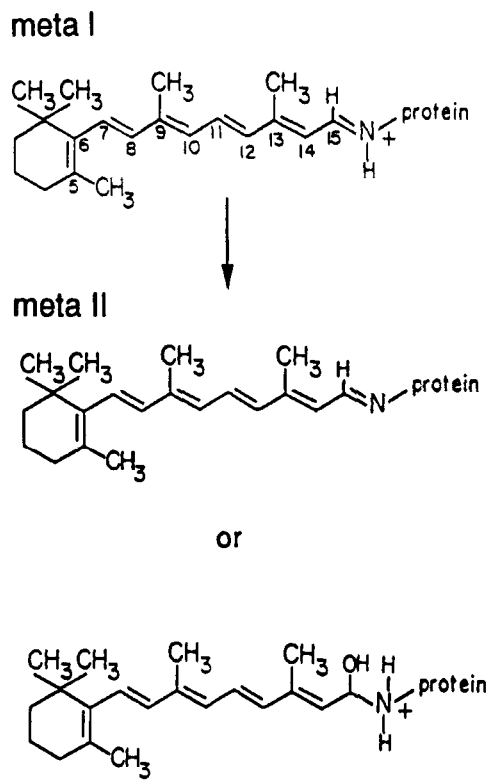

Figure 1. Retinal chromophore structures in the meta I and meta II intermediates of rhodopsin. Meta I (λ_{max} = 478 nm) has an all-*trans* PSB chromophore (top). The formation of meta II (λ_{max} = 380 nm) is thought to involve either deprotonation of the Schiff base (middle) or the onset of Schiff base hydrolysis generating a carbinolamine intermediate (bottom).

capability of determining the protonation state of the Schiff base, since deprotonation leads to a characteristic shift of the ^{13}C-13 resonance to 141–145 ppm and to a small shift of the ^{13}C-15 resonance to 157–159 ppm[20]. Furthermore, the proposed carbinolamine intermediate has a ^{13}C-15 chemical shift in the range of 70–90 ppm, well below that of both protonated and unprotonated Schiff bases[22]. In this paper, MAS NMR spectra have been obtained of the meta II intermediate using rhodopsin regenerated with ^{13}C-13 and ^{13}C-15 retinal. The observed ^{13}C resonances in meta II clearly show that the retinal chromophore is bound to the protein as an unprotonated Schiff base.

MATERIALS AND METHODS

The synthesis of the 15-^{13}C- and 13-^{13}C labeled retinals have been described by Lugtenburg[23]. Rhodopsin was isolated from bovine retinas (Lawson, Lincoln, NE), purified by sucrose gradient ultracentrifugation and hydroxyapatite chromatography, and regenerated with 9-*cis* retinal as previously described[19,24]. The regenerated rhodopsin was reconstituted into membranes by detergent dialysis[25]. Dipalmitoleoylphosphatidylcholine was added to rhodopsin solubilized in Ammonyx-LO in a 100:1 lipid to protein ratio. The mixture was allowed to incubate for 6 h at 4°C and the detergent was subsequently removed by dialysis against either pH 7.4 phosphate buffer or pH 5.5 MES buffer containing 1 mm dithiothreitol over three days. The dialysis buffer was changed every 4–8 h. Absorption spectra were taken of the rhodopsin samples at 4°C after illumination using a 540 nm long pass filter. These spectra exhibited a 380 nm absorption maximum characteristic of the meta II intermediate. The reconstituted protein was concentrated by ultracentrifugation and low speed centrifugation using Amicon centriflo cones. Excess water was removed by blowing N_2 gas over the sample.

^{13}C MAS NMR spectra were obtained on a Chemagnetics CMX 360 MHz spectrometer using a Doty Scientific (Columbia, SC) double-resonance probe equipped with a 7 mm spinning system. The ^{13}C and ^{1}H frequencies were 90.4 and 359.4 MHz, respectively. A standard ^{1}H-^{13}C cross polarization pulse sequence was used with a 4 μs 90° pulse length, a 2 ms contact time, and a 34 ms acquisition time. The data were typically zero-filled to 4K points and line-broadened by 20–30 Hz.

The meta II intermediate was trapped at low temperature using rhodopsin equilibrated at pH 5.5 or pH 7.4. The rhodopsin sample was first loaded into an NMR rotor in the dark and evenly distributed in a thin layer along the rotor walls. The rotor was then illuminated at −5 to −15°C using a 540 nm long pass filter for ~30 s and placed into the precooled NMR probe. NMR spectra were obtained in the dark at −50°C.

RESULTS

Our previous NMR studies on rhodopsin and bathorhodopsin were on detergent-solubilized samples[19,26] since the absorption spectra and primary photochemistry are not significantly different between the detergent and membrane-reconstituted proteins[25]. Several studies have shown that the nature of the lipids in reconstituted systems can greatly influence the meta I–meta II transition, and that unsaturated acyl chains may be necessary for the stabilization of the meta II intermediate[27–29]. Mitchell *et al.* have indicated that the meta I–meta II equilibrium favors meta II in 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) bilayers relative to dimyristoylphosphatidylcholine (DMPC) and that maximal G-protein activation is observed in POPC vesicles at temperatures above the phase transition[30]. In the experiments below, rhodopsin was reconstituted into dipalmitoleoylphosphatidylcholine bilayers where the lipid phase transition is below −30°C allowing for the conversion to meta II in the liquid crystalline phase of the lipid.

The MAS NMR spectrum of 15-^{13}C isorhodopsin reconstituted in bilayers is presented in Fig. 2(a). The large resonance at ~130 ppm results from the natural abundance methylene carbons of the unsaturated palmitoleoyl chain, while the line at ~174 ppm is due to the natural abundance backbone carbonyls of rhodopsin. The sharp resonance at 166.7 ppm is due to the enriched 15-^{13}C label on the retinal chromophore. Within the resolution of the experiment, the frequency (166.7 ppm) and linewidth (90 Hz) of the 15-^{13}C resonance is the same as that previously obtained of detergent-solubilized rhodopsin at low temperature[19].

Isorhodopsin was converted to the meta II intermediate above the phase transition temperature of

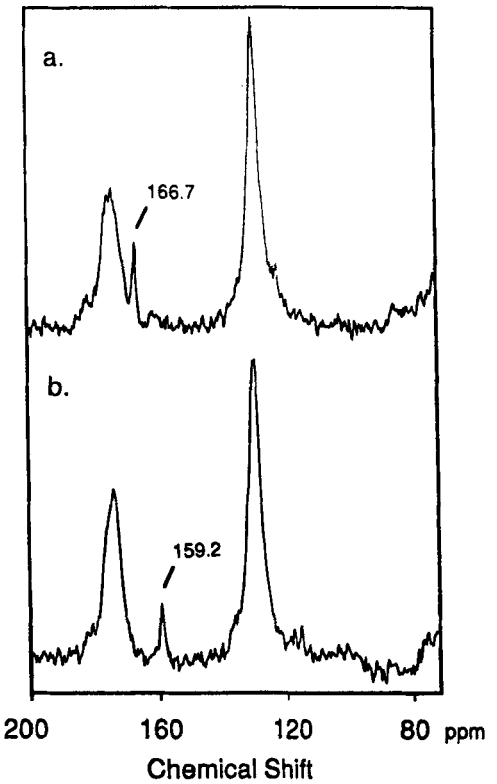

Figure 2. MAS NMR spectra of 15-^{13}C isorhodopsin (a) and meta II (b) at pH 5.5. The sample was converted to meta II at −5 to −15°C. For both the isorhodopsin and meta II spectra, the temperature was maintained at −50°C during for data collection.

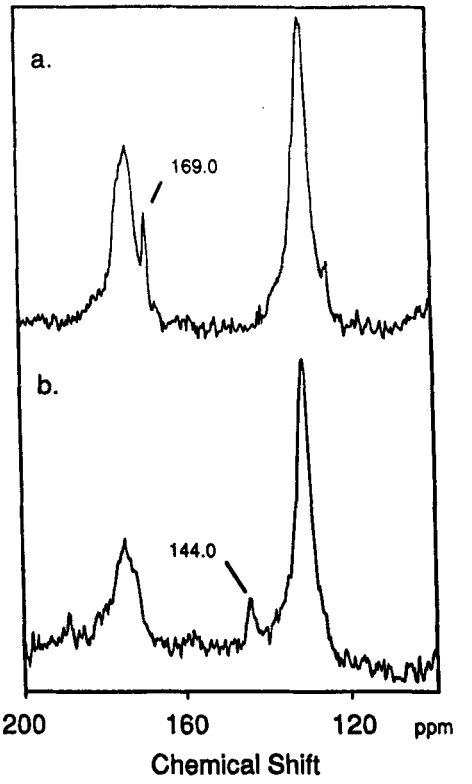

Figure 3. MAS NMR spectra of 13-^{13}C isorhodopsin (a) and meta II (b) at pH 5.5. The sample was converted to meta II at −5 to −15°C. For both the isorhodopsin and meta II spectra, the temperature was maintained at −50°C during for data collection.

dipalmitoleoylphosphatidylcholine. The meta II intermediate was trapped by subsequently lowering the temperature to −50°C. Figure 2(b) presents MAS NMR spectra of meta II produced from rhodopsin at pH 5.5 containing 15-^{13}C retinal. The resonance at 159.2 ppm is assigned to the meta II intermediate. There is no indication of a residual isorhodopsin resonance at 166.7 ppm. The meta II resonance at pH 7.4 (data not shown) is broadened relative to that at pH 5.5 suggesting greater heterogeneity in the environment around the Schiff base.

Figure 3 presents spectra of 13-^{13}C isorhodopsin (a) and meta II (b) at pH 5.5. Model compound studies have shown that the 13-^{13}C resonance is sensitive to the protonation state of the Schiff base[20]. The sharp 13-^{13}C centerband is observed at 169.0 ppm in isorhodopsin and at 144.0 ppm in meta II. The meta II resonance is at a characteristic frequency for an unprotonated Schiff base chromophore.

DISCUSSION

The absorption spectra of the rhodopsin intermediates clearly reflect changes in protein–chromophore interactions that occur during the photoreaction cycle. The red-shifted absorption maximum in rhodopsin and the intermediates prior to meta II argue for a protonated Schiff base linkage. The nature of the chromophore in meta II, however, has been difficult to establish since the absorption maximum of the meta II intermediate (380 nm) is close to that of free retinal (380 nm) and at slightly longer wavelengths than model unprotonated Schiff bases (360–370 nm). Meta II was originally thought to contain an unprotonated Schiff base on the basis of the photoreversibility of meta II to rhodopsin[2]. Cooper and co-workers subsequently have argued that free retinal noncovalently bound in the retinal binding site or a carbinolamine form of retinal are consistent with the experimental data on meta II[16,17]. These competing interpretations have not been resolved by resonance Raman or FTIR spectroscopy since it has not been possible to assign the -C=N-Schiff base or -CHOH—NH2- carbinolamine vibrations. One advantage of NMR over absorption or vibrational spectroscopy in addressing such questions is the ability to characterize unambiguously the electron density at single sites along the polyene chain.

Comparison of the MAS NMR chemical shifts of the meta II intermediate with those of retinal model

compounds clearly indicates that meta II has an unprotonated Schiff base. Both the 15-^{13}C (159.2 ppm) and 13-^{13}C (144.0 ppm) resonances are within the range of chemical shifts observed for unprotonated Schiff bases. Furthermore, the 15-^{13}C resonance is much higher in frequency than that expected for a tetrahedral carbinolamine group (70–90 ppm) and much lower in frequency than expected for retinal (191 ppm).

What do these results reveal about the meta I to meta II transition? First, deprotonation argues that the Schiff base pK_a has decreased during the meta I–meta II transition. Such a shift can result from moving the Schiff base into a more hydrophobic environment, by the approach of a positively charged amino acid, or by a change in the intermolecular angle between the hydrogen bond donor and acceptor[31]. Based on hydropathy plots of the rhodopsin amino acid sequence, His 211 is the only residue other than the lysine 296 with potential positive charge in the transmembrane portion of the bilayer. However, the lack of conservation of His 211 between rhodopsin and the cone pigments argues that this residue is not an integral part of the deprotonation mechanism. As a result, the role of isomerization of the retinal is most likely to change the location or orientation of the Schiff base (or rearrange the protein environment) to facilitate proton transfer at meta II. In this regard, our previous NMR studies have provided converging evidence that a large (6–7 ppm) downfield chemical shift of C-13 in rhodopsin, isorhodopsin, and bathorhodopsin results from a negative counterion in the retinal binding site, whereas the chemical shift for position 13 meta II is not significantly perturbed from the chemical shift observed in model unprotonated Schiff bases, suggesting a different protein conformation in this intermediate. Second, Schiff base deprotonation and the stability of meta II at low pH argues for the protonation of a protein group in the retinal binding site. The simplest explanation would be protonation of glutamate 113, the counterion to the Schiff base[32–34]. Khorana and co-workers have shown that mutagenesis of glutamate 113 to glutamine results in activation of transducin *in the dark* upon addition of all-*trans* retinal[34]. Considering that glutamate 113 is the counterion in native rhodopsin, it is not unlikely that all-*trans* retinal binds to this rhodopsin mutant as an unprotonated Schiff base generating a meta II-like intermediate. Both protonation of glutamate 113 or site-directed mutagenesis to glutamine effectively remove the counterion charge. These results set the stage for NMR studies of lumirhodopsin and metarhodopsin I in order to characterize the protein environment of the retinal chromophore in these intermediates.

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