Rhodopsin and 9-Demethyl-retinal Analog

EFFECT OF A PARTIAL AGONIST ON DISPLACEMENT OF TRANSMEMBRANE HELIX 6 IN CLASS A G PROTEIN-COUPLED RECEPTORS

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Rhodopsin is the visual pigment of rod cells and a prototypical G protein-coupled receptor. It is activated by cis → trans photoisomerization of the covalently bound chromophore 11-cis-retinal, which acts in the cis configuration as an inverse agonist. Light-induced formation of the full agonist all-trans-retinal in situ triggers conformational changes in the protein moiety. Partial agonists of rhodopsin include a retinal analog lacking the methyl group at C-9, termed 9-demethyl-retinal (9-dm-retinal). Rhodopsin reconstituted with this retinal (9-dm-rhodopsin) activates G protein poorly. Here we investigated the molecular nature of the partial agonism in 9-dm-rhodopsin using site-directed spin labeling. Earlier site-directed spin labeling studies of rhodopsin identified a rigid-body tilt of the cytoplasmic segment of helix transmembrane helix 6 (TM6) by ~6 ¯, A as a central event in rhodopsin activation. Data presented here provide additional evidence for this mechanism. Only a small fraction of photoexcited 9-dm pigments reaches the TM6-tilted conformation. This fraction can be increased by increasing proton concentration by anticipation of the activating protonation step by the mutation E134Q in 9-dm-rhodopsin. These results on protein conformation are in accord with previous findings regarding the biological activity of the 9-dm pigments. When the proton concentration is further increased, a new state arises in 9-dm pigments that is linked to direct proton uptake at the retinal Schiff base. This state apparently has a conformation distinguishable from the active state.

Rhodopsin, the visual pigment and photoreceptor in the vertebrate rod cell, is the best-studied member of the large family of rhodopsin-like (class A) G protein-coupled receptors (GPCRs).3 The crystal structure of the rhodopsin ground state (Fig. 1) confirmed the proposed seven transmembrane helix structure (TM1–TM7) and revealed an eighth helix (helix 8) after TM7 lying parallel to the cytoplasmic surface of the membrane (1–3). The pigment consists of the apoprotein opsin and the chromophore 11-cis-retinal, which is covalently bound to Lys-296 in TM7 through a protonated retinal Schiff base (RSB; Fig. 1C). The inactive ground state of rhodopsin is stabilized by a salt bridge between the protonated RSB and its counterion Glu-113 as well as the interactions of 11-cis-retinal, which acts as an inverse agonist. Absorption of a photon results in isomerization of retinal into the all-trans configuration and triggers a series of subsequent conformational rearrangements in the protein (4). After milliseconds, an equilibrium of meta-rhodopsin (M) states is reached that is dependent on pH, temperature, and the lipid environment of rhodopsin. These metarhodopsin states are characterized by their absorption maxima at 478 nm (MI) and 380 nm (MII), respectively. The blue shift in absorption is a result of deprotonation of the RSB (5) and protonation of the counterion (6). The pH dependence of the MI/MII equilibrium arises from a proton uptake reaction in MII, which involves the conserved (D/E)RY region in TM3 (Glu-134/Arg-135/Tyr-136 in rhodopsin) near the cytoplasmic surface of rhodopsin rather than the Schiff base region. The two regions are thermodynamically coupled, so that proton transfer from the Schiff base and proton uptake appear as two simultaneous steps that mark one species, MII. Kinetic studies have identified two subspecies, MIIa and MIIb, that are separated by motion of TM6 and the proton uptake reaction (4, 7, 8, 34).

Here we will start from a two-state model of GPCR activation which assumes that GPCRs exist in equilibrium between inactive and G protein activating states (corresponding to intermediates MI and MII, respectively), with the latter being stabilized by agonists (9, 10). In such a model both the Schiff base region and the (D/E)RY region simultaneously contribute to stabilizing the inactive rhodopsin conformation and provide the constraints that are broken when the active state forms. Retinal in its all-trans configuration is a strong agonist since under phys-
the partial agonism of 9-demethyl-retinal is due to a shift in the equilibrium between an inactive and an active rhodopsin state. We have now employed site-directed spin labeling (SDSL) to investigate if and how this equilibrium appears in local protein structural changes. This method revealed that the major light-induced conformational change is in both solubilized and membrane-bound rhodopsin a ~6 Å rigid-body motion of the cytoplasmic end of TM6 toward TM5 (indicated by the red arrow in Fig. 1A (17–20)). SDSL requires introduction of a “sensor” nitroxide side chain, designated as R1 (Fig. 1B), into the cytoplasmic rhodopsin surface (21, 22). The EPR spectra of the R1 sensor at position 227 (TM5, Fig. 1A) or 250 (TM6) are most sensitive to TM6 motion out of the helix bundle (18). The EPR spectral changes for R1 at these positions arise from changes in nitroxide mobility and can be used as diagnostic tools to verify the effect of the 9-dm-retinal partial agonist on equilibrium between different receptor states.

The salient result of the present study is that all-trans 9-dm-retinal in light-activated rhodopsin reduces the population of displaced TM6 relative to the native all-trans retinal. Consistently, the activating mutant E134Q results in an increased population of displaced TM6 in the 9-dm pigment and restoration of activity. At low pH a new state is found that represents the RSB-protonated MI species (MI_pb) identified by UV/visible and Fourier transformation infrared spectroscopy analysis (12–14). MI_pb bears spectral similarities to other identified Meta products with protonated Schiff base (Meta-Ib (23–25)). In MI_pb the protein apparently has a conformation similar to that in MII but with specific differences.

**EXPERIMENTAL PROCEDURES**

**Preparation and Spin Labeling of Rhodopsin—** The recombinant mutant opsin genes were derivatives of the synthetic opsin gene cloned into an eukaryotic expression vector (26, 27) and were constructed using standard fragment replacement and subcloning techniques. All gene products contained the C140S and C316S mutation in addition to the V227C or V250C mutation designated by the sequence number followed by R1. Thus, V227R1 and V250R1 designate single reactive cysteine mutants designated by the sequence number followed by R1. Thus, V227R1 and V250R1 designate single reactive cysteine mutants.

From previous work using UV/visible and Fourier transformation infrared spectroscopy (12–15), it was concluded that...
**UV/visible Spectroscopy**—Spectra were taken at 20 °C with a Varian Cary 50 UV/visible spectrometer with a resolution of 2 nm. Samples were illuminated for 15 s by using a 150-watt fiber optic light source equipped with a 480-nm cut-off (GG495 Schott, Mainz, Germany) and a heat protection filter. Buffers used were 50 mM BTP (pH 6.0–8.0) or acetate (pH 4.5–5.5), 0.03% DM.

**EPR Spectroscopy**—EPR spectra were collected on a Bruker E-580 spectrometer under dim red light. All spectra were measured at X-band using a high sensitivity resonator with a nitrogen flow-cooled Dewar manufactured by Bruker. Samples were adjusted to the designated pH by adding 1/3 volume 250 mM BTP buffer (pH 5.0, 6.0, or 7.5) and filled into a flat Suprasil cell (20 μm) of 250-μm thickness, optimized for illumination of the sample. Spectra were collected over 100 gauss with a 4-gauss modulation (100 kHz) and 10-milliwatt incident microwave power; typically, 16 scans were signal averaged. After collection of a spectrum in the dark, a second spectrum was collected after illumination of the sample for 20 s with a 150-watt halogen light source fitted with a 515-nm cut-off and heat protection filter.

**RESULTS**

**Formation of the MII State in 9-dm Pigments Is Accompanied by Changes in Mobility of the 227R1 Sensor**—Pigments containing the R1 spin label side chain at position 227 were generated by reconstitution of mutant opsin with 11-cis-retinal or 11-cis-9-demethyl-retinal (Fig. 1C) followed by spin labeling and purification in DM detergent solution. By this procedure the pigments V227R1 and 9-dm-V227R1 were obtained. UV/visible spectra of V227R1 yielded λ<sub>max</sub> values of 500 nm in the dark and 380 nm after illumination corresponding to the normal absorption maxima of rhodopsin and MII in DM detergent solution (Fig. 2A). 9-dm-V227R1 showed the same spectra as reported previously for WT-regenerated pigments with λ<sub>max</sub> values of 464 nm in the dark (9-dm-rhodopsin ground state) and 466 nm after illumination (12, 13) (Fig. 2B, measured at pH 7.5 and 20 °C). Approximately two-thirds of the latter absorption is due to 9-dm-MI, and the remainder is caused by 9-dm-rhodopsin and 9-dm-isorhodopsin (containing 9-cis-9-dm-retinal) in the photoequilibrium (see supplemental Fig. S1). The amount of the 380-nm absorbing species is very low due to the weak agonism of 9-dm-retinal (12, 13).

To monitor structural changes, EPR spectra of these pigments were recorded under the same conditions (Fig. 2C). Detailed analysis of the V227R1 pigment containing 11-cis-retinal has been discussed previously (18, 20). The R1 sensor at position 227 reports a shift of the nitroxide population to a more immobile state in MII as revealed by increases and decreases in the intensity of components corresponding to immobilized (im) and mobile (m) nitroxides (Fig. 2C, left, enlarged view of the low field part of the spectrum). This is presumed to arise from increased tertiary interactions of R1 with TM6 as it moves outward by ~6 Å (Fig. 1A (17, 18)).
Report pH Sensitivity of 9-dm Pigments

SDSL Studies of 9-Demethyl-rhodopsin

R1 at position 227 allows identification of the light-induced motion of the cytoplasmic part of TM6 out of the helix bundle. In the dark, 9-dm-V227R1 (blue line) shows an EPR spectrum identical to V227R1 (black line, Fig. 2C). After illumination, however, very little spectral change is observed for the 9-dm pigment (green line) in contrast to the V227R1 pigment (red line). This indicates little motion of TM6 upon isomerization of 9-dm-retinal, although the majority of the retinal is isomerized to all-trans-retinal (see the supplemental data).

We showed previously by UV/visible spectroscopy that the E134Q mutation could rescue MII formation in the 9-dm-E134Q pigment yielding the same amount of MII as the 11-cis-retinal-containing E134Q pigment (12, 14). We repeated the UV/visible measurements with spin-labeled pigments E134Q/V227R1 and 9-dm-E134Q/V227R1 and obtained the same rescue effect (Fig. 2, D and E). At pH 7.5 and 20 °C, the extents to which 380-nm absorbing species form from 9-dm-E134Q/V227R1 or V227R1 are comparable with one another, indicating nearly quantitative retinal isomerization. Moreover, formation of MII in 9-dm-E134Q/V227R1 is also reflected in the EPR spectra (Fig. 2F). The corresponding EPR spectra of 9-dm-E134Q/V227R1 and E134Q/V227R1 in the dark and after illumination are very similar, indicating the same basic light-induced motion of TM6 in 9-dm-E134Q/V227R1.

The Schiff Base Protonation State and the 227R1 Sensor

The Schiff base protonation state of the 227R1 sensor as function of pH. Pigments containing either 11-cis-9-dimethy1-retinal (A) or 11-cis-retinal (B) were derivatized with the spin-label at position 227. UV/visible (left) and EPR (right) spectra were taken as described in Fig. 2 at various pH as indicated. UV/visible spectra measured in the dark were independent of pH and are shown for pH 7 (black). All other spectra shown were measured directly after illumination of the pigments. The inset in the UV/visible spectra shows the absorption at 466 or 500 nm as a function of pH. For the EPR spectra only the low field peak of each spectrum is shown. Mobile (m) and immobile (im) components are indicted by arrows. All EPR spectra are normalized to the height of the central resonance line in order to compare the line shapes.

For comparison with structural changes involving TM6, EPR spectra of the pigments containing the 227R1 sensor were measured at pH 7.5, 6.0, and 5.0. No effect of pH was observed when the spectra were measured in the dark. In the spectra of illuminated 9-dm-V227R1 pigment, the fraction of the immobile nitroxide component increased slightly with decreasing pH (Fig. 3A, top right). However, the EPR spectral change at pH 5.0 is substantially smaller than the spectral change of the V227R1 pigment (Fig. 3B, top right), consistent with the UV/visible spectra that show less MII formation in the former case. This may be explained by the remaining 9-dm-rhodopsin and 9-dm-isorhodopsin in photoequilibrium (see the supplemental data) and the spectral difference between MII and MII PSB (see below).

At low pH, RSB-protonated MII PSB absorbing at 466 nm is formed that was identified earlier for unlabeled 9-dm-E134Q pigment by UV/visible and Fourier transformation infrared spectroscopy (12, 13). To examine the correlation between TM6 motion and MII PSB formation, we investigated 9-dmE134Q/V227R1 in the pH range 5.0–7.5. No pH dependence
protein in MIIPSB is different from that of either the dark or
not large, this difference suggests that the conformation of the
line shape are different, particularly in the region corre-
vant in the spectrum at pH 5 (Fig. 3

Taking this
was observed for the dark state as in the corresponding E134Q/
V227R1 pigment (not shown). In the illuminated state of 9-dm-
E134Q/V227R1 at pH 7.5, the fraction of the immobile nitrox-
and the same was observed for E134Q/V250R1 (Fig. 5

The V250R1 Mutation Influences the MI/MII Equilibrium—
We used a second sensor mutation, V250R1, at the cytoplasmic
end of TM6 to test the results obtained with the 227R1 sensor
(Fig. 1A). The sensor 250R1 was shown previously to be able to
sense TM6 motion, since the R1 nitroxide at position 250
undergoes a large change in mobility upon illumination of the
pigment (18). In this case the dominant spectral change due to
an immobile (Fig. 5, im) nitroxide population present in the
dark state decreases upon light activation, whereas the mobile (m)
population increases. The mobility change is, therefore,
opposite compared with the 227R1 sensor. Because the 250R1
sensor is oriented toward the helix bundle (Fig. 1A), an outward
motion of TM6 releases steric constraints on R1, leading to an
increased mobility of the R1 nitroxide. The pH dependence of
the UV/visible spectra of V250R1 in the dark and after illumi-
nation (Fig. 5B, top left) is similar to V227R1 (Fig. 3B, top left),
and the same was observed for E134Q/V250R1 (Fig. 5B, bottom
left) compared with E134Q/V227R1 (Fig. 3B, bottom left). How-
ever, the spectra of the illuminated 9-dm-V250R1 pigment
showed increased A_{380} values relative to 9-dm-V227R1, indi-
cating that the 250R1 sensor facilitates MII formation and shifts
the MI/MII equilibrium toward MII. Increasing the proton
concentration again resulted in a gradual increase of the MIIPSB
form (Fig. 5A, top left).

The corresponding changes in the EPR spectra for the 250R1
sensor are shown in Fig. 5, right panel. In the dark state pH
changes had no influence on the spectra of pigments V250R1 and
9-dm-V250R1. Upon illumination, V250R1 showed a large
increase in mobility of the nitroxide as seen in the change of
relative intensities of the mobile (m) and immobile (im) com-
ponents of the spectra (Fig. 5B, top right), as previously reported
(18, 20). This change was slightly increased at pH 5 and 6 com-
pared with pH 7.5. The R1 mobility change of 9-dm-V250R1
(Fig. 5A, top right) upon illumination was reduced in magnitude
compared with V250R1 and had smaller pH dependence com-
pared with 9-dm-V227R1 (Fig. 3A, top right).

We also tested the effect of the E134Q mutation on pig-
ments with the 250R1 sensor. The UV/visible spectra of illu-
minated E134Q/V250R1 and 9-dm-E134Q/V250R1 were
shifted completely to MII at pH 7.5, as for the corresponding
227R1 sensor mutants. MIIPSB was formed in the 9-dm pig-
mment with a pK_{a} of 6, whereas the 11-cis-retinal reconsti-
tuted pigment showed an onset of MIIPSB at pH < 5.5 (Fig. 5,
A and B, bottom left). The EPR spectra of 134Q/V250R1 and
9-dm-E134Q/V250R1 in the dark state are independent of pH
(data not shown). The spectral change of 134Q/V250R1 and
9-dm-E134Q/V250R1 upon illumination (Fig. 5, A and B,
bottom right) was similar to V250R1, although slightly
smaller for 9-dm-E134Q/V250R1. In contrast to 9-dm pig-
mments with the 227R1 sensor, the EPR spectra of illuminated
pigments with the 250R1 sensor have only weak pH depend-
ence in the range 5.0–7.5. The weak pH effect and the facili-
Partial Agonism of 9-dm-retinal Is Reflected in Helix Motion—GPCRs are differently activated by full and partial receptor agonists. Partial agonists are, by definition, unable to elicit full maximal response. Photoisomerization of 11-cis 9-dm-retinal in rhodopsin mainly yields the all-trans isomer that is a partial agonist, whereas a small and pH-dependent fraction is found in the 11-cis and 9-cis configurations (see the supplemental data). Available spectroscopic and biochemical data were found to be in agreement with an equilibrium between an inactive and an active receptor form, MI and MII, respectively (12–15), but with the equilibrium strongly shifted toward the MI state relative to the native pigment. Unlike these previous studies, the observations in the present study provide site-specific structural information and allow us to relate protein conformation to different photochemically and functionally defined states in 9-dm pigments.

In previous SDSL studies on rhodopsin containing the native 11-cis-retinal, evidence was provided to show that TM6 is held within the helix bundle in the inactive MI state, whereas in the active MI state the cytoplasmic end of TM6 is tilted outward, and these conformational states are in equilibrium (19, 20). The results presented above show that the same two-state MI/MII equilibrium is reflected in the EPR spectra of 9-dm pigments, although on a background of a small fraction of pigment molecules staying in the ground state (see the supplemental data). Moreover, the fraction undergoing TM6 movement and activity toward the G protein (12, 14) are parallel (restored in the 9-dm-E134Q mutant), both being reduced to a similar extent when rhodopsin is activated by the 9-demethyl analog instead of the native retinal. This correlation provides strong support for the activation model derived from the previous SDSL work. Partial agonism of 9-dm-retinal is, thus, reflected in the fraction of TM6 moved, in agreement with the notion that the full movement of TM6 originally described is necessary to constitute the active form of rhodopsin and presumably GPCRs in general (17, 19, 31).

Proton Uptake to the Retinal Schiff Base Affects Rhodopsin Conformation—Although the MI/MII two-state model is sufficient to describe the relationship between activity and helix motion in a qualitative way, it does not account for a new state which forms only when 9-demethyl-retinal is present in the binding site of rhodopsin. This is MIIPSB, a MI conformation that appears to be related to this species. MIIPSB as a third state (forming with a pK below 6) has to be added, in agreement with the notion that the full movement of TM6 originally described is necessary to constitute the active form of rhodopsin and presumably GPCRs in general (17, 19, 31).

Partial Agonism of 9-dm-retinal in 9-dm pigments is photoexcited. In native rhodopsin such proton uptake to the RSB is observed only at much lower pH, and therefore, a two-state model of MI/MII or TM6 inward/TM6 outward is sufficient to describe the gross conformational changes. Because proton uptake causes a shift of the MI/MII equilibrium due to formation of protonated MIILb, tilt of TM6 appears to be related to this species.

In 9-dm pigments, a two-state model is only justified at high pH (near physiological) where MIIPSB is not present. At low pH, MIIPSB as a third state (forming with a pK below 6) has to be taken into account to describe all the details of the experimental data. An intriguing finding in the present study is that this species, which has the MI signature bands in the infrared spectrum (13, 14), shows an EPR spectral line shape similar to MI but with slight differences. It is not possible to interpret these
differences in terms of protein structure at this time, but they imply that MII_{PSB} has a conformation distinct from MI or MII. Further study using direct distance mapping provided by double electron resonance (DEER) spectroscopy may provide a better appreciation of the structural differences. Such experiments are in progress. It is interesting to note that for rhodopsin containing another partial agonist, namely an acyclic retinal, a MII_{PSB} product was found to interact with the C terminus of the α-subunit of transducin (14). Similarly, a photoproduct with protonated RSB, termed Meta-Ib, was described to also interact with this C terminus (24) and GDP-bound transducin (25). Furthermore, a mutant rhodopsin with protonated RSB in the light-activated state, was reported to activate transducin (32). With further progress using SDSL, we may come closer to an understanding of the structural differences behind this complex behavior.

**Influence of the 250R1 Sensor on Rhodopsin Activation**—The simple MI/MII two-state model also fails to explain the data in yet another, instructive way. This is the behavior of the 250R1 sensor. Relative to 227R1, it reflects a shift in the MI/MII equilibrium toward MI as revealed by the UV/visible spectra of 9-dm pigments. This shift was also paralleled in the EPR spectra. A similar effect was observed for 250R1 rhodopsin in lipid bilayers (20). The reason for this may be the proximity of the 250R1 sensor to Glu-247 and Thr-251. These two residues interact with Arg-135 of the (D/E)RY motif in TM3 as seen in the crystal structure. Moreover, a recent crystal structure of the rhodopsin ground state shows Val-250 in van-der-Waals contact with Arg-135 (2). It is, therefore, possible that the 250R1 modification disturbs these interactions, influences the pH dependence resulting from proton uptake involving Glu-134 in the (D/E)RY region (8), and facilitates TM6 displacement. Alternatively, the shift in equilibrium may be the result of strain energy of the 250R1 side chain in the highly constrained environment of the helix bundle interior, as suggested earlier (20).

The 250R1 sensor did not indicate a mobility change when MII_{PSB} was formed in the 9-dm-V250R1 pigment at low pH. This may be explained by two counterbalancing effects in the equilibrium between MI, MII, and MII_{PSB}. The amount of MI in equilibrium at pH 7.5 is replaced at pH 5 by MII_{PSB} and 9-dm-rhodopsin and 9-dm-isorhodopsin (see the supplementary data, Fig. S1C). The spectral line shapes of MI, the ground state, and MII_{PSB} are such that changes in the relative amounts of these states may have little influence on the EPR spectra and explain the weak pH dependence observed. It is interesting to note that in salamander red cone pigments MI formation is facilitated, and the agonism of 9-dm-retinal is sufficient to fully form MII (33), similar to the behavior of the 9-dm-V250R1 pigment.

**Conclusion**—In summary we have shown that the outward tilted position of the TM6 mirrors the active state, metarhodopsin II, in both the native receptor and in pigments containing the partial agonist 9-dm-retinal. Partial agonism appears to be related to the fraction of TM6 moved rather than a partial movement. Consistently, the full activity of the 9-dm-E134Q pigment is reflected in a maximal fraction of TM6-tilted mole-

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