A Methyl Group at C7 of 11-*cis*-Retinal Affects Rhodopsin Activation (VI) IV.3

IV.3.1. 11-*CIS*-RETINAL CHROMOPHORE AND ARTIFICIAL VISUAL PIGMENTS

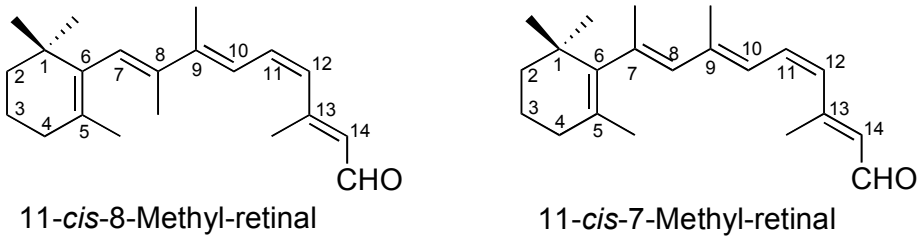
The 11-*cis*-retinal chromophore is exquisitely designed to occupy the binding site of rhodopsin acting as an inverse agonist, and thus to abrogate its activity. This is most likely due to its distorted conformation around the C6-C7 and C12-C13 bonds because of the steric interactions between C5-CH_3/H8 and C13-CH_3/H10. Being adapted to the chiral binding pocket, these distorsions in polyenes define an absolute sense of twist that affects the absorption maxima of the visual pigments and the movements of the chromophore after the initial photoisomerization.

There is an ongoing debate regarding the movements of the chromophore following the 11-*cis* to *trans* photoisomerization in Rho, in particular whether a ring flip of the C6-C7-s-*cis* to the C6-C7-s-*trans* conformation occurs and its effect in the activation step of the visual process [Albeck et al., 1989; Jang et al., 2001]. Photocross-linking experiments by Nakanishi using (11*Z*)-3-diazo-4-oxo[15-^{3}H]retinal suggested a flip-over of the ionone ring in the Batho to Lumi transition [Fishkin et al., 2004], which appears to be consistent with computations [Ishiguro, 2000]. However, solid-state ^{13}C-NMR observations are compatible with intermediates retaining the cromophore in its original location in Rho up to the active MetaII state [Spooner et al., 2002; Spooner et al., 2003; Spooner et al., 2004].

The C6-C7 bond of retinal in native Rho was recently established to be in the s-conformation through binding studies of two enantiomeric 6-s-*cis*-locked analogues (*R*)-**3** and (*S*)-**3** [Fujimoto et al., 2001; Fujimoto et al., 2002]. On the other hand, the 2.8 Å resolution X-ray structure [Teller et al., 2001] indicates that the C12-C13-s-bond helicity is most likely positive [Chan et al., 1974; Rando, 1996], in keeping with recent theoretical analysis [Buss et al., 1998; Buss, 2001], thus correcting earlier estimations of negative

helicity [Kakitani et al., 1977; Han and Smith, 1995; Han et al., 1997; Tan et al., 1997; Lou et al., 1999).

Artificial visual pigments, generated by treatment of the apoprotein opsin with synthetic analogues of retinal [Nakanishi and Crouch, 1995], in particular when combined with mutations of opsin, have been invaluable in the study of the role that conformational changes of the chromophore play in the visual cycle. We have recently reported the consequences of sterically perturbing the C6-C7-conformational equilibrium by incorporating methyl groups at the C8 position [Alvarez et al., 2003] (Figure IV.3.1), with the finding that the perturbation extends to the C9-C10 bond, thus impairing reconstitution. It was considered that the methyl group at the C7 position could hinder the C6-C7 free rotation due to steric interactions with C1-2CH_3, thus becoming the non-locked analogue of bicyclic systems, and allowing the examination of a region of the chromophore which has been shown previously to be important for the stability of early rhodopsin photointermediates [Randall et al., 1991; Lewis et al., 1995].



11-*cis*-8-Methyl-retinal 11-*cis*-7-Methyl-retinal

Fig IV.3.1. 11-*cis*-8-methylretinal and 11-*cis*-7-methylretinal chemical structures. The 8-methyl derivative was previously studied (Alvarez et al., 2003). The 7-methyl analogue of 11-*cis*-retinal used in the present study has been newly synthesized.

Dr de Lera's group has synthesized 11-*cis*-7-methylretinal (Figure IV.3.1). We studied 11-*cis*-7-methylretinal regeneration in binding experiments with native and mutated opsins. We find that opsin can be regenerated with the 7-methyl analogue (7-methyl-Rho) to a similar extent as with native 11-*cis*-retinal (Rho) and forms a chromophore that is blue-shifted to 490nm. The spectroscopic properties of the dark state of 7-methyl-Rho are similar

to those of Rho except for a slightly lower thermal stability of the protein. In constrast, the photobleaching behaviour is clearly altered indicating an effect of the methyl group at C7 in the photoactivation pathway of rhodopsin. Molecular modelling studies suggest that this methyl group is in the vicinity of Met-207 in transmembrane helix V of rhodopsin. Accordingly, this part of the retinal molecule, particularly the β-ionone ring, would come in close contact with Met-207 after illumination in agreement with recent theoretical [Saam et al., 2002] and experimental NMR studies [Crocker et al., 2006].

Our results, taken together with previous studies on other retinal analogues, reinforce the current model of rhodopsin function, where photoactivation is very effectively controlled by the tight molecular coupling between opsin and 11-*cis*-retinal. 11-*cis*-retinal is a unique molecule with a chemical design that allows optimal interaction with the opsin apoprotein in its binding pocket, and this is essential for the formation of the light-activated conformation of the receptor.

IV.3.2. ROS RHODOPSIN REGENERATION WITH 11-*CIS*-7-METHYLRETINAL

Rhodopsin, purified from bovine retinal rod outer segments (ROS) by immunoaffinity chromatography, was regenerated with 11-*cis*-7-methylretinal (7-methyl-Rho) (Figure IV.3.2). The 11-*cis*-retinal analogue was added to the purified rhodopsin in dodecyl maltoside (DM) detergent solution and the sample was subsequently illuminated. Spectra of the regeneration process were recorded in a time course experiment (Figure IV.3.2, inset). The initial spectrum in the dark shows the typical absorbance bands at 280nm and 500nm corresponding to native rhodopsin (with 11-*cis*-retinal, Rho) and a band at 376nm corresponding to the free 11-*cis*-7-methylretinal added to the sample. After 10s illumination with light of $\lambda > 495$nm, rhodopsin was bleached and the chromophore band at 500nm was converted to a species absorbing at 380nm. The spectrum recorded 2h after illumination - the last one recorded after illumination- shows clear regeneration of the chromophoric band in the visible region in the vicinity of 500nm. A small but definite blue-shift of the visible band to 490nm can be detected.

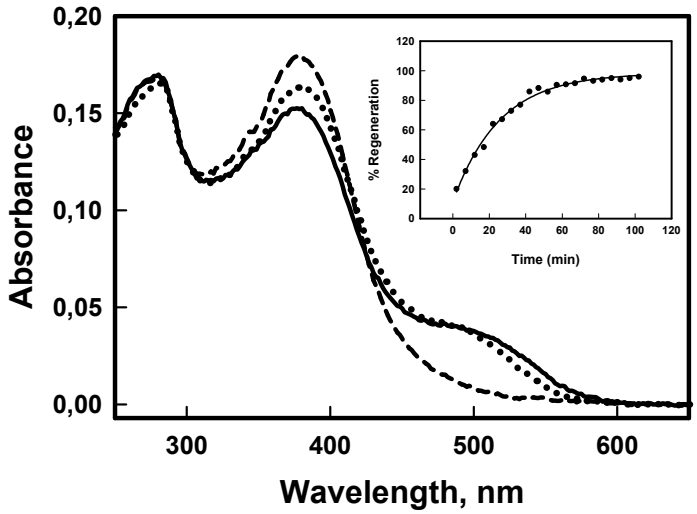


Figure IV.3.2. Chromophore regeneration of purified rhodopsin from native ROS in DM detergent solution (2mM Na_2HPO_4, pH 6, 0.05% DM, buffer C). UV-vis absorption spectrum of dark ground-state rhodopsin regenerated with 11-*cis*-7-methylretinal plus free added 11-*cis*-7-methylretinal (—). Absorption spectrum of the rhodopsin sample after illumination for 10 s with light of λ>495nm (--). Absorption spectrum of the sample 2 h after illumination showing the regenerated visible chromophoric band (•••). Temperature, 20ºC. (Inset) time course of the regeneration process of purified ROS rhodopsin with 11-*cis*-7-methylretinal..

IV.3.3. EXPRESSION AND CHARACTERIZATION OF 7-METHYL-RHO

Wt protein expressed in transiently transfected COS-1 cells and purified by immunoaffinity chromatography was regenerated with 11-*cis*-retinal (Rho) and 11-*cis*-7-methylretinal (7-methyl-Rho). The UV-vis absorption spectra in the dark, after illumination and acidification for these proteins are shown in Figure IV.3.3. The 7-methy-Rho formed normal chromophore and UV-visible absorption spectra in the dark with a λ_{max} at 490nm and a A_{280}/A_{490} 1.8, the same as the A_{280}/A_{500} of Rho indicating that the binding pocket is formed and able to accommodate the retinal analogue. This result is in agreement with what was observed in the case of the ROS rhodopsin regenerated sample (Figure IV.3.2). The regeneration experiments indicate that, after incubation of opsin with 11-*cis*-7-methylretinal (λ_{max} = 368nm in MeOH), regeneration is observed with both native ROS and

with purified recombinant Wt rhodopsin, and the chromophoric yield is similar to that of 11-*cis*-retinal.

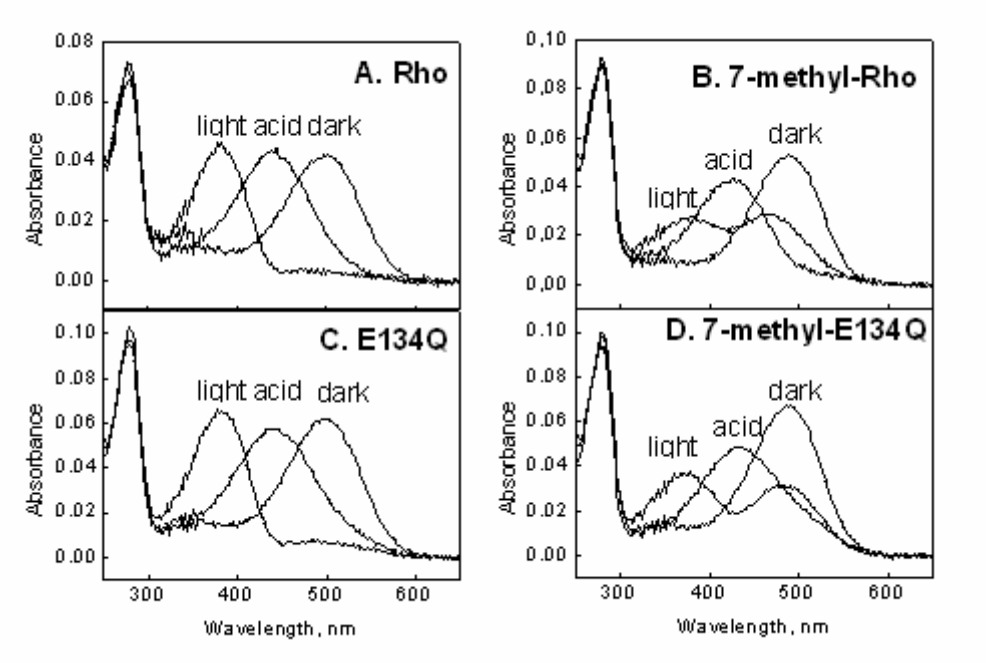


Figure IV.3.3. UV-visible absorption spectra in the dark in buffer C (dark), after illumination for 10s with light of λ>495nm, and acid denaturation (acid). A. Rho; B. 7-methyl-Rho; C. E134Q; and D. 7-methyl-E134Q. The spectra were recorded at a temperature of 20ºC.

After 10s illumination of 7-methyl-Rho, with light of λ>495nm, the visible band at 490nm was not fully converted to MetaII species with maximum at 380nm, but rather two bands of similar intensity are detected at 373nm and 467nm respectively (Figure IV.3.3B), indicating that the methyl group introduced at C7 in retinal causes a stability change in photointermediates. Acidification of the sample after illumination resulted in formation of a band with λ_{max} at 427nm with concomitant decrease in the bands at 373nm and 467nm. This indicates that the two bands, at 373nm and 467nm, correspond to species that are covalently linked to opsin through an unprotonated and protonated Schiff base linkage respectively. An altered photobleaching behavior has been previously observed for rhodopsin mutants in the transmembrane domain and mutations associated with the degenerative disease of the eye retinitis pigmentosa, like G51V and G89D [Hwa et al., 1997; Bosch et al., 2003, chapter IV.1.]. A similar alteration in the native photointermediate pathway formation has been also

recently reported for rhodopsin regenerated with other retinal analogues, like an acyclic retinal [Bartl et al., 2005] or polyene methylated retinals [Vogel et al., 2006].

Rho did not show hydroxylamine reactivity in the dark, indicating that hydroxylamine could not access the Schiff base linkage, and this reflects that the protein has a compact structure in the dark. A similar result was obtained for 7-methyl-Rho suggesting that 7-methyl-Rho has a compact structure around the Schiff base linkage like Rho. Thermal stability at 55ºC of 7-methyl-Rho in the dark was reduced ($t_{1/2}$= 4.6min, Rho $t_{1/2}$=13.1min), indicating that the dark state was less stable when the protein is regenerated with the methylated analogue (Figure IV.3.4). Spectra of the regeneration process were recorded in a time course experiment for Rho and 7-methyl-Rho and the total regeneration and regeneration kinetics of both proteins were similar (data not shown).

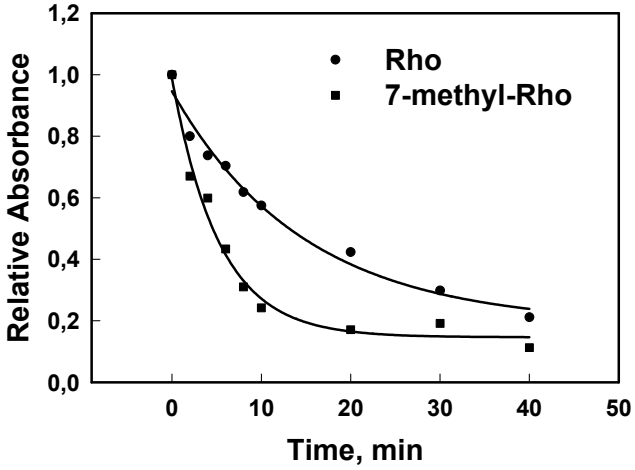


Fig. IV.3. 4. Dark-state stability at 55 °C of Rho (•) and 7-methyl-Rho (■) determined by measuring the decay of the absorbance band at 500 and 490 nm, respectively.

Further illumination of 7-methyl-Rho for 1min or 5min only caused a small decrease in the 467nm band with a concomitant slight increase in the 373nm band (Figure IV.3.5A). The altered photointermediate with λ_{max} at 467nm decayed with time to a species with λ_{max} at 485nm (Figure IV.3.5B). The figure shows representative spectra at different times after

illumination and the difference spectrum between those at 60min and 0min (Figure IV.3.5B, inset). It is interesting to note that two different MetaI photointermediates, MetaIa and MetaIb have been recently proposed to absorb at 485nm and 465nm respectively [Shichida and Morizumi, 2006], very close to the absorbances observed in our experiments (Figure IV.3.5B). In our case, the altered photointermediate pathway is a consequence of a methyl group introduced at the C7 position of 11-*cis*-retinal, causing a stability change in activation photointermediates.

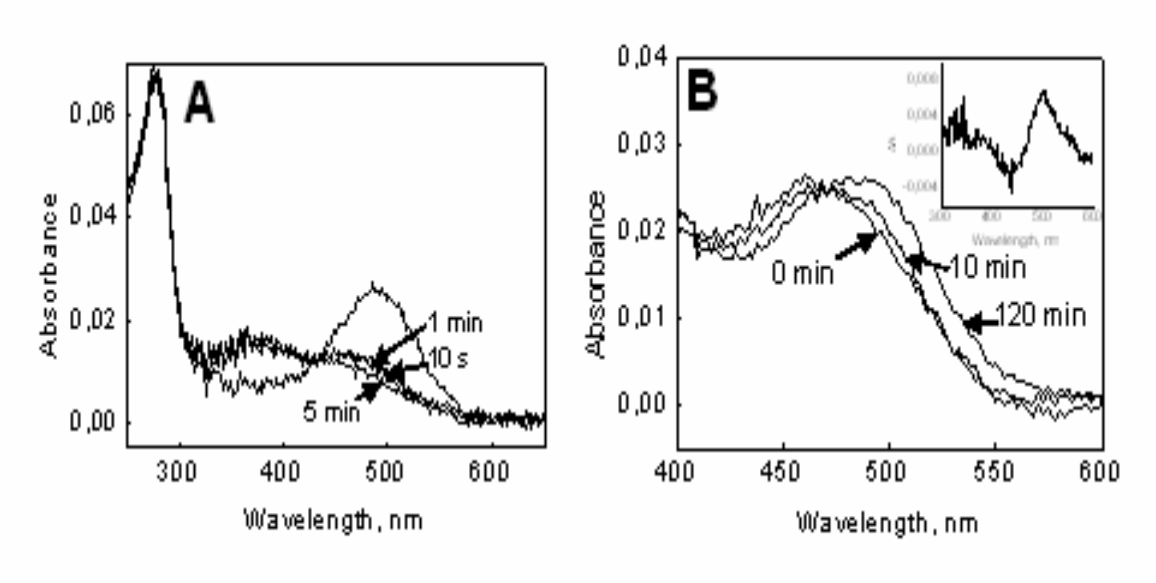


Fig IV.3.5. Photobleaching and decay of the 467nm species at a temperature of 20ºC. (A) The photobleaching of 7-methyl-Rho was further explored by illuminating the sample for different time periods after the first initial 10s illumination as indicated in the figure. (B). The band formed after 10s illumination was allowed to decay in the dark at 20ºC. The band decayed to form a new band at 485nm as shown in the figure where representative spectral time points are depicted. The presence of an isosbestic point indicates a change between two distinct conformational state. The difference between the initial illuminated spectrum (after 10s illumination) and that after 120min shows a maximum at about 500nm and a minimum at about 450nm (inset).

We have also analyzed the E134Q mutant, -Glu-134 belongs to the D(E)RY triplet which is highly conserved through the GPCR superfamily- which mimics the protonated state in photoactivated rhodopsin and which facilitates the MetaII conformation [Vogel et al., 2006; Bartl et al., 2005; Fahmy et al., 2000]. The UV-vis spectrum after illumination of E134Q mutant regenerated with 11-*cis*-7-methylretinal (7-methyl-E134Q) (490nm band in the dark) showed two bands, one at 373nm and the other at 485nm. In this case, the E134Q

mutation, in contrast to the results reported with other retinal structures [Bartl et al. 2005], could not facilitate light-induced MetaII formation (no shift of the protonated species at 485nm to 380nm was detected) (Figure IV.3.3C and 3D). A summary of the data for the dark, light and acidified spectra of wt Rho and the mutant E134Q is depicted in Table IV.3.1.

Recombinat protein	λ_{max} (dark)	λ_{max} (light)	λ_{max} (acid)	A_{280}/A_{500}*
Rho	500 nm	380 nm	440nm	1.8
7-methyl-Rho	490 nm	373/467nm	427nm	1.8
E134Q	500nm	380 nm	440nm	1.6
7-methyl-E134Q	490nm	373/485nm	435nm	1.6

Table IV.3.1. λ_{max} of the absorption bands in the Uv-vis absorption spectra of rhodopsin proteins in the dark, and after illumination and acidification. *In the case of the 7-methyl-containing pigments, and because of the blue-shift observed for the visible band, the A_{280}/A_{490} ratio is used.

In order to elucidate if the different species formed in the light activation process are in equilibrium, 100μM of the high affinity peptide Gtα-HAA (VLEDLKSCGLF) was added before sample illumination [Bartl et al., 2000]. No change in band formation or intensity could be appreciated after illumination of 7-methyl-Rho in the presence of the Gtα-HAA peptide (Figure IV.3.6). Illumination of 7-methyl-E134Q in the presence of Gtα-HAA peptide shifted most of the 485nm band to 373nm. Higher concentration of peptide (1mM Gtα-HAA) was required to shift most of the protonated band of 7-methyl-Rho to 373nm (Figure IV.3.6D). This suggests that the species formed after illumination of pigments regenerated with 11-*cis*-7-methylretinal are in equilibrium and that this equilibrium is shifted with the Gtα-HAA peptide to the 373nm species.

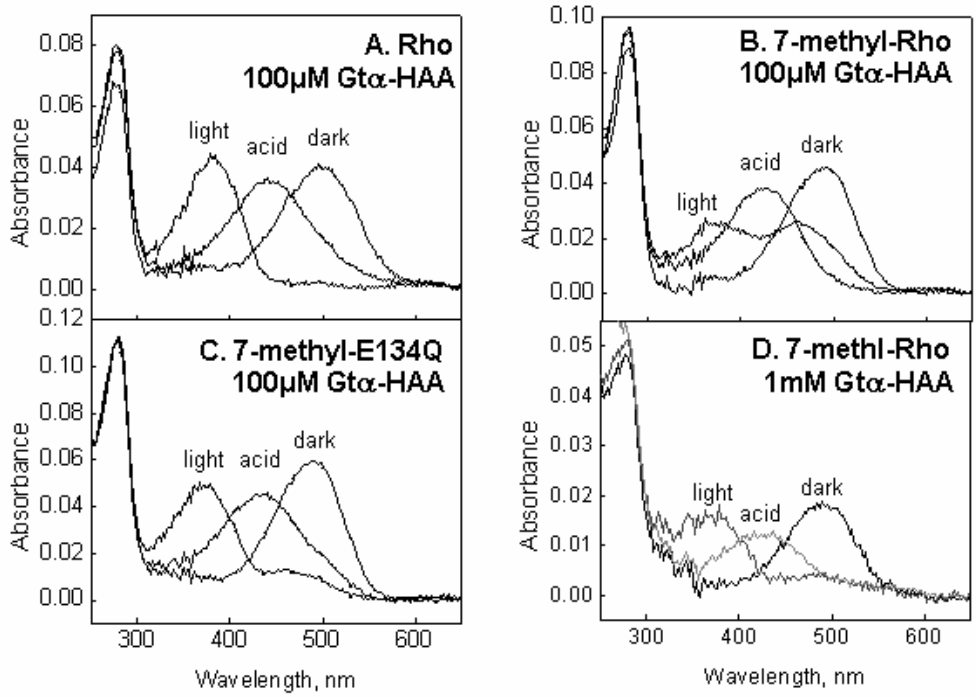


Fig IV.3.6. UV-visible absorption spectra in the dark in buffer C (dark), after illumination for 10s with light of λ>495nm, and acid denaturation (acid) of Rho, 7-methyl-Rho and 7-methyl-E134Q in the presence of different concentrations of the high affinity peptide Gtα-HAA (VLEDLKSCGLF), a peptide of the C-terminal domain of the α-subunit of G_t. A. Rho with 100µM Gtα-HAA; B. 7-methyl-Rho with 100µM Gtα-HAA; C. 7-methyl-E134Q with 100µM Gtα-HAA; and D. 7-methyl-Rho with 1mM Gtα-HAA. The spectra were recorded at a temperature of 20ºC.

IV.3.4. G PROTEIN ACTIVATION

The functionality of 7-methyl-Rho and E134Q-7-methyl-Rho was also determined by measuring the transducin activation by means of a fluorescence assay which monitors intrinsic tryptophan fluorescence changes in the G protein resulting from GTPγS uptake. The initial rates of activation for 7-methyl-Rho and 7-methyl-E134Q are similar to those of the same proteins regenerated with the native 11-*cis*-retinal chromophore (Table IV.3.2). However, the total level of activation for the methylated proteins was reduced to about half of that of Rho. This result suggests that one of the two species formed (373nm or 467nm band with similar intensities) is responsible for the total G_t activation observed while the other species is not active. A first interpretation could be that the 373nm corresponds to the active species and that the 467nm band corresponds to and inactive MetaI-like species.

However, it is tempting to speculate that the 373nm can be an inactive unprotonated Schiff base linked species –typically active MetaII conformation absorbs at 380nm- while the 467nm band could correspond to a protonated Schiff base linked species that may be active. There are previous reports of protonated Schiff base species capable of transducin activation, like G90D congenital night blindness mutant [Rao et al., 1994; Fahmy et al., 1996]. Further experiments are required to clarify this point. It is also possible that the decreased ability to activate G_t of the methylated protein is due to a faster rate of MetaII decay for the sample as has been experimentally observed (data not shown).

Recombinat protein	Relative total G_t activation[1]	Relative initial activation rate[2]
Rho	1.00	1.00
E134Q	0.95	0.95
7-methyl-Rho	0.55	0.91
7-methyl-E134Q	0.50	0.85

Table IV.3.2. G_t activation measured, by a fluorescence assay of wt Rho and the E134Q mutant regenerated with 11-*cis*-retinal and with the 7-methyl analogue. [1]Relative total G_t activation measured as the maximal fluorescence increase; and [2] initial activation rates derived from the first 60s of the fluorescence curves.

IV.3.5. MOLECULAR MODELLING (Molecular Modelling was done at Dr Perez's lab by Arnau Cordomí).

MD was carried out to get further insight into the understanding of the experimental results obtained. To this aim, four models of the retinal binding sites for both Rho and 7-methyl-Rho, with the retinal in the 11-*cis* and the all-*trans* configurations respectively were obtained (Figure IV.3.7). These models were extracted from a set of MD of rhodopsin in the dark-state and after the isomerization of the retinal from the 11-*cis* to the all-*trans* configuration. Despite that these simulations do not lead to the active state of rhodopsin (the time-scale required for such a calculation is still beyond the current possibilities of

atomistic MD simulations), they can be useful in order to give insight into the understanding of the activation process. The isomerization was performed using a procedure similar to that previously described [Lemaitre et al., 2005; Rohrig et al., 2002; Saam et al., 2002].

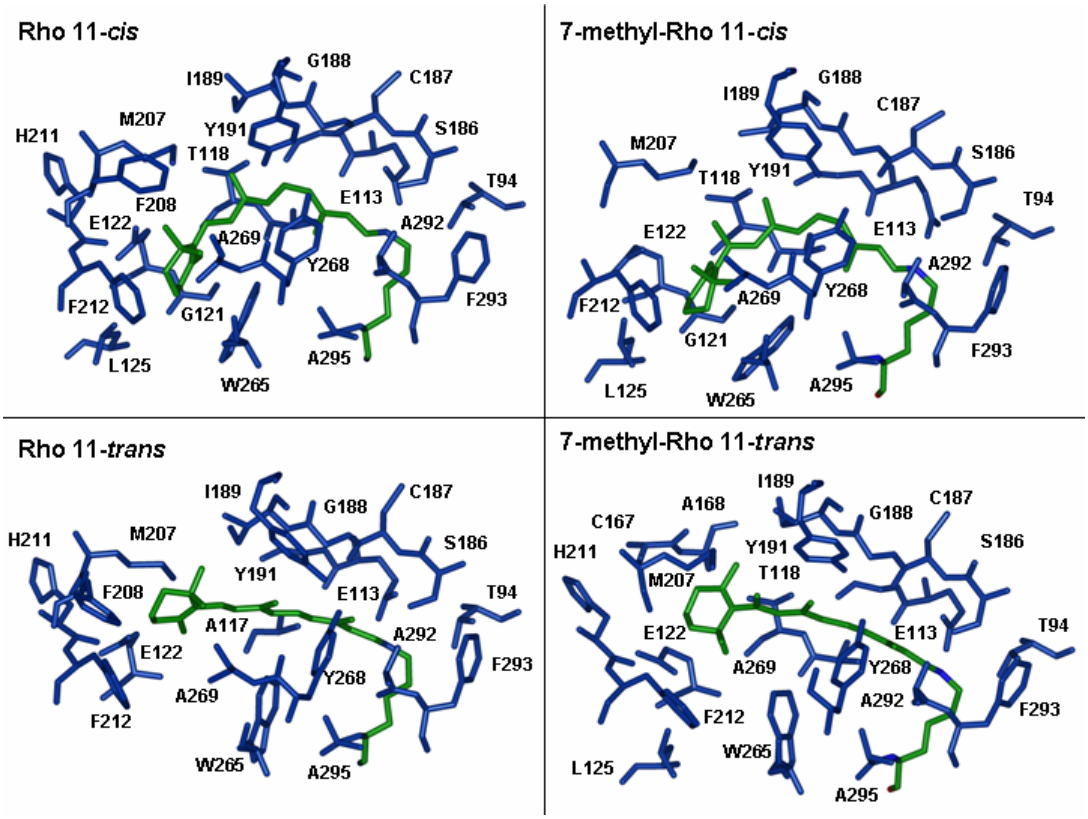


Fig IV.3.7. Model of the rhodopsin binding pocket for either retinal or 7-methylretinal extracted from MD simulations. The top panels correspond to opsin with retinals in the 11-*cis* form and the bottom panel to opsin with retinals isomerized to 11-*trans*.

The analysis of these MD suggests that the methylation at position C7 not only introduces an additional steric volume in the cavity, but also modulates significantly the conformation of the β-ionone ring relative to the polyene chain. Indeed, when comparing the simulations corresponding to Rho and 7-methyl-Rho we observe, in both the dark-state and the isomerized from, that the orientation of the β-ionone ring relative to the polyene chain changes from a more co-planar conformation in the case of Rho to an almost perpendicular conformation in the case of 7-methyl-Rho. This difference is associated with the steric

repulsion between the methyl group adjacent to C7 and the methyl groups in the β-ionone ring.

In the dark-state, both retinal and residues forming the binding site exhibit subtle differences when comparing the MD simulations of Rho and 7-methyl-Rho (Figure IV.3.7, top panels). Accordingly, the β-ionone in Rho is located in a pocket between residues Trp-265, Phe-212, Glu-122, Leu-125 and Met-207. Interestingly, retinal occupies the space available because of the absence of side-chain in Gly-121. These findings are in agreement with the available crystallographic structures of rhodopsin in the dark-state [Li et al., 2004; Okada et al., 2002; Okada et al., 2004; Palczewski et al., 2000]. Moreover, no differences are detected in the conformations observed depending on the retinal form used from the α-carbon of Lys-296 up to the C8 of the covalently bonded retinal. The existence of a methyl group adjacent to position C7 has a direct influence on Met-207, and on Glu-122 to a lesser extent. This is an interesting observation since Met-207 has recently been found to interact with the β-ionone ring in the MetaII intermediate [Patel et al., 2004]. However, the change in the orientation of the β-ionone ring induces additional changes in the residues around the ring which are mostly hydrophobic. Residues whose side-chains are moved or re-oriented more significantly are Leu-125, Phe-212, Trp-265 and Glu-122. In all cases the differences observed are subtle and in agreement with the findings that the retinal binding site can accommodate the retinal analogue similarly to the native 11-*cis*-retinal chromophore.

After isomerization, the "L"-type shape which the 11-*trans* retinal adopts is far different from the "U"-type shape that characterizes the 11-*cis* configuration. This new "L"-type shape can no longer allow the β-ionone ring to be accommodated within the pocket formed by residues Trp-265, Phe-212, Leu-125 and Glu-122. In this way, the ring is forced to occupy other sub-pockets. Interestingly, the simulations performed with both Rho and 7-methyl-Rho in the all-*trans* configuration suggest a different localization of the β-ionone ring due to the conformational peculiarities of the methylated analogue. In Rho, the β-ionone ring interacts, after isomerization, with the hydrophobic residues Phe-208, Phe-212, Tyr-191 and Met-207, whereas in the 7-methyl-Rho the β-ionone ring interacts with Glu-122, Cys-167, His-211, Thr-118, Ile-189 and Met-207. Trp-265 is in all cases moved apart

from the β-ionone ring and becomes closer to the polyene chain. This is in accordance with a recent experimental study which proposed that the interaction of Trp-265 with the retinal chromophore is responsible for stabilizing the inactive conformation in the dark, and that the motion of the β-ionone ring allows Trp-265 and transmembrane helix IV to adopt the active conformations upon illumination [Crocker et al., 2006]. Moreover, it is important to highlight that the main difference between Rho and 7-methyl-Rho is that the β-ionone ring is located in opposite sides around Met-207, suggesting the importance of the methylation at position C7 in the conformations achieved after the isomerization. Regarding the interactions of the methyl group at C7 in the methylated chromophore, in the dark state the methyl group lies close to Glu-122 and Met-207, whereas after isomerization it becomes closer to Thr-118 maintaining the interactions with Met-207 (Figure IV.3.7, bottom panels).

Significant changes regarding the orientations of the C19 and C20-methyl groups can be observed after retinal isomerization, not only because of the different configuration of the double bond itself, but also due to the different localization of the β-ionone ring that leads to a rearrangement of neighboring side-chains interacting with the polyene chain in both Rho and 7-methyl-Rho. Thus, the C20-methyl group interacts with Ala-295 in the dark state in all cases, and with Ala-292 after isomerization. Positions of the C20-methyl group are similar in Rho and 7-methyl-Rho. That is not the case of the C19-methyl group which adopts an slightly different orientation, being closer to Thr-118 in the case of Rho. The C19-methyl group, which is interacting with Ile-189 and Thr-118 in the dark in both Rho and 7-methyl-Rho is moved towards Ty-268 and Tyr-191, occupying the space between them after isomerization. Another aromatic residue, Tyr-268, exhibits a significant displacement due to the existence of both the C19 and C20-methyl groups pointing to the same side. This displacement is larger in the case of Rho because of the different location of the β-ionone ring.

IV.3.6. ROLE OF MET-207 IN THE CONFORMATIONAL CHANGE UPON RHODOPSIN ACTIVATION

The methyl group at C7 is positioned close to Met-207 in transmembrane helix V of rhodopsin (Figure IV.3.7). Interestingly, Met-207 has been proposed to come in close

contact with the β-ionone of the retinal in the activated MetaII state both by molecular dynamics [Saam et al, 2002] and NMR studies [Crocker et al., 2006]. In our case the observed altered photobleaching behaviour could be reflecting the steric constraint imposed by the added methyl group at C7. This methyl group is likely affecting the conformational change in the protein induced by the *cis-trans* isomerization of the retinal chromophore, and could be partially blocking the transition to the active MetaII conformation. This would result in a decreased G_t activation, as experimentally observed (see Table IV.3.2). Evidence of the important role of the β-ionone ring and its methyl groups in rhodopsin photoactivation, particularly in the MetaI-MetaII transition, has been obtained by using retinals with ring modifications in a recent FTIR study [Vogel et al., 2005].

Amino acid residues at the equivalent position of Met-207 in rhodopsin, like Ser-203 in the β-adrenergic receptor generally have strong propensities to mediate helix or ligand interactions [Liu et al., 2004; Patel et al., 2005]. For instance, in the H2-histamine receptor, this position is occupied by an Asp that has been suggested to interact electrostatically with the positively charged histamine ligand [Birdsall, 1991]. In addition, Met-207 causes adRP when mutated to Arg in the M207R mutant [Farrar et al., 1992] and patients with this mutation show a several clinical phenotype. It was recently found in a theoretical study that M207R mutant has an altered pattern of light absorption based on quantum mechanical simulations [Padron-Garcia et al., 2004] but no molecular evaluation of the mutation has been reported so far.

We are currently constructing mutants at Met-207 (the RP mutant M207R and others) to further dissect the role of this residue in the coupling of opsin to the retinal and in the activation process. The specific study of M207R mutation can provide new insights into the molecular defects associated with retinitis pigmentosa mutations in rhodopsin. Further work is also in progress to increase the size of the alkyl group at C7 that could hinder the rotation of the C6-C7 bond and thus affect the structural and functional behaviour of the native pigment. The combined study of Met-207 mutants and other retinal analogues with bulkier substitutions at C7 should add relevant information to the unravelling of the detailed mechanism of the rhodopsin photoactivation process.