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The Ring of the Rhodopsin Chromophore in a Hydrophobic Activation Switch Within the Binding Pocket

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The current view that the β-ionone ring of the rhodopsin chromophore vacates its binding pocket within the protein early in the photocascade has been adopted in efforts to provide structural models of photoreceptor activation. This event casts doubt on the ability of this covalently bonded ligand to participate directly in later stages involving activation of the photoreceptor and it is difficult to translate into predictions for the activation of related G protein-coupled receptors by diffusable ligands (e.g. neurotransmitters). The binding pocket fixes the formally equivalent pair of ring methyl groups (C16/C17) in different orientations that can be distinguished easily by ^{13}C NMR. Solid-state NMR observations on C16 and C17 are reported here that show instead that the ring is retained with strong selective interactions within the binding site into the activated state. We further show how increased steric interactions for this segment in the activated receptor can be explained by adjustment in the protein structure around the ring whilst it remains in its original location. This describes a plausible role for the ring in operating a hydrophobic switch from within the aromatic cluster of helix 6 of rhodopsin, which is coupled to electronic changes within the receptor through water-mediated, hydrogen-bonded networks between the conserved residues in G protein-coupled receptors.

Keywords: rhodopsin chromophore; receptor activation; NMR

Introduction

The light-transducing protein, rhodopsin, is a prototypical G-protein-coupled receptor (GPCR). It is a member of the largest sub-family (type A), comprising ~90% of all GPCRs.[1] This family consists of a wide variety of sensory and ligand-activated receptors, which are expected to share a highly conserved process of activation.[2] Although these receptors require structural diversity in their ligand-binding sites, the molecular mechanism of their activation by ligands may well share common principles. The retinylidene chromophore of rhodopsin has a dual role, first as inverse agonist in its 11-*Z* (*cis*) conformation (Figure 1(A)) to realize extremely low levels of basal activity in the ground ("dark") state of the protein. Secondly, on photoisomerization to the 11-*E*(all-*trans*) chain configuration, the chromophore initiates rapid progression of the protein through a sequence of spectrally well-characterized intermediates until, from the late inactive metarhodopsin I state (Meta-I), its role as agonist culminates in the formation of the activated signalling form of the receptor, Meta-II. The nature or existence of any direct role for the chromophore in this final activation step has been brought into question by evidence from photo-crosslinking studies that, prior to formation of the inactive Meta-I state, the ring of the chromophore is extruded from its binding pocket within the protein and extends across the helical bundle (between helix 4 and 7).[3] From this location, it is difficult to deduce any further role for the chromophore ring in

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Abbreviations used: GPCR, G protein-coupled receptor; Meta-I, metarhodopsin I; Meta-II, metarhodopsin II; FTIR, Fourier transform infrared; MAS, magic-angle spinning.

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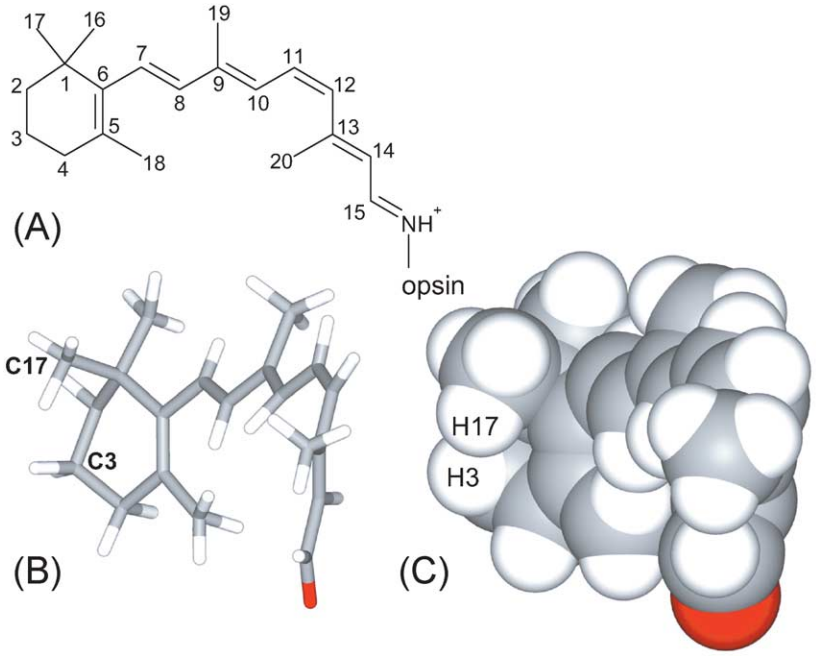

Figure 1. (A) The carbon skeleton chemical structure of the retinylidene chromophore. (B) Model of the chromophore, represented as retinal, showing the C17 methyl group in axial orientation on the ring. (C) Space-filling model of the structure in (B) in which the proton on C3 (H3) penetrates into the van de Waals space of protons on the C17 methyl group (H17).

activation of the receptor or propose any functional relationship with the activation of related receptors by diffusible ligands. The photoaffinity labelling of protein residues was achieved from a chromophore that was chemically modified in the ring (3-diazo-4-oxo) to cross-link adjacent polypeptide residues following its selective photoactivation. These unexpected results and their problematic implications raise the question of whether these early changes are reproduced for the native structure of the chromophore. Studies conducted to resolve this issue are reported here on a rhodopsin-containing chromophore that is selectively enriched only with non-perturbing ^{13}C nuclei for direct NMR observation.

In a previous ^{13}C NMR study, we recognised that the formally equivalent C16 and C17 methyl groups attached to C1 on the ring (Figure 1(A)) are spectrally distinguishable in rhodopsin due to the distinct conformation in which they are held within the binding site.[4] In the ground-state structure of the chromophore (Figure 1(B)), C17 is tilted out of the ring plane into axial orientation whilst C16 remains close to the ring plane in equatorial orientation. The intramolecular contact with the proton at C3 (H3 in Figure 1(C)) polarizes the CH bonds in the C17 methyl group to increase electronic shielding around the carbon atom, and its chemical shift decreases by >4.2 ppm. This "steric" shift, or so-called γ-effect (C3 is in the γ-position from C17), was the largest that had been observed for this ring system.[4] The shift for C17 is therefore viewed as diagnostic of the specific ring contacts within the binding pocket of rhodopsin and provides a sensitive means of monitoring any change in the ring location as rhodopsin progresses along the photoactivation pathway. Photoactivation of previous NMR samples has indicated that these ring contacts remain unperturbed[5] but this observation was limited by a number of factors arising from preparation of protein samples in the native membranes. Firstly, the procedure used to remove excess ^{13}C-labelled retinals reduced the lipid content in the membranes. In these motionally restricted membranes, the formation of the active Meta-II state was suppressed,[6] and so only the inactive photostates (up to Meta-I) were observable. Even then, the possibility that lipid-depletion had prevented some movement of the ring up to the Meta-I state could not be ignored. Finally, the spectra were complicated by the presence of minor components,[4] and so were not optimal for detecting and describing conformational changes in the samples. Although this work indicated clear differences between behaviour of the native chromophore and the photo-affinity analogue, it has not prevented the appearance of further studies that model photoactivation changes in the protein based around the presumption of early ring movement away from its binding pocket.[7] In order to address the limitations of our previous study and provide a conclusive account of the fate of the ring within the photocascade, we have conducted measurements on protein with a ^{13}C-labelled chromophore that was reconstituted into proteoliposomes with native lipids at their full complement found in the native membranes ($\sim$60 lipid molecules per protein molecule). Crucially, this enables the ring contacts to be monitored (1) through to the signalling (Meta-II) and even into later photostates of rhodopsin, (2) for the native structure of the chromophore and (3) with the protein in a non-restricted environment, equivalent to the native membrane.

Results

Characterization of rhodopsin activation in proteoliposomes

Firstly, it was important to demonstrate that the reconstituted protein exhibits its activation behaviour found in the photoreceptor membranes and establish conditions required for effective observation of the active state, Meta-II, that had not previously been converted from ground-state rhodopsin for observation by NMR. Both these aims were best achieved by forming Meta-II directly by brief illumination of small amounts of the reconstituted protein for optical analyses. The reconstitution of rhodopsin with native lipids formed large, predominantly unilamellar proteoliposomes,[8] which showed characteristic UV/visible absorption spectra with a maximum at 498 nm from the chromophore in the ground state of the protein (Figure 2(A)). Following photoactivation at 283 K, the majority of the protein converts to Meta-II, with an absorption maximum at 380 nm, that activates the G-protein, transducin, with the same rate as native photoreceptor membranes (data not shown). Subsequently, the active Meta-II state decays to a population of species, partly absorbing at longer wavelength (Figure 2(A)), with a $t_{1/2}$ of 19 minutes at pH 6.0. The reduced pH favours formation of Meta-II and further UV/visible analysis showed that the Meta-II to Meta-I ratio was around 4 : 1. These measurements establish that, following photoactivation of the membrane-reconstituted protein, the active Meta-II state is generated rapidly with high yield and is sustained for a few minutes at this pH and temperature. This is indistinguishable from the behaviour of protein in its native membrane.[9]

Since Meta-II decays rapidly under these conditions, effective observation by NMR was possible only if the active state could be trapped and maintained stably at low temperature. The Fourier transform infrared (FTIR) difference spectra in Figure 2(B) (blue) show that when Meta-II is cooled to liquid nitrogen temperatures where the primary intermediate bathorhodopsin is stable (80 K) and then warmed to temperatures associated with conversion to subsequent early intermediates,[9] its characteristic changes in the carboxyl region from Glu residues (1767(−), 1750(+), 1728(−), 1710(+) cm^{-1}) are well preserved and are clearly distinct from difference spectra for the earlier intermediates (red). The positive amide I band from Meta-II at 1686 cm^{-1} is preserved also, and this region shows no accumulation of Meta-I, as is evident from the absence of positive bands at 1664 and 1182 cm^{-1}. The same result is obtained if a sample is illuminated at 80 K to generate bathorhodopsin, as used for NMR samples, warmed to 283 K to convert bathorhodopsin to Meta-II and then cooled again to the transition temperatures of the earlier intermediates. Therefore, even during the relatively slow cooling of Meta-II in the FTIR cryostat (initially, 2.5 K min^{-1}), its conversion back to Meta-I is prevented and the active state can be maintained stably for NMR observation at temperatures less than 253 K.

NMR observation of the chromophore ring segment on photoactivation

Magic-angle spinning (MAS) NMR spectra recorded from the proteoliposomes samples at pH 6.0 show distinct resonances from the ^{13}C-enriched C16 and C17 methyl groups superimposed within the alkyl region of the spectra (Figure 3(A), stacked to the left). The natural-abundance ^{13}C contribution from the membranes is effectively subtracted using a spectrum from proteoliposomes prepared with non-labelled chromophore to reveal only the C16 and C17 resonances (Figure 3(A), stacked to the right). The C16 and C17 resonances are well resolved with 4.8 ppm separation and no evidence of minor components as observed previously in samples prepared in native membranes.[4] The large amount of membrane-reconstituted protein used for NMR observation meant that these optically dense samples could not be rapidly photoactivated directly into the Meta photostates, as achieved with near quantitative results on the small samples used in the optical analyses. It has been reported recently that high levels of direct photoconversion to Meta-II could be achieved for dense NMR samples of protein in its solubilized state.[10] This sacrifices the option of observing sequential changes through the earlier intermediates with protein in a native membrane environment but the approach enabled important observations that will be discussed below (see Further recent NMR observations). As established previously,[5] the optimal photoactivation conditions for membrane preparations for NMR were achieved following lengthy photoactivation at low temperature into the primary photointermediate, bathorhodopsin from which the later photostates can be formed on warming (Materials and Methods). This unavoidably generates a photoequilibrium mixture in which only a portion of the rhodopsin is photoconverted properly but does prevent non-equilibrium photoconversion or decay of the metarhodopsin intermediates.[5] Despite adopting different photoactivation conditions, the prior optical analyses established that the reconstituted protein should reproduce the optimal levels of photointermediates achieved previously in the NMR samples of photoreceptor membranes.[5]

Following photoconversion at the bathorhodopsin stage and progression into the Meta-I state, the spectra remain unchanged, apart from some broadening of the C17 resonance (Figure 3(A)). The unperturbed spectral splitting between C16 and C17 indicates that these methyl groups retain their characteristic conformation in Meta-I and that the β-ionone ring is not displaced from its binding pocket. The previous result in

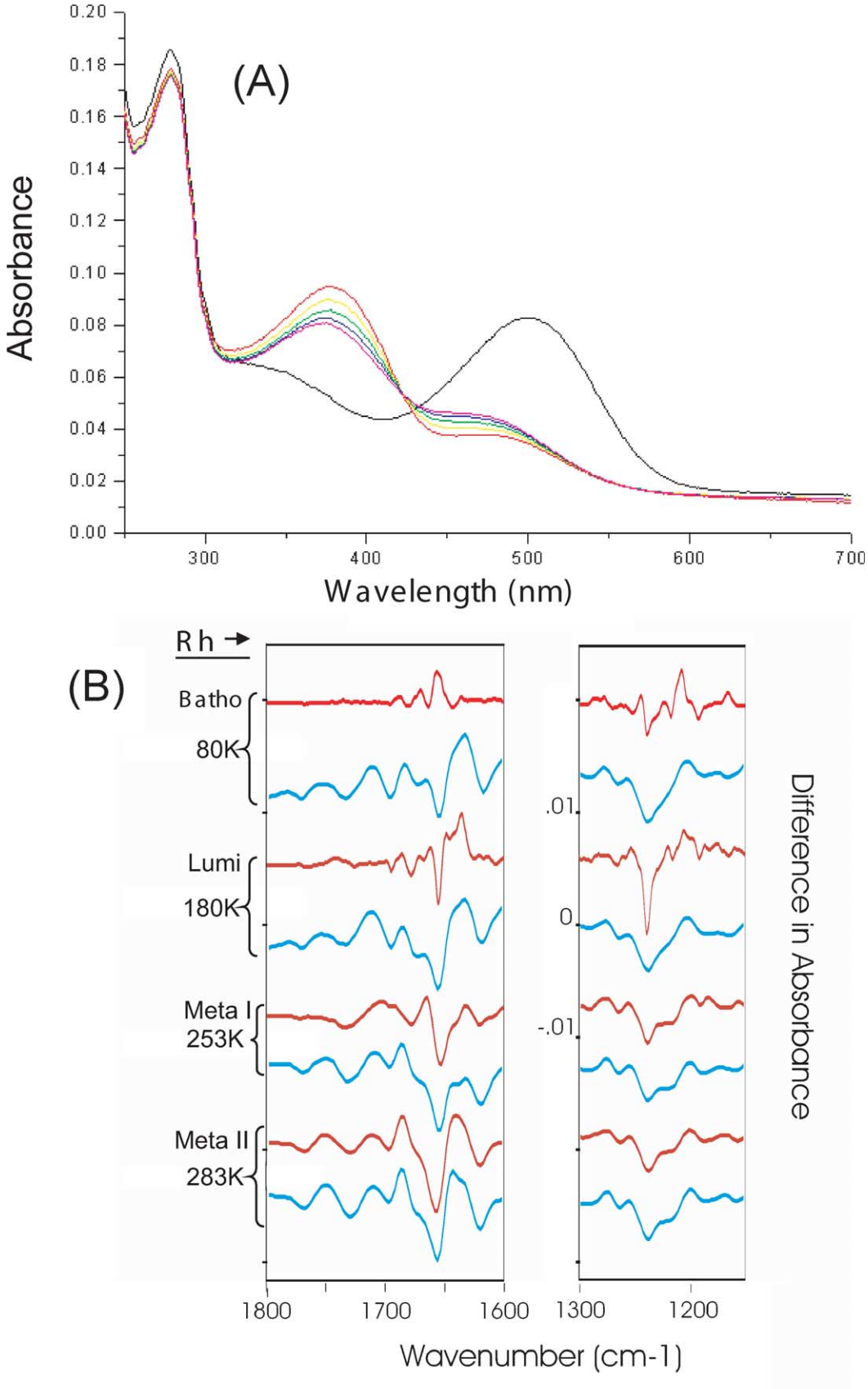

Figure 2. (A) UV/visible spectra recorded at 283 K from the proteoliposomes with protein in the ground state (black trace, λ_{max} = 498 nm) and after illumination (red trace) showing mainly Meta-II (λ_{max} = 380 nm) and then decay of Meta-II after ten minutes (yellow trace), 20 minutes (green trace), 30 minutes (blue trace) and 40 minutes (purple trace). (B) FTIR difference spectra in red recorded from the proteoliposomes for the transition from rhodopsin to (Rh→)

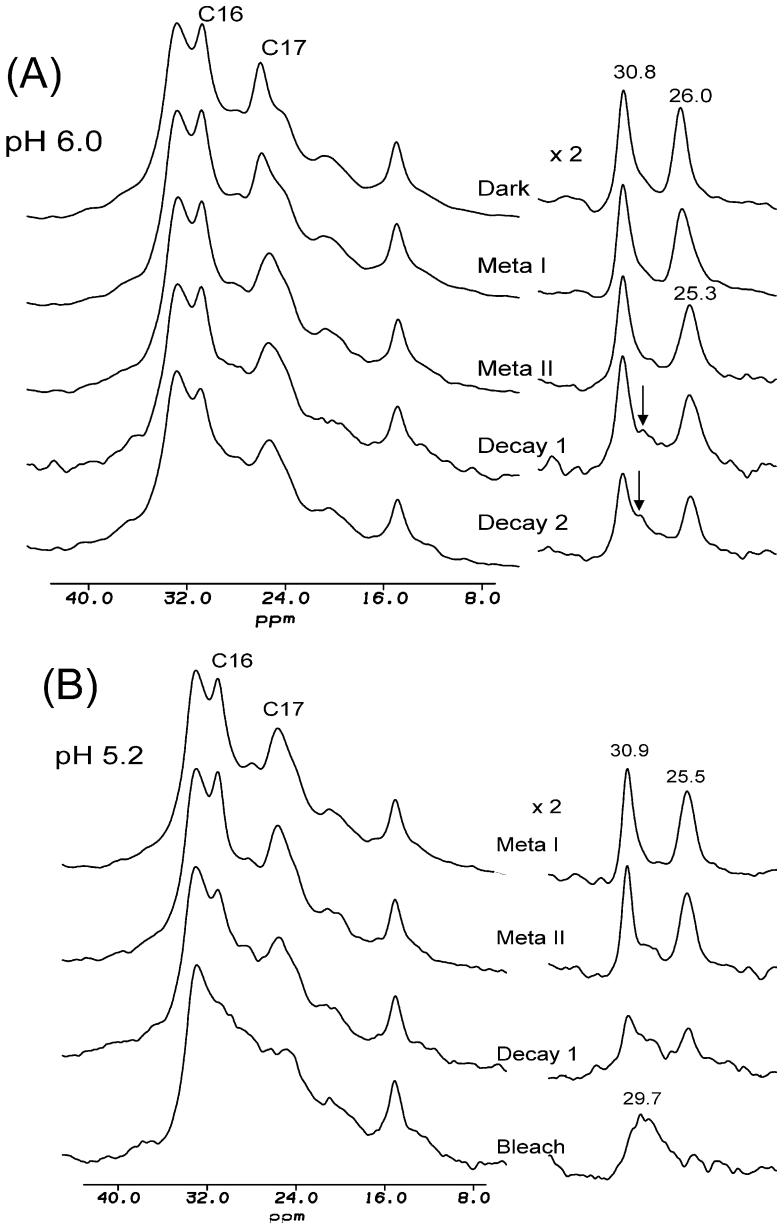

Figure 3. ^{13}C MAS NMR spectra recorded from the proteoliposomes prepared with [16,17-^{13}C-retinylidene]rhodopsin at (A) pH 6.0 or (B) pH 5.2. Alkyl regions of the spectrum (stacked to the left) show superimposed signal from C16 and C17 and after subtraction of background signal (stacked to the right). From the top down are shown the spectra obtained before illumination (Dark) and subsequently obtained from trapping Meta-I, Meta-II and then to follow Meta-II decay (Decay I: 293 K for ten minutes or Decay 2: 293 K for 60 minutes). (B) The result finally from continuous illumination at 293 K (Bleach). Decay of Meta-II results in a reduction in the high-resolution signal and the emergence of an indistinct broad spectral component representing "released" chromophore (maxima arrowed in (A)).

lipid-depleted native membranes[5] is thus reproduced at optimal resolution from a protein sample that appears homogeneous with respect to the conformation of the chromophore in the ground state and is fully solvated in the membrane. On conversion to Meta-II, the C16 and C17 resonances again remain as two distinct components, separated now by an increased chemical shift difference of 5.5 ppm (C17 chemical shift reduced from 26.0 to 25.3 ppm). Rather than showing any signs of structural relaxation in the activated Meta-II state, the NMR observations indicate that the segment of the ring around C17 experiences even stronger interactions within the binding pocket, forcing C17 to lie closer to the proton at C3 on the ring. Release of the chromophore can only begin to be observed by a moderate loss of high-resolution signal to a very broad underlying component after warming to 293 K for ten minutes (Decay 1) and achieved limiting levels (~40–45% of the C16 and C17 intensity) after incubation for 60 minutes (Decay 2). The broad featureless signal produced from the decay of the Meta-II component illustrates that once the β-ionone ring is released from its binding pocket, C16 and C17 lose their distinctive conformation and resonate at around 29–30 ppm (arrowed

bathorhodopsin (Batho), lumirhodopsin (Lumi), and metarhodopsin intermediates. Spectra in blue were recorded after formation of Meta-II and then following cooling to 80 K for recording at this and subsequent transition temperatures. Segments are shown from the C–C stretch fingerprint region (1300–1150 cm^{-1}) and the combined amide I (1700–1600 cm^{-1}) and carboxyl region of C=O stretch modes (1800–1700 cm^{-1}).

in Figure 3(A)), which represents an average equatorial conformation for both. The shift in the C17 resonance on activation appears to persists in the residual components not undergoing decay (Figure 3(A), Decay 2) even though these correspond roughly to the levels of ground state components (rhodopsin and isorhodopsin) generated in the original photoequilibrium mixture.[5] However, this observation may be unreliable because the spectra are complicated by the broad, non-resolved underlying component from "released" chromophore that could not be deconvoluted and subtracted with any reasonable certainty. The spectral lineshapes with Meta-II are not complicated in this way, and so the decreased chemical shift in C17 can be relied upon as reflecting an enhanced steric interaction at this location within the ring binding pocket upon activation.

The series of photointermediates were examined in proteoliposomes equilibrated at pH 5.2, which, apart from further favouring formation of Meta-II, may accelerate its decay. Now the increased splitting (5.5($\pm$0.1) ppm) between C16 and C17 associated with the active state at pH 6.0 was observed under conditions expected to generate Meta-I (Figure 3(B)). No further change is observed under conditions used for trapping Meta-II but this was followed by a rapid decay that was complete within ten minutes at 293 K (Figure 3(B), Decay 1). In comparison with the sample at pH 6.0 (Figure 3(A), Decay 2), the extent of this release would appear to be greater at the lower pH value, which may suggest an increase in bathorhodpsin levels generated in the photoequilibrium mixture at the lower pH. The spectral intensity observed from C16 and C17 following complete photoactivation and decay (Figure 3(B), Bleach) illustrates clearly the dramatic loss in spectral resolution and structural homogeneity that occurs on displacing the ring from the binding pocket. However, this lineshape appears to be less heterogeneous (narrower) than the underlying intensity formed immediately on decay of Meta-II and so could not be used to assist in deconvolution for reliable quantification of the non-decayed components.

The finding that conformational changes identified with the activation process, as represented by the increased steric interaction at C17, can be observed in earlier intermediates at low pH is consistent with the view that the activated state is an intrinsic property of the opsin protein that can be induced independently by protonation.[11,12] It is thus reasonable to assume that these stronger interactions at C17 that accompany receptor activation are not a direct result of increased torsional forces on the ring from isomerization within the chromophore but instead originate from conformational changes in the protein occurring around the ring region of the binding pocket. In fact, the torsional strain from isomerization within the chromophore is expected to be released by structural adjustments within the protein-binding pocket prior to activation. This is consistent with the increase in structural heterogeneity observed in the chain adjoining the ring in Meta-I,[5] which can be interpreted as a more relaxed conformation in this segment. A recent interpretation of time-resolved UV resonance Raman measurements[13] indicated that key adjustments in the contacts between aromatic residues in the binding pocket and the ring of the chromophore are initiated from the onset of photoactivation. Such adjustments in certain contacts around the chromophore, along with FTIR evidence for additional structural changes occurring within the binding pocket,[9] are important in resolving the precise molecular details leading to activation. However, adjustments in the binding pocket leading to receptor activation can be regarded as nominal around the ring segment observed here and should still allow the ground-state crystallographic structure of the protein in this region to be used as the starting point for describing a conformational switch to the signalling state in which the ring can play an important role.

Table 1. Chemical shifts (ppm) of the C16 and C17 methyl groups on the ring of the rhodopsin chromophore and the steric shift (δ_C) taken as their separation, in native photoreceptor membranes (lipid-reduced) and in proteoliposomes

	C16	C17	δ_C
A. *In native membranes*			
[8,16/17-$^{13}C_2$-retinylidine]rhodopsin	30.7[a]	26.4[a]	−4.3
[8,16/17-$^{13}C_2$-retinylidine]Meta-I	30.7[a]	26.4[a]	−4.3
[16,17-$^{13}C_2$-retinylidine]rhodopsin	30.8[b]	26.0[b]	−4.8
[16,17-$^{13}C_2$-retinylidine]Meta-I	30.8[b]	26.0[b]	−4.8
B. *In proteoliposomes*			
[16,17-$^{13}C_2$-retinylidine]rhodopsin	30.8[b]	26.0[b]	−4.8
[16,17-$^{13}C_2$-retinylidine]Meta-I	30.8[b]	26.0[b]	−4.8
[16,17-$^{13}C_2$-retinylidine]Meta-I (pH 5.2)	30.8[b,c]	25.4[b,c]	−5.4
[16,17-$^{13}C_2$-retinylidine]Meta-II	30.8[b]	25.3[b]	−5.5

Chemical shifts are referenced against adamantane methylene carbon (38.7 ppm).
[a] Measured previously[4,5] after regenerating rhodopsin with equimolar chromophore:protein.
[b] Measured after regenerating with a 2 : 1 molar excess of chromophore to protein, which avoids the incorporation of low levels of alternative conformers and provides a better-resolved C17 spectral component showing a reduced chemical shift as reproduced here for ground-state rhodopsin and Meta I in proteoliposomes.
[c] At the lower pH (5.2) the binding site may already have entered a conformation reflecting the active state.

Discussion

Geometric restraints on the ring conformation

The lack of structural perturbation around the C16/C17 methyl groups on the ring up to activation is demonstrated in our data with great sensitivity. The chemical shifts measured here for these ring methyl groups are shown together in Table 1 with previous measurements from protein in lipid-reduced native membranes. All data support the overall conclusion that the conformationally sensitive chemical shifts of C16 and C17 are not perturbed on forming Meta-I in native membranes, and that this ring conformation is preserved for rhodopsin and Meta-I in proteoliposomes. The sensitivity of the steric shift to conformational relaxation imposes very strict constraints on the ring segment remaining immobilized within the binding pocket. According to the theoretical predictions (Materials and Methods), an experimentally significant reduction in the steric shift of 0.2 ppm would result from the C17 methyl group moving just 0.02 Å (2 pm) back toward the plane of the ring or a reduction of just 0.5° in its tilt angle (C4–C5–C6–C17 torsion). It is clear, therefore, that the current observations do not allow for any significant adjustment in the contacts around this segment of the ring up to Meta-I and neither is it realistic to expect that these contacts could be reproduced faithfully at any alternative location within the protein. The −4.8 ppm steric shift for C17 in ground-state rhodopsin and Meta-I reported here, agrees with that predicted for the axial methyl group in an idealized structure of methyl cyclohexane[14] but a smaller shift (∼−3 ppm) is calculated from the geometry of our previous structural model,[4] which adopts the ring proton orientations found in the crystallographic structure of retinal.[15] According to these calculations (Materials and Methods), the −4.8 ppm steric shift observed up to Meta-I can be reproduced by the interacting methyl group proton being rotated 8° toward the proton on C3 from its position in the retinal crystal. Similarly, the −5.5 ppm steric shift in the active conformation can be explained by the methyl group being forced to rotated, on average, a further 4° toward the interacting proton. Again, it is highly unlikely that an alternative location within the protein would be able to put such constraints on the ring conformation. It should be stressed that the conformation in this ring segment is not correlated with any conformational transitions within the polyene chain or the torsion angle between the two. This is reflected in the independence of the C16/C17 shifts on the 11-*cis* (rhodopsin) or all-*trans* (Meta-I) chain configuration, and contrasts with the greater structural heterogeneity observed previously in the chain around C8 in Meta-I.[5]

The overall conclusion is that conservation of the C16 and C17 chemical shifts reflect a highly specific immobilization of this segment in the ring-binding pocket of the chromophore-binding site in rhodopsin that is carried through to the active Meta-II state. Only upon decay of Meta-II does the C16/C17 spectral splitting collapse, which coincides with the reported hydrolysis and release of the entire chromophore from its binding site.[9,16] An increase in this spectral splitting (4.8 ppm to 5.5 ppm) under certain condition is interpreted as a temperature and pH-dependent conformational change in the ring-binding pocket, reflecting binding site preparation for, or adaptation to, the fully active state of the receptor. The origins of this change are explored in the following discussion, which anticipates changes in the ground-state structure of the protein in the context of the current results and a wider consideration of biophysical evidence for activation changes in rhodopsin and related GPCRs.

A conformational switch within the aromatic cluster in helix 6

The results shown here support a role for the ring of the chromophore in the final stages of the activation process, exerted from its original location within the binding pocket of rhodopsin. More specifically, the stronger protein interactions at C17 that accompany the activated state suggest some role for this segment of the ring in directing the conformational changes that lead to the signalling state of the receptor. The binding pocket for the C17 face of the ring in ground-state rhodopsin is made up mainly of aromatic residues around Pro267 in helix 6, which is one of the most conserved residues amongst GPCRs.[2] The surrounding "aromatic cluster" is highly conserved amongst neurotransmitter GPCRs, showing identity for Phe261(6.44), Cys264(6.47), Trp265(6.48), and Pro267(6.50). The nomenclature in parentheses describes the helix number (6.) followed by the sequence position where 50 is assigned to the most highly conserved helix residue.[17] The remainder of this sequence differs on the C-terminal side of the proline with Phe(6.51) being replaced in rhodopsin by Tyr268 and Phe(6.52) replaced by Ala269, which lies in close contact to C17 in the ground-state structure of the protein.[1] Within the aromatic cluster, an important contribution to maintaining the inactive state of rhodopsin comes from Trp265, which covers the cytoplasmic edge of the ring at C18 (Figure 4(A)). This interaction is facilitated by backbone flexibility around Pro267, since, from being highly tilted on the extracellular side, helix 6 kinks sharply at Trp265 to proceed almost perpendicular to the membrane, ending close to helix 3 at the cytoplasmic surface. From below the proline-kink (Figure 4(A)), Phe261 points upward to complete the cytoplasmic side of the binding pocket around C3–C4 of the ring.

Structural changes predicted to occur on activation of rhodopsin[18–20] and related GPCRs[21] have mainly highlighted changes in the relative orientation of helix 6 and helix 3, the largest changes

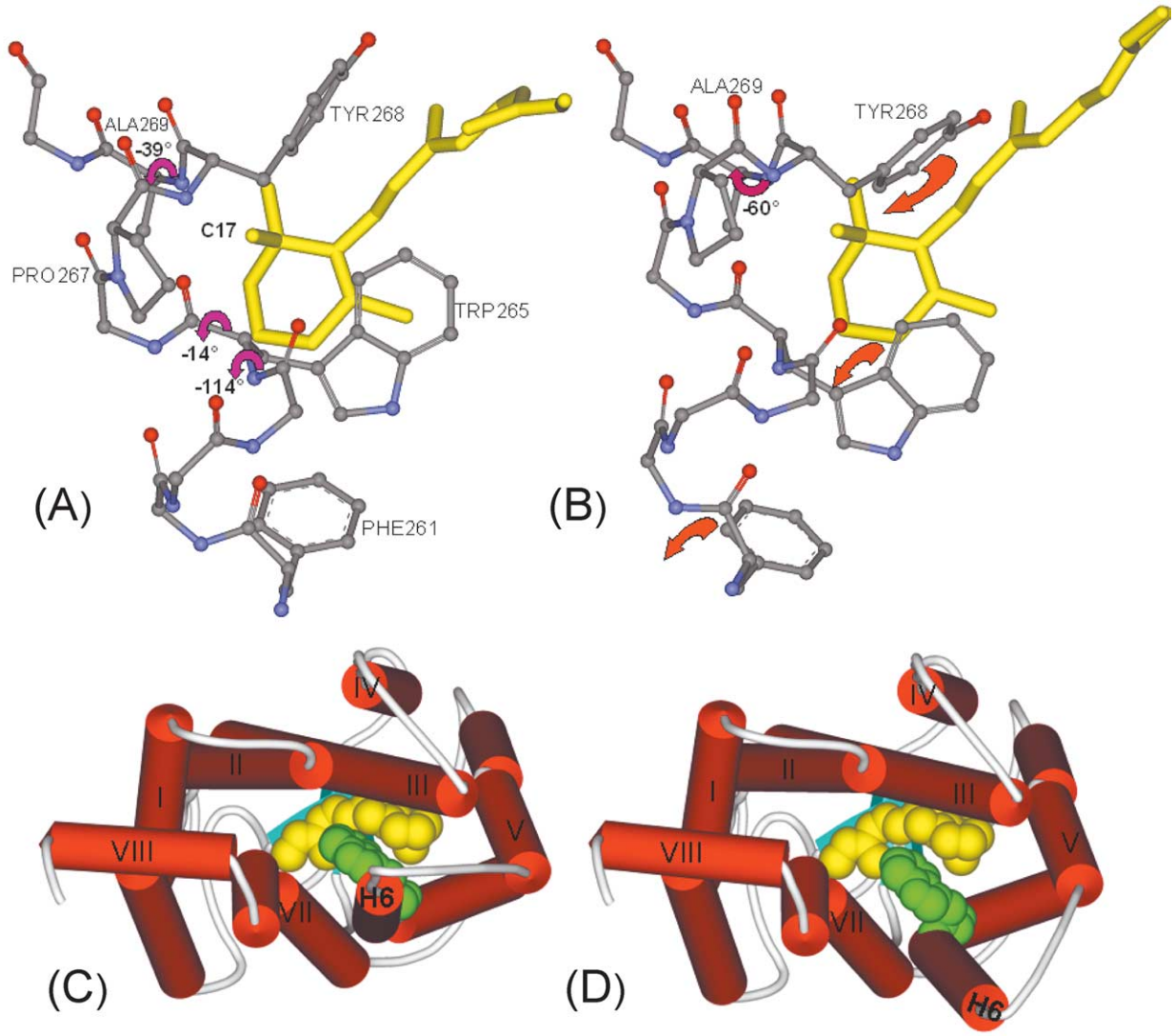

Figure 4. (A) The chromophore (yellow) ring within the aromatic cluster of helix 6. The helix is kinked from irregular ϕ ($-114°$) and ψ ($-14°$) angles (purple arrows) in the Trp265 backbone, is tilted at $\sim 36°$ on the extracellular side of the kink (above) and is roughly perpendicular to the membrane as it proceeds to the cytoplasmic surface (below). (B) Rotation of the reduced ϕ angle for Ala269 to a regular value for an α helix ($-60°$), with the entire cytoplasmic section of helix 6. The aromatic side-chains swing down and out from the cytoplasmic boundary of the binding pocket (red arrows), whilst Tyr268 on the extracellular boundary rotates down to make close contact with C17. (C) Rhodopsin viewed from the cytoplasmic surface showing helix 6 (H6) covering the cytoplasmic side of the binding pocket with van de Waals surface contacts between the Trp265 (green) and the chromophore (yellow, with the ring on the right of the binding pocket) in the inactive state of the protein and (D) showing exposure of the chromophore and tryptophan following displacement of the cytoplasmic portion of helix 6 (H6) by the torsional adjustment in.

being a whole-body tilting and rotation predicted for helix 6.[22] This transition can be regarded as pivotal for exposure of cytoplasmic sites responsible for binding and subsequent activation of G-protein. It is expected that these changes in helix 6 are detected here, as they operate around the β-ionone ring, which remains immobilized within its binding pocket. On the basis of the available evidence and the observations reported here, we postulate that helix 6 is released from critical structural restraints upon protonation of opsin, induced either at low pH or by photoisomerization of the chromophore into the Meta-II state, at physiological pH. Such critical stabilizing restraints have been proposed between the cytoplasmic end of helix 6 and the (D/E)RY motif that is highly conserved in helix 3 of type A GPCRs.[1,23] It has been suggested that, once these constraints are released, helix 6 could move by flexing its cytoplasmic segment about the proline-kink and thus separating from helix 3.[23] This straightening around the proline-kink is equivalent to readjusting the backbone torsion angles to those more appropriate for a regular right-handed helix, the main distortion for the kink being localized to the backbone at Trp265 ($\phi = -114°$, $\varphi = -14°$). Initially, torsional adjustments in this region are highly restricted due to steric clash between the indole ring of Trp265 and

the β-ionone ring, which, according to our data, can no longer be assumed to change its location on progression to the activated state. A specific "rotomer toggle-switch" proposed for activation around the proline-kink depended upon the ring moving to free restraints within the aromatic cluster.[24] Additionally, adjustments in the backbone at Trp265 alone could not account for the stronger interactions occurring at C17. Such interactions can instead be expected to involve the portion of the aromatic cluster (Tyr268) above the proline-kink in Figure 4(A). Here, the sequence Pro-Tyr-Ala-Gly corresponds to PxxG identified as a natural proline-hinge-forming motif found in the alimethacin polypeptide.[25] Restoring the ϕ value for Ala269 ($-39°$), in the middle of this flexible segment to a more typical value for an α helix ($-60°$), can rotate Trp265 and Phe261 below and away from the β-ionone ring avoiding steric clash (Figure 4(B)). Above Pro267 in Figure 4(B), this adjustment rotates the ring of Tyr268 down to make close contact with C17 along with the C^{β} of Ala269, whose position is unchanged if the rotation is restricted to the cytoplasmic segment of the helix backbone. This conformational change, which can account for the increased steric interaction detected at C17, also accomplishes a counter-clockwise rotation in the cytoplasmic segment of helix 6, when viewed from the extracellular face, as is predicted consistently to occur within the activation step for GPCRs.[21] The feasibility of whether a simple torsional rotation can accomplish a whole-body movement in the cytoplasmic segment of helix 6 will depend upon other restraints on this segment during activation. These restraints on helix 6 were predicted to be limited to nominal non-specific hydrophobic and steric restraints with adjacent helices,[26] although its involvement in bound water-mediated hydrogen-bonded networks is now recognized (see below).[27] Steric restrictions would be expected to be minimal, since the rotation carries the cytoplasmic segment of helix 6 directly away from the helical bundle, as shown between Figure 4(C) and (D), which view the cytoplasmic side of the protein. This change appears similar in direction and magnitude to the whole-body movement, combined with the same sense of rotation as predicted for the cytoplasmic end of helix 6 from electron paramagnetic resonance spectroscopy (EPR) measurements of spin-label interactions.[22] The concurrent release of Trp265 from its close contacts in the binding pocket (Figure 4(B)) is consistent with a body of spectroscopic evidence that this residue alters its orientation and becomes exposed to a more polar environment on activation of rhodopsin.[28–30] Nevertheless, the predicted displacement of Trp265 from the "fixed" β-ionone ring is in line with the modest change in fluorescent resonance energy transfer recently reported[31] on rhodopsin activation, as opposed to the much larger effects expected from also displacing the β-ionone ring entirely from the binding pocket.

Specific interactions with helix 6 are apparent only in a recent crystallographic structure of bovine rhodopsin.[27] This revealed that many of the most highly conserved residues in related GPCRs are found to be associated with water molecules within the membrane domain, and that these appear to mediate a network of hydrogen-bonded interactions capable of regulating receptor activity. The ring of Tyr268 in the proposed activation switch lies at the end of one extensive network that connects with the protonated Schiff base attachment on helix 7 and its counter-ion (helix 3). A possible mode for release of Tyr268 from this location is therefore provided from neutralization of the salt-bridge that marks the transition to the active Meta-II state, with consequent disruption of this hydrogen-bonded network. A loss of hydrogen bonding for the side-chain of Tyr268 followed by its displacement toward the β-ionone ring would be consistent with FTIR evidence that a tyrosine residue becomes partly reprotonated or changes its environment on activation of rhodopsin.[32] Also, the backbone of Trp265 forms part of a binding site for a further water molecule[27] located between the proline hinges on helix 7 (Pro291) and helix 6 (also involving Cys264), and this could provide an additional and perhaps even more sensitive means of cooperatively communicating the activation signal to the proposed conformational switch around the β-ionone ring in the binding pocket.

Recent studies have suggested that during the photocascade to Meta-I, the hydrogen bonded network along the length of the binding pocket coordinates a counter-ion switch from Glu113, which forms the primary salt-bridge at the Schiff base end of the chromophore, to Glu181, which lies over the mid-section of the chromophore chain.[33] Although the authors proposed that the Schiff base relocates to interact with the secondary counterion, an overview of this proposal points out that Tyr268 would also be close enough to partly stabilize the Glu181 anion.[34] This interaction with a secondary counter-ion would consolidate Tyr268 in the hydrogen-bonded network and would not interfere with the proposed activation switch around the chromophore ring, providing the new counterion is neutralized as Meta-I proceeds to the active state.

The NMR approach used in the current study has provided a sensitive means of demonstrating that the native ring structure of the chromophore is not ejected from the protein-binding pocket at the early photointermediate stage but is retained with strong binding contacts into the active signalling state of the receptor. This revised view can now be incorporated into structural models of the activation process, as illustrated in the above proposals concerning the major conformational change in helix 6. Although the description of conformational changes involving helix 6 is likely to be over-simplified, it serves to define a feasible role for the β-ionone ring as a pivot point for these changes, operating from within the aromatic cluster of the binding pocket. We suggest that the flexibility

around Pro267 can operate a switch that toggles between aromatic interaction around the C18 methyl group for stabilizing the inactive state and the alternative aromatic interactions around the C17 segment of the ring to help stabilize the signalling, active state of the receptor. The switch between these alternative hydrophobic interactions can be facilitated by rearrangements in the hydrogen bonded networks triggered by proton exchange, which is in turn initiated by the presence of the activating ligand. This complies with most of the available biochemical and biophysical data on rhodopsin, and should have more general relevance to the range of neurotransmitter receptors whose aromatic ligands are predicted[35] to interact within the conserved aromatic cluster in helix 6 (e.g. adrenergic and hydroxytryptamine receptors, dopamine D2 receptor).

Further recent NMR observations

Since submitting this work for publication, important NMR observations have been published that detect light-induced changes between the interactions of ^{13}C in the chromophore chain, with ^{13}C introduced into residues that line the binding pocket.[10] These measurements report the trajectory of labels located in the portion of the chain from the C19 methyl to the Schiff base (C12, C20, C14, C15) and predict a 4–5 Å movement of this segment on receptor activation, toward helix 5 at the ring end of the binding pocket. This was interpreted as indicating a complete translation of the whole chromophore, including the ring toward helix 5. However, unlike the current study, this work included no measurement from sites toward or within the ring itself. Furthermore, even their smallest projected change in ring position would need to overcome close contacts with residues in helix 5, already present in the ground-state structure of the binding pocket. As discussed previously,[5] these contacts are important for retaining the ring within the binding pocket and assist in maintaining the conformation around the C16/C17 ring segment, which we confirm here is unperturbed up to activation. In the same article, the authors refer to unpublished evidence that a hydrogen bonded interaction between His211 on helix 5 and Glu122 on helix 3 is disrupted on activation and suggest that this results from direct interaction with the ring in this region. Although this may require only a modest movement of the ring, our current observations, that the labelled ring segment becomes even more strongly restrained on activation, are not consistent with a change in these contacts with helix 5 that disrupt the local tertiary constraints on this helix within the transmembrane helical bundle. The interpretation adopted by the authors of the new NMR work does not consider the possibility that some of the observed phenomena result from adjustments in the protein around the binding pocket, such as perturbations of a His211-Glu122 interaction from movements and rotations in helix 3. In the light of the current observations and the absence of direct evidence for movement of the native ring structure within the binding pocket, we retain the view that its strong contacts, including those with helix 5, need to be retained in order to support the critical conformational adjustments required around the proline in helix 6, as postulated above.

Materials and Methods

Sample preparation

The complete synthesis and purification of 11-*Z*-[16,17-$^{13}C_2$]retinal used to regenerate rhodopsin with labelled chromophore was as described.[36] Rod outer segment membranes were isolated from fresh bovine retina, bleached with a white light source (250 W) to remove the native chromophore.[8] The opsin in these membranes was then regenerated with a 2 : 1 molar excess of (16,17-$^{13}C_2$)-labelled or non-labelled 11-*Z*-retinal[6] and then purified and reconstituted[8] with total lipids from rod outer segment membranes at a lipid to protein molar ratio of around 60 : 1. The resulting proteoliposomes were equilibrated with 20 mM Mes (pH 6.0 or 5.2), 0.13 M NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.1 mM EDTA, 1 mM DTE, and collected by ultracentrifugation (100,000*g*) for the various spectroscopic analyses.

UV/visible and FTIR spectroscopy

UV/visible spectra were recorded from proteoliposomes suspended in 20 mM Mes (pH 6.0) to a concentration of 1 μM rhodopsin. Measurements were made at a sample temperature of 283 K with a Perkin–Elmer Lamda 15 double-beam spectrometer equipped with an end-on photomultiplier detector to minimize the effects of light-scattering. Sample suspensions were photoactivated with yellow light (40 seconds) from a 20 W halogen lamp fitted with infrared and 530 nm cut-off filters (Schott, Mainz, Germany). FTIR analysis was conducted on thin films of proteoliposomes prepared by iso-potential spin-drying[37] of a pH 6.0 suspension (1 ml) containing 2–3 nmol of rhodopsin onto a AgCl window (Fisher Scientific Co.). The proteoliposome films were hydrated with 2 μl of the Mes buffer and sealed by means of a rubber O-ring spacer and a second AgCl window. Measurements were made with a Bruker IFS-66v/S spectrometer equipped with a liquid nitrogen-cooled, narrow-band, HgCdTe detector and operating at a resolution of 6 cm^{-1}. The sample temperature was controlled using a liquidhelium-cooled cryostat (APD Cryogenics Inc., Allentown, PA, USA). Samples were photoactivated into either bathorhodopsin by illumination (40 s) at 80 K with light at 497 nm (band-pass filter) or into the Meta-II state by illumination (two minutes) at 283 K with yellow light from a 250 W source fitted with infrared and 530 nm cut-off filters. Difference spectra were obtained by subtracting the spectrum recorded (256 scans in ~63 s) before illumination from that recorded subsequent to illumination and temperature equilibration.

NMR measurements

Proteoliposomes containing around 7.5 mg (~190 nmol) of protein were analysed at 193 K by cross-polarization magic-angle spinning (CP MAS) NMR as

described[5] using a rotation speed of 10 kHz. Spectra were recorded typically from 16,000 acquisitions and processed with 60 Hz of exponential line-broadening to improve signal-to-noise in the transformed spectra. The NMR samples were photoactivated by illumination at 83–85 K as described,[5] and then warmed to allow quantitative and sequential conversion of the primary intermediate to the later photostates: 245 K for ten minutes to Meta-I, 283 K for two minutes to Meta-II and at 293 K to accelerate decay of the activated state. These photostates were trapped by rapid freezing in liquid nitrogen for the NMR analysis. Valence bond theory has been used to predict the force between interacting protons as a function of distance and combined with an empirically derived coefficient for the steric shift as a function of the polarizing force component along the H–C bond of the observed carbon atom to arrive at the following expression for the steric shift:[14]

$$\delta_c(\text{ppm}) = -1680 \cos\theta \exp(-2671r)$$

where r is the distance between the interacting protons (Å) and θ is the subtending angle between the interacting proton–proton axis and the H–C bond of the observed carbon atom. The unperturbed chemical shift is taken as that for C16, which assumes an equatorial orientation in the binding pocket. Therefore, the chemical shift difference between the two methyl groups is interpreted simply as the value of the steric shift in C17.

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