Conformations of the Active and Inactive States of Opsin

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Running Title: Conformation of the Active Opsin State
Summary

The signaling state Metarhodopsin II of the visual pigment rhodopsin decays to the apoprotein opsin and all-trans retinal, which are then regenerated to rhodopsin by the visual cycle. Opsin is known to have at neutral pH only a small residual constitutive activity towards its G protein transducin which is thought to play a considerable role in light adaptation (bleaching desensitization). In this study we show with Fourier-transform infrared spectroscopy that after Metarhodopsin II decay opsin exists in two conformational states which are in a pH dependent equilibrium at 30 °C with a pK of 4.1 in the presence of hydroxylamine scavenging the endogenous all-trans retinal. Despite the lack of the native agonist in its binding pocket, the low pH opsin conformation is very similar to that of Metarhodopsin II and is likewise stabilized by peptides derived from rhodopsin’s cognate G protein, transducin. The high pH form on the other hand has some conformational similarity to the inactive Metarhodopsin I state. We therefore conclude that the opsin apoprotein displays intrinsic conformational states which are merely modulated by bound all-trans retinal.
Introduction

Rhodopsin is the visual pigment responsible for dim light vision in the outer segments of rod photoreceptors. It is a seven-helix transmembrane protein and serves as a prototype for the study of the large family of G protein-coupled receptors (GPCRs) (1). Unlike other GPCRs, rhodopsin’s apoprotein opsin is covalently bound to its chromophore, 11-cis retinal, via a protonated Schiff base to Lys\textsuperscript{296} on helix 7, maintaining the receptor in an inactive conformation and giving it an absorption maximum centered at 500 nm. Upon photon absorption, the retinal isomerizes to an all-trans geometry, thereby switching from an inverse to a full agonist, and drives the receptor to an active state conformation, called Metarhodopsin II (MII). This state is capable of catalytically activating the visual G protein, transducin. Formation of MII is accompanied by deprotonation of the Schiff base, thereby shifting the absorption maximum of the pigment to 380 nm with a concomitant protonation of Glu\textsuperscript{113} (2), which forms the counterion to the protonated Schiff base in the MII precursors (3).

In vivo, signaling is shut off by receptor phosphorylation catalyzed by a specific receptor kinase and subsequent binding of visual arrestin (reviewed in (4)). The retinal Schiff base becomes hydrolyzed and retinal is reduced by retinal dehydrogenase. The visual cycle is closed by dephosphorylation of the apoprotein opsin and regeneration to rhodopsin with 11-cis retinal from the retinal pigment epithelium. In isolated washed disk membranes, where these shut-off and regeneration reactions are absent, MII decays by hydrolysis of the Schiff base and dissociation into opsin and all-trans retinal. All-trans retinal ($\lambda_{\text{max}}$ 380 nm) may then form in part Schiff bases with peripheral lysines on the apoprotein or phosphatidyl ethanolamine in the lipid bilayer (5).

Opsin has at neutral pH only a very small intrinsic activity towards the G protein transducin. This small constitutive activity is nevertheless suspected to be involved in adaptation of photoreceptors to high light levels in a process termed bleaching desensitization (6,7).
Presumably, opsin is held in an inactive conformation by the constraint of a salt bridge between Glu\textsuperscript{113} on helix 3 and Lys\textsuperscript{296} on helix 7, as replacement of either of these residues by uncharged sidechains considerably increases the constitutive activity (8). Other mutations resulting in constitutive activity of opsin involve the residues Met\textsuperscript{257} (9), Gly\textsuperscript{121}, and Phe\textsuperscript{261} (10,11), which mainly seem to affect stabilizing interhelical packing interactions, as well as Glu\textsuperscript{134} (12), which is known to play a major role in determining the equilibrium between MII and its still inactive precursor, MI. A second factor determining activity of opsin is the presence of the natural agonist of rhodopsin, all-\textit{trans} retinal. In high retinal/opsin ratios, all-\textit{trans} retinal is known to further increase the activity of native opsin (13-18) and of constitutively active opsin mutants (3,8-10,19) and may in some mutants even reach the activity of the photoactivated mutant pigment.

Infrared difference spectroscopy is a powerful tool to investigate both conformational and functional changes in proteins (20). In the present study, we show with Fourier-transform infrared (FTIR) difference spectroscopy that native opsin may form two distinct conformational states, which are in a pH-dependent equilibrium. The existence of a state with a putatively inactive conformation at neutral pH was characterized earlier by infrared spectroscopy (21). At low pH, however, we show that opsin adopts a conformation which is very similar to that of the signaling state MII and which features a protonated Glu\textsuperscript{113}, in contrast to its inactive counterpart at higher pH. In the framework of a two state model of GPCR activation, a non-vanishing population of this state may be the molecular reason for the very small constitutive activity of wildtype opsin at neutral pH. The population of this state with an active conformation does not depend on, but is slightly augmented by the stoichiomterical presence of all-\textit{trans} retinal being formed as the second MII decay product. We conclude that opsin possesses two intrinsic conformations which are formed in the absence of ligand and which are likely to be regulated by the protonation state of Glu\textsuperscript{113}.
Experimental Procedures

Preparation of the Pigment

All experiments were carried out on bovine rhodopsin in disk membranes, which were prepared from rod outer segments of cattle retinae essentially as described previously (22). All preparations involving pigment were performed under dim red light.

FTIR Spectroscopy

FTIR spectroscopy was performed in transmission mode with a Bruker IFS 28 FTIR spectrometer, equipped with a liquid nitrogen cooled HgCdTe detector.

All data were obtained from well hydrated sandwich film samples prepared by drying 0.5 nmol pigment on specially designed CaF$_2$ bottom windows with 4 µm pathlength as described previously (23). In this sample type, a preferred orientation of the disk membrane normal perpendicular to the window plane is achieved as evident from the insert in Fig. 2C. To ensure accurate pH adjustment, we pre-equilibrated the sample films on the bottom window with 5 µl of 200 mM buffer for a few seconds before squeezing the excess buffer out of the central sample part by sandwiching the bottom with the top window. We used citrate buffer (for pH < 6.0), 2-N-morpholino ethanesulfonic acid buffer (MES, pH 6.0 - 7.0), and bis-tris propane buffer (BTP, pH > 7.0), all supplemented with 200 mM NaCl to reduce potential membrane surface pH effects. The samples were thermostatted (stability 0.1 °C) in the dry air purged sample chamber. Spectra were recorded in blocks of 512 scans at 4 cm$^{-1}$ resolution with 1 min acquisition time. After baseline stabilization, 10 subsequent spectra were recorded, the sample was photolyzed for 30 s by a fiber optic connected to a 150 W slide projector and an OG 530 cut-off filter (Schott, Germany), and up to 30 subsequent spectra were recorded to follow the decay of the initially resolved MII photoproduct.

In the experiments involving the transducin derived high-affinity peptide analog, 10 nmol of
peptide were added to 0.5 nmol of pigment before drying.

All experiments were carried out at 30 °C to ensure rapid decay of MII within a timeframe accessible to infrared difference spectroscopy. At this temperature, no further spectral changes were observed 15 min after illumination, and we therefore used the difference spectrum obtained after this period as *final decay product*. In the presence of hydroxylamine, a stable decay product was already obtained 1 to 5 min after illumination, depending on pH, thereby allowing experiments also at 20 °C.

In additional experiments, we also used squeezed sediment samples (24) with similar results as for the film samples which involve drying of the pigment. As the kinetics of MII decay are similar in sandwich film samples and dilute suspensions (halftime about 2.8 min at 30°C), our results obtained with sandwich samples likely match those expected from dilute suspensions within reasonable deviations.

**Fitting Procedures**

To determine the half time of the decay of MII, a negative exponential was fitted to the decay of prominent bands at 1748, 1711, 1686, 1664, 1645, 1554, and 1530 cm\(^{-1}\).

To determine the titration curve of the opsin states, we defined two normalized basis spectra, which were averages of final decay product spectra of each two experiments at pH 4.0 and 7.0, respectively (shown in Fig. 3A and B). All obtained spectra of the final decay products were normalized by their absorption band at 1238 cm\(^{-1}\) and then fitted to the linear combination \(scale \cdot (a \cdot \text{base}_{4.0} + (1-a) \cdot \text{base}_{7.0}) + offset\) of this spectral basis set over the range from 1800 to 1100 cm\(^{-1}\) by a least square Newton fitting procedure. The parameters \(scale\) and \(offset\) were always very close to 1 and 0, respectively. To eliminate possibly pH dependent contributions from peripheral Schiff bases formed by dissociated retinal, we
excluded their spectral range of absorption, i.e. from 1665-1645 cm$^{-1}$, 1585-1540 cm$^{-1}$, 1248-1228 cm$^{-1}$, and 1210-1170 cm$^{-1}$. This was essentially not necessary for the experiments in the absence of hydroxylamine, as a fit over the entire spectral range gave very similar results. It was, however, necessary for the fitting of spectra obtained in the presence of hydroxylamine (where no peripheral Schiff bases are formed) to our standard basis set, which was obtained without hydroxylamine, and therefore used throughout for consistency.

The pH dependency of the obtained values of $a$ were fitted to the Henderson-Hasselbalch equation \( \frac{e_{p_{\text{high}}} + e_{p_{\text{low}}} \cdot 10^{pK - pH}}{1 + 10^{pK - pH}} \), where $e_{p_{\text{high}}}$ and $e_{p_{\text{low}}}$ are the endpoints of titration at high and low pH. Fitting of the data obtained at 30 °C in the absence of hydroxylamine yielded a lower endpoint parameter \( e_{p_{\text{low}}} = 1.53 \), suggesting that the spectra obtained at pH 4.0 do not yet correspond to a pure state, while $e_{p_{\text{high}}}$ could be set to zero. The pure state \( \text{Ops}^* \) was reconstructed from these data by the linear combination \( \text{Ops}^* = e_{p_{\text{low}}} \cdot \text{base} \, 4.0 + (1 - e_{p_{\text{low}}}) \cdot \text{base} \, 7.0 \).

**UV-visible Spectroscopy**

UV-visible spectroscopy was performed with a thermostatted Perkin-Elmer Lambda 17 UV-vis spectrophotometer either with sandwich samples identical to those used for FTIR experiments or with disk membranes in suspension in 100 µl microcuvettes with 10 mm pathlength (Hellma, Germany).

**Results**

**Decay of the Signaling State MII**

In Figure 1, the time evolution of the FTIR difference spectra photoproduct minus dark state
from film samples after pigment photolysis are shown. The first spectrum of the initial photoproduct, which was recorded immediately after illumination, still corresponds largely to a MII spectrum. At pH 7.0, this spectrum decays, however, rapidly as evident from the decrease of both negative and positive absorption bands. This decay is particularly evident in the region of amide I and amide II vibrations indicative of changes in the protein backbone (around 1650 cm\(^{-1}\) and 1550 cm\(^{-1}\), respectively) and in the region of the protonated carboxyl groups between 1700 and 1800 cm\(^{-1}\), which are also serving as structural and functional markers. This general decrease of absorption bands was reported before and interpreted as a refolding of the MII structure to a structure partly resembling the dark state during the decay (21). At pH 4.0, the situation is very different as the intensities of most bands remain, after an initial small decrease, mostly unchanged (the difference spectrum obtained after 20 min still possesses most features of the initial MII difference spectrum).

(insert Figure 1 near here)

We next examined identical, oriented film samples by UV-vis -spectroscopy (Fig. 2). As evident from Fig. 2A and B, after photolysis of the dark state (\(\lambda_{\text{max}}\) 500 nm) a MII photoproduct is formed (\(\lambda_{\text{max}}\) 380 nm), which then decays by hydrolysis of the Schiff base to opsin and free all-trans retinal, a fraction of the latter forming Schiff bases with peripheral lysines and the amino group of phosphatidyl ethanolamine present in the embedding lipid bilayer (5). The resulting products absorb at 380 nm (free retinal) and around 450 and 370 nm (for the protonated and unprotonated Schiff bases). The relatively low absorption of the decay products is due to a reorientation of the transition dipole moment of the retinal during decay. While in the dark and the MII state, the dipole moment in the oriented film samples is mostly parallel to the electric field vector of the measuring beam (25), this preferential orientation is lost upon decay. This could also be verified by comparing difference spectra of the final decay product minus dark state at pH 4.0 in the presence of hydroxylamine, converting the
retinal quantitatively into oxime absorbing around 360 nm (inset in Fig. 2C), obtained from oriented film samples and from suspension samples. In the film samples the ratio oxime absorption / dark absorption is decreased compared to suspension samples, in line with the above interpretation.

(insert Figure 2 near here)

The 380 nm absorption of MII decays exponentially with a half time of about 2.8 min, both at pH 4.0 and 7.0. A similar half time was obtained for the decay of prominent FTIR bands (see Experimental Procedures) for both pH values in Fig. 1, although the decrease of band intensities at pH 4.0 is quite small. The difference between the decay patterns obtained at both pHs in the infrared can therefore not be due to slower or an only partial hydrolysis of the Schiff base at pH 4.0 and therefore an extended MII lifespan. Instead it must be due to a lack of conformational change during the decay at this pH. The conformation of the decay product opsin at pH 4.0 must therefore be similar to MII, while at pH 7.0, its conformation is closer to the dark state as evident from the overall decrease of bands and in agreement with a previous study (21).

It should be noted, that in the MII spectra obtained at pH 4.0, there is a small contribution of a MII photoproduct with a protonated Schiff base due to anion binding from the solvent to the Schiff base, which is generally observed at low pH (Vogel et al., submitted for publication). This does, however, not interfere with the conclusions drawn in this paper.

Analysis of the Decay Products

In Figure 3A and B, the infrared spectra of the final decay products obtained 15 min after pigment photolysis at pH 4.0 and 7.0 are plotted and compared to the corresponding spectra of the initial photoproducts obtained immediately after photolysis, corresponding largely to MII.

(insert Figure 3 near here)
At pH 4.0, the spectrum of the final decay product is very similar to that obtained immediately after photolysis (Fig. 3A). An important spectral region lies between 1730 and 1770 cm\(^{-1}\) with the difference band pattern of the protonated carboxylic acids Asp\(^{83}\) and Glu\(^{122}\), which may serve as a conformational marker (26). While a difference band located at -1768/+1750 cm\(^{-1}\), which reflects a change of the hydrogen bonding properties of Asp\(^{83}\) during activation, is preserved in the final decay product, there is an absorption decrease of a positive photoprodct band at 1745 cm\(^{-1}\), which due to its position can be assigned to Glu\(^{122}\).

According to the rhodopsin 3D structure, Glu\(^{122}\) is positioned in contact to the \(\beta\)-ionone ring of retinal (27), and the changes therefore reflect likely a changed environment due to dissociation of the chromophore. There is also a very slight decrease in the positive band at 1712 cm\(^{-1}\), which is attributed to protonated Glu\(^{113}\) in MII (2), and which reflects either a marginal deprotonation of this residue in the final decay product at this pH or local changes associated with chromophore dissociation. In the amide I region, there is a small decrease at 1659 and 1643 cm\(^{-1}\) as well as in the amide II region at 1547 cm\(^{-1}\), indicating slight conformational changes. The absorbance increase at 1558 cm\(^{-1}\) may, as we will show below, be partly due to the C=C stretch mode of peripheral protonated Schiff bases of retinal being formed during the decay process.

At pH 7.0, the decay process corresponds to a reduction of almost all difference bands, with the exception of bands related the 11-\textit{cis} retinal chromophore of dark rhodopsin. These are particularly the intense fingerprint band at 1238 cm\(^{-1}\), the C=C stretch vibration around 1549 cm\(^{-1}\), and the Schiff base C=N stretch vibration at 1659 cm\(^{-1}\), which cannot be compensated by the decay product all-\textit{trans} retinal or its Schiff base (Fig. 3B). In the amide I region, there are two positive bands at 1667 and 1632 cm\(^{-1}\) arising in the decay product. Interestingly, there are possibly equivalent bands in the spectrum of MI, the inactive
precursor of MII (Fig. 3C). Indeed, except for the positive bands related to the chromophore of MI at 1539 and 1204 cm\(^{-1}\), the spectrum of MI has some similarity to that of the final decay product at pH 7.0. Importantly, as deduced from the considerable decrease of the band at 1712 cm\(^{-1}\), Glu\(^{113}\) deprotonates during the decay of MII at this pH, thereby decreasing the amplitude of a negative band at 1399 cm\(^{-1}\), attributable to the symmetric stretch vibration of the carboxylate in the decay product.

We can therefore conclude that there are two distinct conformational states of opsin: 1) a state with a conformation somewhat similar to MI and with only minor structural changes compared to the dark state, and, importantly, a negatively charged glutamate at position 113. 2) a state very similar to the active state MII, as judged from amide bands and the band pattern related to Asp\(^{83}\) and in part Glu\(^{122}\), with a neutralized side chain of Glu\(^{113}\), despite apparent hydrolysis and dissociation of the retinal from its binding pocket.

To determine the pH dependency of the conformational equilibrium between these two states, we used a fitting procedure as described in Experimental Procedures with spectra obtained at pH 4.0 and 7.0 as basis spectra. The linear coefficient \(a\) obtained by this method in the pH range 4.0 to 7.0 was found to follow a Henderson-Hasselbalch equation with the lower titration endpoint left open for fitting and the higher titration endpoint set to zero. The results of this fitting procedure give a \(pK\) of the transition of 4.3. The value 1.53 for the endpoint of titration at low pH clearly indicates that the final decay product obtained at pH 4.0 does not yet correspond to a pure state. Instead it is still a mixed state consisting of contributions from the conformation obtained at pH 7.0 (from now on termed \(\text{Ops}\)) and from a MII-like conformation, which should be formed in purity at still lower pH. This also explains why there is a small but significant decay of the initial photoproduct at pH 4.0 (Fig. 3A). As it was not possible to obtain completely stable final decay products at pH values below 4.0, we constructed this pure state, which we will call \(\text{Ops}^*\) as it possesses an active state.
conformation, from the given basis set of spectra and the parameter obtained for the titration endpoint at low pH (see Experimental Procedures). In Fig. 4A, we plot the fraction of Ops* contributing to the final decay products over the pH range 4.0 to 7.0. In Fig. 4B, we show the reconstructed infrared spectrum of Ops* in comparison to the final decay product obtained at pH 4.0 and a pure MII spectrum obtained at 10 °C. Obviously, Ops* is structurally equivalent to the signaling state MII and is therefore very likely also an active state, while Ops corresponds to an inactive conformation as suggested by its conformational closeness to the MI state.

(insert Figure 4 near here)

**Stoichiometric All-trans Retinal Slightly Enhances Formation of the Active State Conformation Ops***

Up to now, all experiments were conducted in the presence of the 1:1 stoichiometry of all-trans retinal being formed as decay product of MII. In several studies, all-trans retinal was shown to exert an agonistic activity on opsin, particularly when applied in higher ratios retinal/opsin (8,15,19). To investigate therefore its role in the equilibrium of the two opsin states, we repeated our experiments in the presence of hydroxylamine, which scavenges the retinal by quantitatively transforming it into retinal oximes. In contrast to the aldehyde, the oxime of all-trans retinal was shown to possess no agonistic activity on opsin (18,28). As evidenced in Fig. 3D, opsin was capable of forming the active conformation Ops* also in the absence of all-trans retinal. The only major difference from spectra obtained in the absence of the scavenging hydroxylamine is a decrease around both 1570 and 1200 cm⁻¹, which can be attributed to the lack of peripherally formed protonated Schiff bases being otherwise formed by the dissociated retinal. A more detailed analysis of the pH dependency of the opsin equilibrium indicates a slightly lower contribution of Ops* to the final decay product, which is expressed in a pK of 4.1 compared to 4.3 in the absence of hydroxylamine (Fig. 4A). Due to the more
rapid decay of MII in the presence of hydroxylamine (Fig. 2C), the equilibrium of the opsin states could also be examined at 20 °C, yielding a pK of 3.8 (data not shown).

**Binding of a Transducin Derived Peptide Stabilizes Ops**

Transducin as well as short peptides corresponding to certain domains of transducin are known to interact specifically with MII and to shift thereby the MI/MII equilibrium towards the active state MII (extra MII effect) (29). We therefore examined the influence on the opsin equilibrium of a modified peptide analog to the C-terminus of the transducin α-subunit, peptide 23 (ac-VLEDLKSCGLF), which was shown to stabilize MII with high affinity (30). We conducted this experiment in the presence of hydroxylamine to ensure efficient decay of MII which was verified by UV-vis spectroscopy (data not shown). In the presence of peptide, a MII-like conformation of opsin was almost fully preserved up to pH 6.0 (Fig. 4C), where in the absence of peptide the opsin conformational equilibrium is already largely shifted towards the inactive state. In comparison to MII spectra obtained in the absence of peptide, there are small deviations in the peptide bound opsin spectra in the region around 1654 and 1550 cm⁻¹, which are similarly observed for the MII-peptide complex (31) and which reflect small conformational changes of peptide and/or receptor upon complex formation. Similarly as MII, Ops* is therefore very likely also recognized by the transducin holoenzyme as an active state, further supporting our above conclusions drawn from the conformational analysis.

**Discussion**

Under bright light, the sensitivity of visual photoreceptors decreases in an adaptation process termed bleaching desensitization. In photoreceptors, where bleached pigment is not regenerated with 11-cis retinal by the visual cycle, the desensitized state persists in the dark and induces a sustained stimulation of the visual cascade similar to that observed under continuous background illumination (7). In the resulting depressed adaptational state, the MII
decay product opsin is accepted to maintain a basal level of G protein stimulation either alone or in combination with all-trans retinal being released as well upon MII decay. Opsin alone has only a slight activity towards the visual G protein transducin, although the reported values vary considerably between e.g. $10^{-6}$ at pH 8 (32) to ~0.1 at pH 6.1 compared to light activated rhodopsin (12). This activity can, on the one hand, be abolished by regeneration with the inverse agonist 11-cis retinal or, on the other hand, be increased by the presence of the agonist all-trans retinal (8). Due to its involvement in bleaching desensitization and its analogy to GPCR activation by diffusible ligands, this retinoid induced activation has attracted much interest recently (17,19). Still its mechanism is presently not entirely clear. An extensive study revealed a pronounced selectivity of opsin for different isomers of retinal and retinal analogues (18), which may suggest a binding of the ligand in the native binding pocket. This study also demonstrated that all-trans retinal has a critical size for binding as its oxime is already excluded. On the other hand, some studies suggest a non-covalent binding of all-trans retinal outside the native binding pocket (16) and exclude formation of a covalent Schiff base bond in the native binding pocket (14-16). This situation is different in constitutively active opsin mutants, which seem to have a considerably increased affinity for all-trans retinal and which in some cases clearly form a stable, active pigment with exogenously added all-trans retinal, corresponding to MII (3,19).

The conformational similarity between Ops* and MII and its specific stabilization by transducin derived peptides strongly suggests that Ops* can activate transducin as well. The previously described constitutive activity of opsin, which becomes detectable upon lowering the pH to pH 6, may therefore be due to an increasing population of Ops*. Importantly, Glu$^{113}$, which forms the counterion to the protonated Schiff base in the dark state of rhodopsin, is clearly protonated in Ops*, but not in the inactive state Ops. This is in agreement with previous studies of MII, which suggested a pivotal role of the protonation state of this residue for the formation of an active receptor conformation (33). Our data do not allow the conclusion
that protonation of Glu$^{113}$ is the determining step in the equilibrium between Ops and Ops*, as e.g. Glu$^{134}$, which is playing a role in the pH dependent equilibrium between MI and MII (34), may be involved as well. However, other studies on opsin mutants strongly suggest that we are really titrating a salt bridge between Glu$^{113}$ and Lys$^{296}$, which constrains opsin to an inactive conformation at neutral pH.

In the opsin mutants E113Q and K296G, where the native charge pair is abolished, the pK for the transition to an active conformation is considerably increased from presumably around 5.1 for native opsin, as extrapolated from transducin activation data (12), to 6.8 and 8.0, respectively (8). In contrast to this, the mutant opsin E134Q shows a considerably smaller increase to only 5.6 (12). Additionally, in the double mutant E134Q/K296G the pK is not further increased compared to the single mutant K296G (12). A different study revealed for the E134Q mutant an activity of only 3.0% (±1.2%) at pH 7.2, while E113Q had an activity of 23% (±0.8%), which was further increased in the double mutant to 31% (±11%) (9). These data suggests that protonation of Glu$^{134}$ is modulating the pK of the salt bridge between Glu$^{113}$ and Lys$^{296}$ by releasing other inactivating conformational constraints rather than being the initial event itself, implying that disruption of the charge pair is the essential step of opsin activation. The difference between the pKs of E113Q and K296G could be shown to reside largely in steric interactions of the sidechain at position 296, as it vanishes in mutants where Lys$^{296}$ is replaced by uncharged residues of similar size as lysine (12).

We further showed in this study, that the formation of Ops* does not depend on the presence of all-trans retinal. Ops* is also formed when the endogenous all-trans retinal is converted to oxime in the presence of hydroxylamine. All-trans retinaloxime, however, was shown to have no influence on the activity of opsin (18,28). The relative increase of the pK of the transition from the inactive to the active conformation by about 0.2 units in the absence of hydroxylamine can therefore be attributed to the presence of the dissociated free all-trans retinal and is quite
small.

These observations allow a description of the conformational equilibria of opsin and the rhodopsin photoproducts similar to that of the two-state model of other GPCRs. In this model first set up by Lefkowitz and colleagues (35), the conformation of a receptor in the absence of ligand is in equilibrium between an inactive state $R$ and an active state $R^*$. This internal equilibrium may be shifted by the binding of either activating or inactivating ligands $L$, forming the respective states $L\cdot R$ and $L\cdot R^*$. In the absence of agonist, opsin forms a similar internal equilibrium with the conformational states Ops and Ops*. Due to the apparently very low affinity of all-trans retinal for the retinal binding pocket of native opsin, the fully agonist bound states are realized only in the rhodopsin photoproduct states MI and MII, which are stable for some time after photolysis of the dark pigment at a decreased temperature. As in the general scheme, the presence of an agonist in the binding pocket of the rhodopsin photoproducts MI and MII also strongly shifts the internal conformational equilibrium to the active state, which becomes evident in a pK shift of 4 units from 4.1 for ligand-free opsin to approximately 8.5 for the MI/MII equilibrium (as extrapolated in ref. 36 in agreement with own data), both at 30 °C (Fig. 4A).

In conclusion, we have shown in this study that the conformations capable of transducin activation are presumably very similar for the rhodopsin photoproduct MII and the apoprotein opsin alone. Both active and inactive conformations are therefore intrinsic properties of the apoprotein itself and are only regulated by the presence of activating or inactivating ligands. The activation process of visual pigments, which involves photon absorption and isomerization of the covalently bound ligand 11-cis retinal, is different from that of other GPCRs, which are activated by binding of diffusible ligands, and puts rhodopsins in a functionally separate class of GPCRs. The conformational equilibria between inactive and active states of visual pigments and other GPCRs, however, are similar and allow the functions of both GPCR categories to be described by a two-state model.
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References

Footnotes

*Abbreviations used:* FTIR, Fourier-transform infrared; MI and MII, Metarhodopsin I and II; Ops and Ops*, opsin state with inactive and active conformation; GPCR, G protein-coupled receptor
Figure Legends

**Figure 1:** Decay of MII in the IR. Rhodopsin in disk membranes was photolyzed at 30 °C (t=0) to form MII and the decay of MII was followed in the IR at pH 7.0 (upper panel) and pH 4.0 (lower panel). The spectral traces are difference spectra photoproduct minus dark state (photoproduct bands point upwards, while bands of the dark state are negative) and were recorded with a time resolution of 1 min.

**Figure 2:** Decay of MII in the UV-vis. A and B: The decay of MII was followed in the UV-vis under similar conditions and with identical, oriented film samples as in Fig. 1. The thin black trace is the spectrum of the dark state, the gray traces represent the photoproduct spectra recorded 0, 2, 4, and 8 min after sample illumination, and the thick black trace is the spectrum of the final decay product obtained 16 min after illumination. The absorption decrease at 380 nm over time reflects the hydrolysis of the retinal Schiff base in MII and the dissociation of retinal from the binding pocket. C: As in A, but in the presence of 100 mM hydroxylamine. The photoproduct spectra were recorded 0 (gray) and 2 min (black) after illumination, no subsequent changes occurred afterwards. The inset in C shows the corresponding difference spectra final decay product minus dark state for oriented film and unoriented suspension samples.

**Figure 3:** Final decay products of MII. A and B: Comparison of the difference spectra photoproduct minus dark state obtained immediately after photolysis of rhodopsin at 30 °C (gray) and after complete decay of MII (15 min, black lines) at pH 4.0 (A) and 7.0 (B). C. Difference spectrum corresponding to the MI state (pH 8.0, 0 °C, partially hydrated film sample). D: Influence of hydroxylamine at pH 4.0. Final decay product as in A (gray) and in
the presence of 100 mM hydroxylamine (black). All spectra are the average of each two experiments.

**Figure 4:** Formation of the active opsin state Ops*. A. The fraction of the active state Ops* in the final decay product was determined in dependence of pH at 30 °C either in the presence of the stoichiometrically released all-trans retinal (black), or with 100 mM hydroxylamine (HA) scavenging the retinal to retinal oxime (gray). Spectra were analyzed by a least square fitting routine as described in the text and the pK of the titration was obtained by fitting to a Henderson-Hasselbalch equation. The respective pK values are 4.3 and 4.1, without and with hydroxylamine, respectively. In comparison, we show a titration curve with pK 8.5 (broken line), corresponding to the MI/MII photoproduct equilibrium at 30 °C. B. Extrapolated theoretical difference spectrum of the pure active opsin state Ops* minus the dark state according to the data in A (black spectrum). In comparison we also show the experimental spectrum obtained at pH 4.0 and 30 °C corresponding, according to A, to a mixed state (dashed), and a pure MII spectrum obtained also at pH 4.0, but at 10 °C to prevent decay (gray). C. A peptide mimic of the transducin α-subunit C-terminus stabilizes Ops*. Difference spectra final decay product minus dark state were recorded in the presence of 100 mM hydroxylamine at pH 6.0, 30 °C, either with (solid) or without (gray) a 20-fold excess of peptide (see text for details).
Absorbance change

pH 4.0

A

1768, 1750, 1712, 1687, 1645

pH 7.0

B

1705, 1667, 1658, 1632

MI

1664, 1634, 1539, 1549

+ HA, pH 4.0

D

1204

1 mOD

wavenumber / cm\(^{-1}\)
Conformations of the active and inactive states of opsin
Reiner Vogel and Friedrich Siebert

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