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Photoactivated state of rhodopsin and how it can form

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Abstract

A variety of spectroscopic and biochemical studies of the photoreceptor rhodopsin have revealed conformational changes which occur upon its photoactivation. Assignment of these molecular alterations to specific regions in the receptor has been attempted by studying native opsin regenerated with synthetic retinal analogs or recombinant opsins regenerated with 11-*cis* retinal. We propose a model for the photoactivation mechanism which defines 'off' and 'on' states for individual molecular groups. These groups have been identified to undergo structural alterations during photoactivation. Analysis of mutant pigments in which specific groups are locked into their respective 'on' or 'off' states provides a framework to identify determinants of the active conformation as well as the minimal number of intramolecular transitions to switch to this conformation. The simple model proposed for the active-state of rhodopsin can be compared to structural models of its ground-state to localize chromophore–protein interactions that may be important in the photoactivation mechanism.

Keywords: Fourier-transform-infrared; G protein activation; Mutagenesis; Proton transfer; Secondary structure; Visual pigment

1. Introduction

The three-dimensional structural model of bacteriorhodopsin (BR) [1] has served as a widely accepted template for the disposition of the seven putative transmembrane alpha-helical segments predicted for G protein-coupled receptors (Ref. [2] and references therein). In particular, it was inferred that helix segments next to each other in the primary structure are also direct neighbors in the three-dimensional arrangement. In addition, the N- and C-termini were expected to be exposed to the extracellular and cytoplasmic domains, respectively, with the helical axes roughly perpendicular to the membrane. These general features appear still valid according to a recent modeling attempt for G protein-coupled receptors based on a large number of amino acid sequence comparisons [3] and the projection structure of bovine rhodopsin at 9 Å [4]. However, differences between the proposed rhodopsin structure and the structure of the proton pump BR are obvious. Modeling based on the existing projection map combined with experimental data on receptor activation allows a tentative

Abbreviations: BR, bacteriorhodopsin; EPR, electron paramagnetic resonance; FT-IR, Fourier-transform-infrared; G protein, guanine nucleotide-binding regulatory protein; PSB, protonated Schiff base

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assessment of intramolecular processes that may be of functional importance during activation of G protein-coupled receptors [5].

Here we focus on the visual receptor rhodopsin where *cis*-to-*trans* photoisomerization [6,7] of 11-*cis* retinal covalently linked to Lys-296 via a protonated Schiff base (PSB) [8–10] induces the active receptor conformation R^*. R^* is spectroscopically defined as *meta*-rhodopsin II (MII) absorbing maximally at 380 nm. R^* activates transducin [11] the G protein in the photoreceptor cell (for reviews see [12–14]). Recent UV-visible, resonance Raman, Fourier-transform-infrared (FT-IR) and transducin activation experiments on recombinant pigments have led to a better understanding of the photoactivation mechanism of rhodopsin. A critical evaluation of the experimentally observed molecular determinants of dark and light-activated rhodopsin in conjunction with refinement of the hypothetical structural model of rhodopsin should help to design additional mutant rhodopsins to address specific questions regarding the activation mechanism.

In order to facilitate later discussions we wish to emphasize two aspects of the receptor conformation:

(a) an inactive receptor conformation must change to an active conformation which catalyzes nucleotide exchange in the G protein. Correspondingly, any amino acid side chain that is involved in conformational changes may exist in two different steric and/or electrostatic states, which can be designated as 'on' or 'off' depending on whether the particular state is observed in the active or inactive receptor, respectively. According to this terminology, the 11-*cis*-retinal chromophore is in its 'off' state, but switches to the all-*trans* geometry, i.e. the 'on' state, by photoisomerization. The defined distinction is irrespective of whether or not a particular group is part of a G protein interaction domain. For simplicity, this approach neglects photointermediates other than MII as well as subsequent decay reactions of the active receptor conformation;

(b) receptor activation involves conformational changes at different locations. For example, transducin recognizes the active cytoplasmic surface of rhodopsin, whereas the primary photochemical event takes place in the hydrophobic core of the receptor. Therefore, an individual amino acid or a structural domain in the chromophore binding pocket may be in its 'on' state without necessarily locking the transducin interaction domain into its active conformation, and vice versa. It will be proposed below that it is likely that the individual 'off'/'on' transitions form a hierarchy. In the native system a more or less concerted transition of individual groups creates the active conformation and it is not clear whether or not all the observed changes are actually essential for the switching process. A minimal subset of molecular groups may exist that is able to govern the transition to an active receptor conformation irrespective of the binary state ('off'/'on') of all possible constituents usually participating in receptor activation. We define this subset of molecular changes as the activating switch for the particular receptor. Of course, the functional hierarchy becomes obvious only in recombinant, or otherwise modified receptors, in which the 'on'/'off' state of individual amino acid side chains can be specifically influenced, or even be locked, into one of the two defined states. Ideally, it should be possible to alter the receptor activation pathway such that certain transitions become decoupled from other transitions.

In this paper we argue that the simplified binary model of individual molecular transitions provides a framework to analyze functional data obtained from recombinant rhodopsins (Sections 2–4). Experimental results can be related to available structural predictions (Section 5) and may serve as a basis for the design of mutations to specifically address structure/function relationships in rhodopsin.

It should be kept in mind that we define the active conformation as a state capable of transducin binding and activation. Therefore, certain structural constraints of this receptor state, such as the cytoplasmic loop conformation, may actually arise only as a result of interactions with transducin [46]. The features of the so defined active receptor conformation may differ from those of free photoactivated rhodopsin. Likewise, a different assay for receptor activity, for example rhodopsin kinase binding, may lead to a different partition of 'on' and 'off' substates. The recent finding that certain rhodopsin mutants activate transducin constitutively but are not

recognized by rhodopsin kinase demonstrates this point [58].

2. Light-dependent molecular changes in the putative membrane-embedded region of recombinant rhodopsins

According to the approach described above, only molecular changes occurring between dark rhodopsin and the R* state are considered. FT-IR difference spectroscopy has proven to be a well suited technique for the study of such light-induced conformational changes in retinal proteins (for a recent review see Ref. [15]). FT-IR difference spectroscopy measures frequency shifts of only those vibrational modes which are affected during photoproduct formation, irrespective of whether they are caused by the protein constituents or by the chromophore. In contrast, resonance Raman spectroscopy measures specifically chromophore vibrations and thereby helps to define the chromophore geometry and the Schiff base protonation state without the problem of potentially overlapping absorptions of amino acid side chains. Both techniques have contributed significantly to our knowledge of molecular changes occurring during MII formation in native rhodopsin. Application of these techniques to site-directed rhodopsin mutants has allowed the identification of a number of 'on'/'off' states of particular amino acids during R* formation. Among the putative membrane-embedded carboxylic acid groups, light-induced changes of protonation states or hydrogen bond strengths were deduced from characteristic frequency shifts of C=O stretching vibrations of protonated carboxylic acid groups in FT-IR difference spectra. Their assignment to specific Asp or Glu residues was based on the disappearance of specific difference bands in site-directed mutants and revealed that Asp-83 [16,17] and Glu-122 [17] are protonated in both dark rhodopsin

Table 1
Light-induced molecular changes occurring during R* formation at specific groups in the hydrophobic core of photoactivatable recombinant visual pigments and their respective absorption changes

Recombinant pigment	Isomeric state	Charge at residue 113	Charge at Schiff base	H-bonding D83	H-bonding E122	Absorption maximum (nm)
Rhodopsin	11-*cis*/all-*trans*	−/n	+/n	vw/w	m/w	500/380
E122D [a]	11-*cis*/all-*trans*	−/n	+/n	vw/w	w/m	475/380
E122Q [b]	11-*cis*/all-*trans*	−/n	+/n	vw/w	−	480/380
D83N [c]	11-*cis*/all-*trans*	−/n	+/n	−	m/w	495/380
D83N/E122Q [c]	11-*cis*/all-*trans*	−/n	+/n	−	−	475/380
E113D [d]	11-*cis*/all-*trans*	−/n	+/n	vw/w	m/w	505/380
E113Q [e] (alkaline)	11-*cis*/all-*trans*	n/n	n/n	vw/w (?)	m/w (?)	380/380
E113Q [e] (acidic)	11-*cis*/all-*trans*	n/n	+/n	vw/w *	m/w *	490/380
E113A [f] (acidic)	11-*cis*/all-*trans*	n/n	+/n	vw/w *	m/w *	506/380
E113A/A117E [g] (acidic)	11-*cis*/all-*trans*	n/n	+/+	vw/w	m/w	490/474
E113A/A117E/D83N (acidic) [h]	11-*cis*/all-*trans*	n/n	+/+	−	m/w	490/~470
E113Q/A117D [i]	11-*cis*/all-*trans* (?)	n/n (?)	+/+	(?)	(?)	493/?
G90D [j]	11-*cis*/all-*trans* (?)	n/n (?)	+/+	(?)	(?)	483/~470
G90D/E113Q [j]	11-*cis*/all-*trans* (?)	n/n	+/+	(?)	(?)	472/(?)
A292D [k]	11-*cis*/all-*trans* (?)	n/n (?)	+/+	(?)	(?)	488/455
A292E [l]	11-*cis*/all-*trans* (?)	n/n (?)	+/+	(?)	(?)	~490/~460

Only those pigments have been included which activate transducin light-dependently and for which the individual molecular changes have been determined or can be expected based on similarities to phenotypes of mutants for which these changes are established. In the latter case a question mark is put behind the respective symbols. For a given molecular feature the first and second symbol in each cell represents the 'off' and 'on' state, respectively. The 'off' states inhibit and the 'on' states promote transducin activation.
n: neutral; +: positively charged; −: negatively charged; vw: very (C=O stretching frequency 1767 cm^{-1}); w: weak (C=O stretching frequency 1750 cm^{-1}); m: medium (C=O stretching frequency 1745 cm^{-1}); *:data refer to mutant E113A only; ?: no data available; −-: H-bonding of the newly introduced amino acid not yet identified.
[a] Refs. [19,26,27]; [b] Refs. [19,28,17]; [c] Ref. [17], [d] Refs. [18,19]; [e] Refs. [19,28,29]; [f] Refs. [18,19,28,30]; [g] Refs. [22,23,31]; [h] Ref. [23]; the absorption maximum has been reported to be lower by 3 nm in Ref. [21]; [i] Ref. [32]; [j] Ref. [24]; [k] Ref. [27]; [l] Ref. [33].

and MII, whereas Glu-113 is ionized in the dark state and becomes protonated in MII [18].

Table 1 defines the 'on' and 'off' states of five individual molecular changes assigned to specific chemical groups in native rhodopsin that occur upon MII formation. These light-induced changes occur in the retinal chromophore, at the Schiff base imine, at the counterion Glu-113, and in the hydrogen-bonding environment of Asp-83 and Glu-122. The first symbol in each cell in the second row of Table 1 defines the 'off' state of the particular constituent, which is the respective state observed in dark native rhodopsin. Each symbol representing the 'off' state is followed by the abbreviation describing the 'on' states as they occur in the native MII photoproduct. For example, Glu-113 is in its 'off' state when it is ionized in dark rhodopsin, where it serves as the counterion to the protonated Schiff base [19–21]. The 'on' state of Glu-113 is created by protonation of its side chain. Based on data obtained with mutant recombinant pigments, we can address two questions which are motivated by the classification of molecular transitions described in (a) and (b):

(1) Which of the 'on' states are crucial to maintain the active receptor conformation?

(2) Which of the five light-induced changes at specific sites in the receptor structure are essential for switching to the active receptor conformation?

Answering the first question helps to define the molecular determinants of the active receptor, which need not be identical with the sum of individual 'on' states as will be shown below. Answering the second question gives an initial estimate of the proposed hierarchy among the contributions of individual molecular states to the energy barrier between the inactive and the active receptor conformations.

2.1. Possible determinants of an active receptor conformation

Among the recombinant rhodopsins listed in Table 1 a single 'off' state, namely the PSB linkage has been 'stabilized by mutation' and shown to be still compatible with the R^* state. Rows 11–17 of Table 1 describe light-induced molecular changes in recombinant pigments likely to form active receptors with a PSB in contrast to the MII state of native rhodopsin. This has been explicitly shown only for the mutant E113A/A117E [22,23] (the phenotype of this mutant is identical with that of mutant E113A/A117E/D83N; our unpublished results). Therefore, the Schiff base protonation state does not impose a crucial restriction on the steric arrangement of the cytoplasmic surface necessary to bind and activate transducin. Interestingly, a variety of mutations seem to belong to this particular phenotype. They are all characterized by the introduction of a carboxylic acid group at positions usually occupied by a hydrophobic residue. It is likely that this group serves as PSB counterion in the active photoproducts as is suggested by the fact that the double mutant E113Q/G90D forms a blue-shifted photoproduct with a PSB [24]. The newly introduced electrostatic interaction between a PSB and an 'artificial' counterion in the photoproduct does not prevent the active receptor conformation if the charged pair is established between helix three and seven (E113A/A117E) and in another case between helix two and seven (G90D). Obviously, the active conformation of the receptor surface seems to be compatible with a variety of electrostatic Schiff base environments and stabilization of a particular protonation state is not required.

Similarly, the hydrogen bonds at protonated Glu-122 and Asp-83, although undergoing bonding energy changes highly characteristic of the R^* formation [25], cannot be essential to maintain the R^* conformation since replacement of either residue does not affect transducin activation [17]. Both groups seem to be well-suited reporter groups for steric changes at helix two and three without being directly involved in transducin activation or proton transfer reactions. Correspondingly, 'on' states that are crucial for the active receptor conformation have to be looked for among the remaining subset of 'on' states common to *all* photoactivated pigments. Based on the limited number of recombinant pigments listed in Table 1, candidates for molecular states essential for R^* are the all-*trans* geometry of the retinal chromophore and a neutral amino acid side chain at position 113. These are the features that all those pigments have in common for which these molecular states have been monitored. In the case of the PSB photoproducts of mutant G90D and A292E further experiments are needed to elucidate whether Glu-113 is neutral as would be expected if the newly intro-

duced carboxyl groups compete with Glu-113 for electrostatic interaction with the PSB [24].

2.2. Possible molecular switches

Inspection of the data in Table 1 reveals that except for the photoisomerization, other individual 'off'/'on' transitions usually occurring in the hydrophobic core of rhodopsin can be omitted for switching from an inactive to an active conformation. For example, during the UV photoactivation of E113Q [29], neither the charge at residue 113 nor the Schiff base protonation state change between the dark and the light-activated state. Both groups are in their respective 'on' states already in the dark pigment. Consequently, 11-*cis*-retinal efficiently abolishes any conformational change that the neutral states of the Schiff base and the Gln-113 side chain may evoke in favor of an active receptor state. This has been demonstrated by the inactivation of the constitutively active mutant opsin E113Q by the addition of 11-*cis*-retinal as is the case with other constitutively active mutants which can be regenerated with 11-*cis*-retinal [34,35]. The free energy of 11-*cis*-retinal binding obviously offsets the increase in the free energy of the mutant opsin due to incorporation of a neutral amino acid at position 113. Therefore, a sufficiently high energy barrier between the dark and photoactivated state of the 11-*cis*-retinal regenerated rhodopsin is reestablished which prevents measurable dark activity. It is this mutant which also demonstrates that in a given 11-*cis*-retinal regenerated opsin, photoactivation may or may not involve a change of the Schiff base protonation state. Depending on pH, a protonated or an unprotonated 11-*cis*-retinal Schiff base photoisomerizes to the unprotonated all-*trans*-retinal chromophore and forms an active state R^* [29]. Since the opsin is the same in both cases it is reasonable that the active states thus created are identical. This mitigates against an essential involvement of the Schiff base protonation state in the switching mechanism, which is also in agreement with the existence of an active receptor conformation with a PSB as shown for E113A/A117E.

It is an astonishing result that a variety of electrostatic transitions occurring in the retinal binding site in the recombinant rhodopsins listed in columns 3 and 4 of Table 1 are compatible with the light-dependent formation of a cytoplasmic receptor surface that is recognized by transducin and catalyzes nucleotide exchange. The different electrostatic chromophore environments obviously allow spectral tuning of the photoreceptor (column 6 of Table 1) without interfering with the photoactivation mechanism itself. Most surprisingly, molecular substates typical of the inactive receptor can be mutationally stabilized in the hydrophobic core and yet coexist with an active cytoplasmic surface conformation. This has not been anticipated on the basis of biochemical modification of native rhodopsin, showing that Schiff base deprotonation is required for the formation of MII [37]. Similarly, a model which elegantly unifies the possible causes of constitutive activity in a large number of recombinant opsins suggests breakage of a salt bridge between Glu-113 and Lys-296 as a key event in receptor activation [34–36].

Do the more recent mutational data argue against the importance of an electrostatic switching mechanism in the hydrophobic core of rhodopsin or other G protein-coupled receptors? Site-directed mutation has allowed otherwise necessary electrostatic transitions in the hydrophobic domain of rhodopsin to be modified or even abolished. Only four of the 16 theoretically possible transitions among four combinations of Schiff base and Glu-113 protonation states in the dark and photoactivated pigments have been mimicked by recombinant pigments. To date, site-directed mutagenesis has not created a pigment in which the 'off' state of Glu-113 is stabilized in the photoproduct, i.e. no pigments have been described in which Glu-113 is known to be ionized before and after photoisomerization of the retinal chromophore. In perfect analogy to the mutant E113A/A117E, in which Schiff base deprotonation is decoupled from receptor activation, this hypothetical recombinant pigment would answer the question of whether Glu-113 protonation is indeed essential. The available data favor the necessity of a neutral side chain of Glu-113 in the active conformation. This is primarily based on two results: Glu-113 is the Schiff base proton acceptor in MII [17,18] and Schiff base deprotonation is required for R^* formation [37,38]. In addition, mutational incorporation of a neutral side chain at position 113 causes receptor activity in the

absence of chromophore [34–36]. This of course does not imply that Glu-113 has to be ionized in the inactive receptor, as shown by the lack of dark activity of 11-*cis*-retinal regenerated mutants listed in Table 1 rows 8 to 17.

However, if Glu-113 is ionized in a dark pigment as is the case with native rhodopsin, then an electrostatic switch has to exist which neutralizes Glu-113 in order to create a putatively essential determinant of the active receptor conformation. Therefore, it is likely that in native rhodopsin an electrostatic switch at Glu-113 exists in addition to the steric switch provided by photoisomerization. Both are coupled via proton transfer from the Schiff base to Glu-113. Mechanistically, however, the 'on' state of Glu-113 can be anticipated in the inactive receptor without causing dark activity, due to 11-*cis*-retinal being a strong antagonist. This opens the possibility that naturally occurring UV-pigments with a neutral amino acid at the position homologous to 113 may use the same molecular activation mechanism as rhodopsins containing a protonated retinal Schiff base chromophore. The UV-pigments may use the steric switch alone by anticipating the presumably essential 'on' state of the amino acid at position 113 already in the dark state.

The fact that insect UV-pigments form photoproducts with PSB chromophores does not indicate an activation mechanism different from that in rhodopsin. The results with mutant E113A/A117E [22,23] and E113Q [29] show that the Schiff base protonation state per se is an essential determinant neither of the active nor of the inactive receptor, and therefore does not have to be part of a molecular switching mechanism. However, Schiff base deprotonation does participate in receptor activation in native rhodopsin for the reasons explained above.

2.3. Other molecular groups involved in regulation of receptor activity

In the preceding paragraphs we have focused attention on rhodopsin mutants for which light-dependent changes of the protonation state or physical environment of individual amino acids have been characterized by FT-IR-spectroscopy and on mutants that have phenotypes similar to those already studied by FT-IR-spectroscopy. Of course, additional amino acids have been identified in the hydrophobic core of rhodopsin which affect the absorption properties, the photoreaction, and the transducin activation efficiency of rhodopsin [39]. Only a few of these amino acid residues have been suggested to undergo physical or chemical alterations upon MII formation. His-211, although not generally required for MII formation reduces the amount of MII formed in digitonin-membrane micelles and it has been suggested that protonation of His-211 may explain the pH dependency of the MI–II equilibrium [40] described for rhodopsin in disc membranes [12,41–44]. In a functional assay, however, the H211F mutant was found to activate transducin with similar efficiency as wild-type [34] which was not expected on the basis of a MI-like photoproduct absorption spectrum. However, the compatibility of an active receptor conformation with 'off' states of particular molecular groups, renders the UV-spectroscopic characterization of the rhodopsin states ambiguous. Although the photoproducts of the pigments in rows 11 to 17 of Table 1 exhibit MI-like absorption spectra, a MII-like conformation may co-exist as shown for E113A/A117E. Therefore, further experiments which allow more direct assessment of the protein conformation are needed to elucidate the impact of His-211 on receptor conformation.

In a recent FT-IR study, cysteines have been suggested to be implicated in the photoactivation mechanism as well but site-specific band assignment is not yet available [45].

3. Light-induced molecular changes on the cytoplasmic surface

Synthetic rhodopsin-derived peptides have been shown to compete with native rhodopsin for transducin binding [46]. This has allowed the identification of the cytoplasmic loops connecting helix three with helix four and helix five with helix six as well as a putative loop between the cytoplasmic termination of helix seven and the palmitoylated Cys-322 and Cys-323 as transducin binding sites. Site-directed mutagenesis has further characterized groups of amino acids in these regions implicated in transducin binding and activation [47,48]. However, light-dependent alterations of their physical or chem-

ical states are not well characterized rendering a distinction of 'on' and 'off' states of individual amino acid side chains difficult even if the importance of specific amino acids (e.g. the charged pair Glu-134, Arg-135 [49]) for transducin activation is established. Infrared spectroscopic determination of protonation states or hydrogen bonding of specific amino acid side chains on the cytoplasmic surface of rhodopsin is to date less advanced than for the hydrophobic core of the receptor. This is in part due to the relatively small contribution of water exposed amino acids to infrared absorption changes in typical FT-IR hydrated film samples [50].

Other techniques have been more successful to monitor light-dependent structural alterations occurring during MII formation. It has been shown by time-resolved [51] and static electron-paramagnetic-resonance studies [52] on site-specific spin labeled rhodopsin that the cytoplasmic terminations of helices three and seven undergo structural rearrangements in the vicinities of Cys-140 and Cys-316, respectively. These changes have been specifically assigned to the MII conformation. Cys-140 is close to the highly conserved Glu (Asp), Arg, Tyr triad at the cytoplasmic border of helix three (position 134–136 in rhodopsin) which has attracted attention in earlier studies because of its possible general importance for the function of G protein-coupled receptors. It has been shown that replacement of Glu-134 by Gln renders the photoactivated pigment about 8-fold more efficient in activating transducin at alkaline pH than recombinant native rhodopsin [53]. Therefore, it has been suggested that Glu-134 is a good candidate for regulation of the transducin binding region and may undergo a light-induced transition from an ionized to a protonated state [35,53]. Recent measurements of light-induced pH changes in the bulk water phase monitored simultaneously with R^* formation of the mutants E134Q and E134D showed the involvement of Glu-134 [54] in proton uptake reactions [55]. According to these results it is likely that Glu-134 itself is a group which becomes protonated in MII. However, FT-IR difference spectroscopy has to date failed to detect any C=O stretching vibration assignable to the putative protonation of Glu-134 in detergent (our own unpublished results) or reconstituted membranes [16]. The structural change detected by EPR may be directly related to protonation of Glu-134, which is expected to significantly alter the hydrogen-bonding properties of this amino acid. A rearrangement of neighboring hydrogen-bonding partners may then explain the conformational change. There is no straightforward approach to identify surface groups which interact with Glu-134 although the pH profile of transducin activation [53] as well as the abolishment of the uptake of two protons in E134Q [54] suggests the existence of other titratable groups influenced by Glu-134. Since Glu-134 is at the cytoplasmic border of helix three in a region demonstrated to undergo structural alterations upon MII formation, it is not clear from the helical arrangement model which amino acids may interact with Glu-134. Interestingly, His-65 and to a lesser extent His-152 have been shown to affect the receptor conformation since their replacement favors MII formation as measured by 380 nm absorbance [40], and this phenotype is similar to the one reported for Glu-134 mutations [56].

4. Hybrid states of a receptor containing combinations of 'on' and 'off' states of individual molecular groups

The most striking result of the more recent spectroscopic and functional studies on recombinant rhodopsins is the mutationally mimicked coexistence of 'on' and 'off' states of specific amino acid side chains for which light-dependent chemical or physical transitions have been characterized in native rhodopsin. The question arises, how does the transducin activation by the respective R^* states correlate with the counteracting molecular determinants of the receptor conformation? It has already been shown that a PSB is compatible with an active receptor, although transducin activation is reduced by about 30%. Obviously, other 'on' states (including those listed in Table 1) efficiently determine the active conformation, rendering the Schiff base protonation state a weak restriction for the conformation of the transducin interaction domain on the cytoplasmic surface. In analogy, mutational stabilization of an 'on' state may, under certain conditions, enhance transducin activation as has been demonstrated for the photoproduct of E134Q. In agreement with this

notion of a weighted additivity of molecular substates, mimicking the 'on' state of Glu-134 by substituting Gln in a given mutational background is expected to raise transducin activation in the particular system. This has been indeed observed for the pair of constitutively active mutants K296H and K296H/E134Q [35]. Even in the absence of retinal, the cytoplasmic surface seems to maintain at least a partial structural integrity in the sense that mutations within the transducin binding domain change the phenotype in the same way as in the light-activated recombinant pigment. This agrees with a previously suggested partial conformational independence between transitions occurring in the hydrophobic core (photoisomerization and proton transfer to Glu-113) and on the cytoplasmic surface (proton uptake, probably by Glu-134 and a yet unidentified group) [55].

An exciting question is whether this can be exploited to physically or mutationally create even more severe mismatches between 'on' and 'off' states. As shown above, the limited number of individual 'on' or 'off' states, which had been genetically introduced in a dark pigment, were each still weak enough to be offset by the activity-inhibiting potential of 11-*cis*-retinal. Only after photoisomerization was activity observed, although it was modulated by the proposed 'weighted additivity' of individual molecular states (e.g. alkaline hyperactivity of E134Q, or hypoactivity of E113A/A117E). But is the free energy of 11-*cis*-retinal binding large enough to counteract all of the 'on' states of the remaining molecular groups? Since these are not entirely known, this question cannot be addressed by a mutational approach. Physically however, a trivial way to 'switch on' all molecular groups sensitive to MII formation is merely by photoactivation. Due to the free energy of transducin binding to MII at least part of the produced 'on' states can be stabilized by transducin binding (this constitutes the physical counterpart to the more specific and efficient mutational blockade of 'on' states). Therefore, the question posed can be experimentally addressed by reisomerizing the all-*trans* chromophore in transducin-bound MII in order to create the desired mismatch between the isomeric 'off' state 11-*cis*-retinal and a partially stabilized MII conformation. This approach has been applied by Arnis and Hofmann [57] and has revealed that transducin stays bound to the cytoplasmic surface of R^* even if all-*trans*-retinal is replaced by 11-*cis*-retinal by photoconversion.

Although the data in Table 1 suggest that the all-*trans* chromophore and a neutral residue at position 113 may be essential determinants of an active receptor conformation, the photoconversion experiment shows that there is no tight steric coupling between the transducin binding loops and the all-*trans* chromophore geometry once transducin has bound. This agrees well with the existence of a large number of constitutively active mutant opsins carrying amino acids of variable sizes at position 296 [35]. It should be emphasized, however, that this result does not at all contradict the notion of photoisomerization being a necessary switch to reach the conformation of the *free* receptor which allows transducin binding. Only the latter step provides additional energy which introduces intermolecular interactions that may reorganize the hierarchy of intramolecular 'on'/'off' transitions.

Our proposition that amino acid replacements as well as the described photoconversion create hybrid structures of activity-promoting and activity-inhibiting substates rather than mimicking a well-defined conformation of the native receptor (e.g., the conformation of a spectroscopically defined photointermediate of rhodopsin) is paralleled by a recent study on constitutively active opsin mutants. Although Lys-296 replacements allow transducin binding and activation in the absence of chromophore, the corresponding 'active receptor conformation' is not necessarily recognized by rhodopsin kinase [58]. This supports the importance of specific steric interactions of all-*trans*-retinal with the protein environment in native MII (see below).

5. A mechanistic interpretation of specific light-dependent intramolecular changes in rhodopsin based on structural models

The light-dependent alterations of the protonation or hydrogen-bonding states of specific molecular groups in the hydrophobic core and on the cytoplasmic surface, discussed in Sections 2 and 3, allow some conclusions regarding possible activation mechanisms in rhodopsin when combined with structural information. The structural information includes

the recently proposed helical arrangement [3,5] and FT-IR data obtained with retinal analogs, which allowed identification of sterically important regions of the chromophore. A characteristic feature of the projection structure of rhodopsin is the smaller distance between the axes of helices three and five as compared to bacteriorhodopsin where these helices are clearly separated by helix four. Rhodopsin seems to have a more compact arrangement of helix three, four and five than the more elongated projection structure of BR in which an all-*trans* isomer is incorporated. In a first approximation, photoisomerization in rhodopsin may be expected to cause chromophore–protein interactions which introduce steric perturbations near the three–four and/or the four–five helical interface. Although this notion agrees with an earlier suggestion of relative movements among helices three to five [51] or three to six [59,60] it does not necessarily imply global movements of helices. Our hypothesis rather suggests that at these helical interfaces, amino acid side chains may be expected to experience changes in van der Waal contacts or hydrogen-bond strengths upon photoisomerization of the retinal chromophore.

A possible functional importance of the relative positions of helices three and four of rhodopsin is also suggested on the basis of a conserved disulfide bond between both helices on the extracellular receptor domain [61–63] which stabilizes the MII conformation [64]. As to the chromophore, the suggested localization of light-induced steric changes would be expected mostly to affect parts distal from the Schiff base linkage, because the latter is located at the interfaces of helices two, three and seven [3,5,24]. Such a partitioning of crucial protein–chromophore interactions along the retinal is supported by FT-IR studies on rhodopsin regenerated with retinal analogs showing that the steric impacts of the two methyl groups of retinal are not equivalent. The more distal 9-methyl group is essential to induce those conformational changes which give rise to the typical MII absorption changes [65]. Drastic reduction in transducin activation is observed in 9-desmethyl retinal rhodopsin. In contrast, the 13-methyl group which is closer to the Schiff base linkage does not play a crucial role to sustain the MII conformation [66].

Further evidence for the importance of steric interactions distal from the Schiff base comes from FT-IR studies using ring-modified retinal analogs. Increased flexibility, as in 5,6-dihydro [67] or 7,8-dihydro analogs [68] reduces the usually observed torsions along the retinal chain in the batho intermediates at 80 K. In addition, the protein conformational changes observed at temperatures which stabilize the MI or MII intermediates differ from those observed in native rhodopsin. In an extreme case, illumination of opsin regenerated with a retinal analog lacking the β-ionone ring fails to induce the complete set of infrared absorption changes typical of the MII conformation and shows reduced transducin activation [68]. Therefore, the β-ionone ring must transmit important steric changes to the protein. According to the proposed model of light-induced steric perturbation at the helix three–four interface and the proposed anchoring of the β-ionone ring near helix four, one would expect some effect of ring modifications on infrared absorption changes from amino acids at the helix three–four interface. The model proposed by Baldwin [3,5] locates Glu-122 at the helix three–four interface. The infrared absorption change assigned to the reduction of hydrogen bonding of Glu-122 upon MII formation was indeed not observed in rhodopsin regenerated with a retinal analog which lacks the β-ionone ring, whereas Asp-83 showed a normal absorption change. The proposed vicinity of the ionone ring and Glu-122 is also in agreement with resonance Raman data from mutant E122Q [28]. Interestingly, infrared absorption changes of Glu-122 occur prior (in MI) to those of Asp-83 (in MII). This is even more pronounced in mutant E122D which exhibits hydrogen bond alterations of Asp-122 already in the batho intermediate at 80 K when most of the protein conformation is thermally fixed. This suggests even tighter steric coupling of Asp-122 to the primary photochemical event than is the case for Glu-122 [26].

Taken together it seems likely that the primary steric effect of photoisomerization is mainly localized at the helix three/four interface where it is sensed by Glu-122. Ensuing thermal relaxation would transmit the steric alteration to the cytoplasmic surface and may thus explain why a structural change upon MII formation is particularly observed in the loop connecting helices three and four [51,52].

6. Conclusions

The combination of well-established spectroscopic methods with site-directed mutagenesis has revealed an unexpected variety of active receptor states created by photoisomerization of 11-*cis*-retinal regenerated mutant opsins. Some mutants display hybrid states in which features of the active receptor conformation are anticipated before photoactivation. Other mutants represent the complementary case. The efficiency of transducin activation seems to be roughly determined by a weighted sum of individual molecular substates which promote and inhibit activity. Among these, retinal isomerization contributes through steric interactions to overcome the energy barrier between the dark and photoactivated pigment conformation, whereas the protonation changes of Glu-113 and most likely Glu-134 are expected to determine the amount of electrostatic energy contributions. A specific interaction of the β-ionone ring and the 9-methyl group of all-*trans*-retinal with amino acids at the helix three–four interface seem to be important to induce proton transfer from the all-*trans* PSB to Glu-113 as a prerequisite for transducin activation. However, the decoupling of electrostatic changes in the retinal binding pocket from the photoactivation process in a number of recombinant pigments calls for additional experiments to elucidate the actual role of Glu-113 protonation for activation of native rhodopsin.

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