the structures of these compounds is expected to be accompanied by an increase in the use of such compounds for industrial applications mainly as anionic polymerization catalysts, as has already been proven for selected derivatives.[8d, 27] However, we are still at the beginning of a vastly developing chemistry of the heavy homologous Grignard reagents.

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The Light Shall Show the Way—Or: The Conformational Changes of the Retinal Chromophore in Rhodopsin upon Light Activation

Wolfgang Gärtner*

The Visual Pigment Rhodopsin—A Paradigm for the G-Protein-Coupled Receptors (GPCRs)

Only a few molecules can be considered as biochemical "classics" which have also made their way as distinctive examples into biochemistry textbooks. Amongst these is the archetype of visual pigments, bovine rhodopsin.[1] The strong scientific interest in understanding the structure and function of rhodopsin is not only a consequence of its fascinating conversion of absorbed light into a biological signal, but is also a consequence of the fact that it serves as a paradigm for a large and continuously growing number of other receptors with seven transmembrane α-helices. Common to all these membrane-intrinsic receptors is their capability to respond to external stimuli such as other sensory (olfactory and gustatory), hormone-mediated, or neurotransmitter-mediated signals, and to mediate the signal by activating a heterotrimeric G-protein in the interior of the cell.[2]

Scientific investigation of visual pigments started more than a century ago with work by pioneers such as W. Kühne, who demonstrated image formation on the retina-embedded "Sehpurpur"[3] when illuminated patterns were exposed to the eye. Since then, many multidisciplinary approaches have been used to understand the function of visual proteins, and have included the efforts of pharmacologists, biologists, biophysicists, medicians, neurologists, and even theoreticians. *Chemists* became involved in visual research through the identification of the vitamin A aldehyde retinal as the chromophoric principle of visual proteins by George Wald

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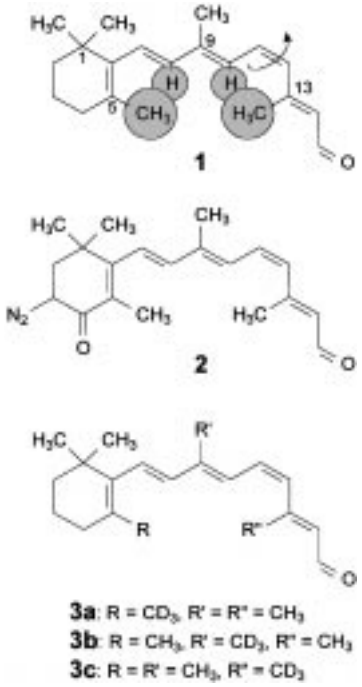
in the early 1930s.[4a] He demonstrated that an 11-*cis* to all-*trans* photoisomerization was the initial reaction of rhodopsin, and was also able to show that—similar under physiological conditions—bleached visual proteins can be reconstituted in vitro by incubation with either the (naturally occurring) 11-*cis* (**1**, Scheme 1) or the 9-*cis* isomer of retinal.[4b]



Scheme 1. Structural formula of 11-*cis*-retinal (**1**) and 3-diazo-4-ketoretinal (**2**), as well as 5-, 9-, and 13-trideuteriomethylretinal (**3a–c**).

The generation of modified chromophores by the then available broad repertoire of retinoid and carotenoid chemistry[5] allowed detailed studies of the precise description of the chromophore conformation and of the interactions between the retinal molecule and the protein moiety.[6] In particular, the wide range of wavelength sensitivity of visual proteins, which span the whole range of the "visible" (sic!) light and are based on only one type of chromophore,[7] represented a challenge which evoked experimental as well as theoretical efforts. The identification of a covalent attachment resulting in the formation of a protonated Schiff base between the chromophore and the protein (Lys296 in bovine rhodopsin) causes a high polarizability in the π-electron system of the polyene chain. This observation was fundamental for Honig and Nakanishi's explanation of the wavelength tuning of visual pigments by an "external two point charge model".[8]

Chromophore–Protein Interactions in Rhodopsin at the Atomic Level

Despite the many attempts to obtain detailed knowledge of the structure and function of rhodopsin by employing chemical, spectroscopic, recombinant, and theoretical techniques, a description of the rhodopsin structure at the atomic level remained elusive for a long time. Only recently a three-dimensional (3D) structure with sufficient resolution was presented[9] which now allows the evaluation and critical revision of many of the results obtained and reported previously.

However, the crystal structure still does not allow a precise description of the interactions between the chromophore and protein, and also can not reflect the dynamics of the conformational changes which both components experience upon light activation. Two recent publications both address this aspect, namely, the conformation of the protein-embedded chromophore in the rhodopsin resting state and its conformational changes during the bleaching process.[10, 11]

Investigation of Chromophore Movements by Cross-Linking Experiments

Borhan et al.[10] make use of the chemically modified retinal derivative 3-diazo-4-ketoretinal (**2**; Scheme 1) to probe the "movement of retinal along the visual transduction path", as their title says. Although the visual transduction still comprises more than just the light-induced reactions of the visual pigment itself, the results give a detailed and unexpected description of the interactions between the chromophore and its protein environment, and allow the conformational changes which the chromophore undergoes upon light excitation to be followed. The data highlighted in the *Science* paper[10] are presented in greater detail in two accompanying publications[12, 13] that deal with the same problem, namely, formation of new covalent bonds between the chromophore and protein by cross-linking during the bleaching process. This study is based on the structure of retinal derivative **2**, which is one of a series of retinoids of this type prepared by Nakanishi and his co-workers. All of these compounds can undergo several different reactions. Besides their ability to form a covalently linkage to the protein through a protonated Schiff base in an identical manner to the native chromophore, these compounds carry a diazo group on the cyclohexene ring as a second reactive group. This diazo group expels nitrogen upon irradiation with UV light and rearranges into a highly reactive carbene moiety, which intercalates into C–H bonds of nearby amino acids. The high reactivity of the carbene results in the covalent bond only being formed directly to adjacent molecules and thus probes the direct environment (for purposes of identification, these cross-linking molecules were furnished with a tritiated aldehyde group (-CTO) to enable the radioactivity in the cross-linked peptides to be followed more conveniently during the sequence analysis of the protein[12]). Thus, these compounds in the opsin-bound form can be activated with respect to their photoisomerization (11-*cis* → all-*trans*) by long-wavelength irradiation, and can then be converted at any time after thermal reactions into the reactive carbene by exposure to ultraviolet light. The authors started their analysis of retinal–opsin interactions by first verifying that the artificial chromophore occupies the binding site of the native chromophore, and showed that the diazo group remains intact in the protein-bound chromophore in the dark. In order to probe physiologically relevant states of the light-activated rhodopsin, the photochemistry of rhodopsin assembled from **2** was initiated at low temperatures where the first stable photoproduct, bathorhodopsin, can be trapped,

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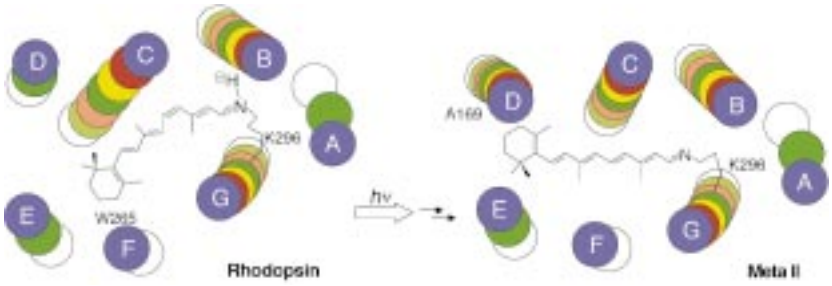
and the resulting products of the reaction pathway can be obtained by a stepwise warming of the sample. By following this well established concept, bathorhodopsin was generated at $-196\,^{\circ}C$ and was then irradiated with UV light to form the carbene retinoid which was then allowed to react with amino acids in its proximity. Similarly, samples were subjected to the bleaching process at temperatures where lumi or alternatively a mixture of meta-I and meta-II rhodopsin can be trapped. The formation of the various stabilized intermediates was proven by difference UV/Vis spectroscopy (intermediate minus the parent state of rhodopsin) prior to the initiation of the cross-linking reaction.

Initiation of the cross-linking reaction in the dark (parent state of rhodopsin without long-wavelength irradiation of the pigment), and also cross-linking in the batho form yielded the same target amino acid, namely Trp 265 in helix F. The position of the cyclohexene ring apparently remains nearly unperturbed, irrespective of the *cis*–*trans* photoisomerization of the chromophore (rhodopsin-to-batho). This finding, according to the authors, is in accordance with other results, such as those from vibrational spectroscopy studies, which indicate that despite adopting the *trans* geometry, the chromophore hardly changes its overall structure, presumably because of the fact that the photoisomerization of the 11,12 double bond is compensated to a large extent by conformational changes of single bonds.

When the protein was transformed into the lumi state, the authors found a surprising new target, Ala 169, which is located in the fourth helix. This result at first glance causes confusion. Following the schematic drawings of the protein as given by the authors (Scheme 2; it should be kept in mind that this publication was submitted, before the rhodopsin structure[9] had been published), the fourth helix (D) in the parent state of rhodopsin is extruded from the chromophore binding pocket by the two helices C and E, which approach each other and are part of the chromophore binding site. Thus, the authors had to propose a remarkable change in the conformation of the chromophore and an even greater one of the protein during the conversion from the batho to the lumi form of rhodopsin in order to interprete their data. In their view, the helices C and E, formerly arranged close to each other, are now pushed aside allowing access for helix D (carrying the later cross-linked amino acid Ala 169). Moreover, as the crystal structure shows, Ala 169 is located on the outward directed side of the rhodopsin protein, and requires not only the movement of the helix towards the reactive site of retinal, but also a rotation around the long axis of the helix. This same amino acid (Ala 169) is also found as the sole modified residue in the meta-I and meta-II forms, which indicates that no gross changes in the conformation of the chromophore occurs during the conversion into these intermediates. The finding of a selective cross-link of an amino acid, formerly believed as being "outward" directed, is a remarkable surprise, even more so when the experimental conditions reported by the authors are taken into account, that is, with only about 50 % of the lumi intermediate generated. It is argued that apparently the movement of the ring site during formation of the lumi selectively directs the reactive carbene towards helix D. The authors claim that all these results from chemical studies, when taken together, indicate a remarkable structural and conformational flexibility of the chromophore and the protein during the light-induced bleaching of rhodopsin.



Scheme 2. Layout of the seven transmembrane helices in lumi- and metarhodopsin according to Borhan et al.[10] (Copyright© American Association for the Advancement of Science 2000).

Characterization of the Chromophore Conformation by Using Solid-State Deuterium NMR Spectroscopy

The publication by Gröbner et al.[11] also deals with the conformation of the chromophore in the resting state and its changes when the pigment is converted into the meta-I intermediate. This paper is the most recent in a series of publications dedicated to the determination of the conformation of the chromophore by NMR spectroscopy.[14, 15] During their studies, the authors have applied solid-state NMR methods to several rhodopsin samples, each reconstituted with selectively deuterium-labeled retinal chromophores. The principle of structure determination by this technique is based on the immobilization of the rhodopsin samples on solid supports (glass plates) within the NMR rotor at a precise angle with respect to the rotor axis. The orientation of the deuterium quadrupole coupling tensor of the deuterated substituents with respect to the membrane normal and the externally applied magnetic field (magic angle oriented sample spinning NMR, MAOSS-NMR) can be identified with this method. NMR analysis of rhodopsin samples assembled with retinals carrying trideuterated methyl groups (CD_3) at positions 5, 9, or 13 (**3a**–**c**, Scheme 1) thus allows the conformation of the chromophore within the binding site and its changes during the photobleaching process to be determined. Gröbner et al.[11] give special emphasis to the movements of the cyclohexene ring during the rhodopsin–metarhodopsin conversion by investigating a 5-CD_3 chromophore. Similar as described by Borhan et al.,[10] the meta-I state was prepared and stabilized below $0\,^{\circ}C$, and the conformation of its retinal chromophore was compared to that of the rhodopsin parent state. On the basis of a previously determined topological arrangement of the polyene chain (derived from 9- and 13-CD_3-containing retinals[14] which yielded angles of 44 and $30 \pm 5^{\circ}$ with respect to the membrane normal for these two substituents), the NMR data obtained with C5-

CD_3-labeled retinal indicate a 6-s-*trans* conformation of the cyclohexene ring for both the rhodopsin parent state and the Meta-I form (a value of $21 \pm 5^\circ$ was determined for the C5–CD_3 bond vector in the parent state, and of about 60° for the meta state). The polyene chain in the rhodopsin parent state exhibits a remarkable twist along its long axis (44 and 30° for the two substituents, see above) and an additional distortion between the cyclohexene ring and the polyene chain. The chromophore adopts a nearly completely relaxed all-*trans* conformation/configuration on arriving at the meta-rhodopsin state. In this intermediate, the bond vectors of all three methyl groups exhibit an angle of $60-65^\circ$ to the membrane normal. These findings indicate that not only the cyclohexene ring, but also the polyene chain of the retinal undergoes conformational changes as a consequence of the photoisomerization of the 11–12 double bond. Besides the finding of a strong distortion of the polyene chain around the 11–12 *cis* double bond, which has already been postulated by other experiments, for example, circular dichroism,[16, 17] to partially compensate the strong steric hindrance between the hydrogen atom at C10 and the methyl group at C13, the twist of the ring moiety and its overall 6-s-*trans* conformation is surprising. A steric interaction between the methyl group at C5 and the hydrogen atom at C8, which was first seen in crystals of retinoids, had also been suggested for the conformation of the chromophore in the rhodopsin binding pocket. Yet, in former studies the cyclohexene ring was assumed to adopt a distorted 6-s-*cis* conformation—this picture was interestingly also derived from solid-state NMR experiments.[18] Gröbner et al.[11] now argue that the former determination of the conformation as 6-s-*cis* had to rely on indirect evidence in which the NMR data from labeled retinal in rhodopsin were compared with model compounds measured in organic solvents, and the signals "were found at the upper end of the region for 6-s-*cis* model compounds, close to the range for the 6-s-*trans* derivatives" (footnote [18] in ref. [11]).

The arrangement of the chromophore in the binding pocket, based on the NMR-derived conformation, places the ring site of retinal as a sandwich between Trp 265 (which was identified by the cross-link experiments, see above) and Tyr 268. Both residues are highly conserved in all opsins. Whereas a conformational change of the ring moiety (from distorted 6-s-*trans*oid to fully relaxed 6-s-*trans*), as proposed from the NMR work, appears feasible, such arrangement would render a rotation of the ring part, as postulated from the cross-linking experiments, highly improbable—a high energy barrier for such motion can be assumed because of the strong interactions between the three ring structures (cyclohexenyl and two aromatic amino acids).

The Three-Dimensional Crystal Structure of Rhodopsin: Consequences for the Proposed Chromophore Conformations

The results of both papers discussed here have to be considered using the knowledge provided by the recently presented structure of bovine rhodopsin.[9] Although the structure is currently available only in the resting (Rho) state at 2.8-Å resolution, the overall arrangement of the seven transmembrane helices is crucial and can not be neglected in the discussion of conformation and conformational changes. Whereas the proximity of the ring part of the chromophore and helix F, which was proposed from both the cross-link and the NMR experiments, is in agreement with the 3D structure, the strong conformational changes of the protein backbone as proposed by Borhan et al. when the receptor arrives at the lumi and meta state have to be discussed more critically. Not only, as outlined above, do the two helices C and E have to move apart from each other and allow helix D to approach the chromophore, but also helix D itself, which carries the cross-linked Ala 169 residue, has to rotate around its long axis, since the target alanine (in the parent state) points away (outwards) from the protein core and the chromophore binding site (Figure 1). The more gradual change of the ring part, as

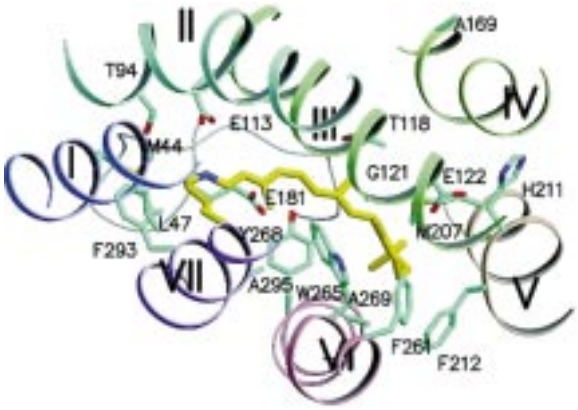


Figure 1. Three-dimensional crystal structure of rhodopsin from Palczewski et al.[9] viewed from the cytoplasmic side of the protein. Note the strong tilt of helix III with respect to the membrane plane (Copyright© American Association for the Advancement of Science 2000).

suggested from the NMR studies by Gröbner et al.[11] (from the formerly believed *cisoid* conformation into the *transoid* structure, and later in the meta state into the relaxed 6-s-*trans* conformation) is more agreeable with the crystal structure (but see below). One expects further NMR studies to be performed which may fill the gap beetween the parent state and the here-presented meta intermediate. Still, one might also expect slight changes of the protein backbone arrangement to be evident in a more refined crystal structure determination with higher atomic resolution, which probably might also indicate whether there is conformational freedom for large structural changes of the protein. It should be kept in mind that a large conformational flexibility is not unlikely for a photoreceptor such as rhodopsin, which has to be absolutely "silent" in the resting state and which undergoes large conformational changes in the cytoplasmic loops to generate a G-protein interaction site upon light activation. In fact, alternative methods can be used to detect conformational freedom in various regions of the protein. Steady-state and even time-resolved EPR measurements have been performed on rhodopsins, which carry spin labels at distinct positions. The most frequently used spin label is the 1-oxyl-2,2,5,5-tetramethylpyrrolidine group which can be covalently attached to cysteine residues, either at their naturally ocurring

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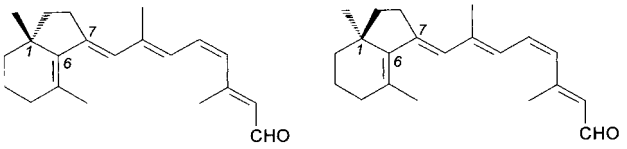
positions in the protein or at different sites by site-directed mutagenesis. This method yields information as to whether a particular domain of a protein gains or loses conformational flexibility during its function, and allows, upon the introduction of more than one label, distance measurements to be performed between adjacent protein regions.[19]

Chemistry Strikes Back

The most recent report of modified chromophores, synthesized again by Nakanishi et al., however, offers a striking new viewpoint on the conformational flexibility between the ring and the chain in rhodopsin-bound retinal: The authors present the results of two retinal derivatives that were prepared in which the 6-s-*cis* configuration is fixed by a methylene bridge between one of the two geminal methyl groups of the ring and the C7 atom of the chain (Scheme 3).[20] This new chemical



Scheme 3. Structural formula of the 11-cis isomers of 7,16- and 7,17-methanoretinal, the 1*R*- (left) and 1*S*-locked (right) analogues of retinal, respectively.

approach does not only allow unambiguous identification of the 6-s-*cis* conformation, but also provides even more subtle information. The authors succeeded in generating the methylene-bridged structure for both methyl groups (which are located below and above the ring plane) to yield the 1*R* and 1*S* enantiomer, of which only one (the 1*R* form) generates a visual pigment upon incubation with rhodopsin. The physiological activity of this new visual pigment, as determined by G-protein activation, reaches about 80 % of that of rhodopsin from natural sources.

It appears that although great progress has been made recently in the elucidation of the structure and function of rhodopsin, the number of unanswered questions and unsolved problems is still growing.

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