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Role of Non-covalent *β*-Ionone-ring Binding Site in Rhodopsin: Historical and Physiological Perspective

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Abstract

Bleached rhodopsin regenerates by way of the Schiff base formation between the 11-*cis* retinal and opsin. Recovery of human vision from light adapted states follows biphasic kinetics and each adaptive phase is assigned to two distinct classes of visual pigments in cones and rods, respectively, suggesting that the speed of Schiff base formation differs between iodopsin and rhodopsin. Matsumoto and Yoshizawa[1] predicted the existence of a β-ionone ring-binding site in rhodopsin, which has been proven by structural studies. They postulated that rhodopsin regeneration starts with a non-covalent binding of the β-ionone ring moiety of 11-*cis*-retinal, followed by the Schiff base formation. Recent physiological investigation revealed that non-covalent occupation of β-ionone ring binding site transiently activates the visual transduction cascade in the dark. In order to understand the role of non-covalent binding of 11-*cis*-retinal to opsin during regeneration, we studied the kinetics of rhodopsin regeneration from opsin and 11-*cis*-retinal and found that the Schiff base formation is accelerated $\sim 10^7$ times compared to that between retinal and free amine. According to Cordes and Jencks[2], Schiff base formation in solution exhibits a bell-shaped pH dependence. However, we discovered that the rhodopsin formation is independent of pH over a wide pH range, suggesting that water solvents do not have access to the Schiff base milieu during its formation. According to Hecht *et al.*[3] the regeneration of iodopsin must be significantly faster than that of rhodopsin. Does this suggest that the Schiff base formation in iodopsin is favored due to its structural architecture? Iodopsin structure once solved would answer such question as how molecular fine-tuning of retinal proteins realizes their dark adaptive functions. In contrast, bacteriorhodopsin does not require occupancy of a distinct β-ionone ring-binding site, enabling an aldehyde without the cyclohexene ring to form a pigment. Studies of regeneration reaction of other retinal proteins, which are scarcely available, would clarify the molecular structure-

phenotype relationships and their physiological roles.

1. Background

Dark adaptation assessed by recovery of visual threshold in humans follows biphasic kinetics for which two distinct classes of retinal proteins, iodopsin in cone cells and rhodopsin in rod cells, are responsible[3]. Both rhodopsin and iodopsin are prototypical retinal proteins consisting of 11-*cis* retinal (11-*cis*-R) and apoprotein moiety opsin, which are covalently linked through Schiff base between 11-*cis*-R and the ε-NH$_2$ group of opsin polypeptide. In order for these visual pigments to function as a photon sensor, the once hydrolyzed Schiff base during visual excitation needs to be reestablished through a process called regeneration, a re-synthesis of the Schiff base. It is likely that the biphasic recovery of visual threshold reported by Hecht *et al.*[3] reflects the kinetic parameters of the two distinct visual pigments during regeneration. These opsins are encoded by distinct genes[4, 5] and, therefore, their expression must be responsible for the difference in the recovery of visual threshold.

Rhodopsin consists of 11-*cis*-R attached to the ε-amino group of lysine in opsin[6]. The regeneration of visual pigments, both rhodopsin and iodopsin, is a biochemical process in which Schiff base forms between the ε-amino group of lysine in opsin and the C15 aldehyde group of 11-*cis*-R. Since retinal has 4 carbon-carbon double bonds in the polyene side chain, there are 16 potentially existing isomers among which all-*trans*, 11-*cis*, and 9-*cis* isomers were established when George Wald's group initially characterized them and their binding to opsin[7]. Based on the fact that certain groups of retinal isomers and analogs form pigments with opsin, while others do not, Matsumoto and Yoshizawa[1] conjectured that opsin recognizes the longitudinal dimension of the ligand and predicted the existence of a β-ionone

ring binding site in an hydrophobic pocket in opsin , and proposed a rhodopsin regeneration

mechanism occurring in two discrete steps:

Step 1: Fast non-covalent binding of 11-*cis*-R to the β-ionone ring-binding pocket

$$\text{Opsin + 11-}cis\text{-R} \leftrightarrow \text{opsin·11-}cis\text{-R non-covalent complex} \qquad (1)$$
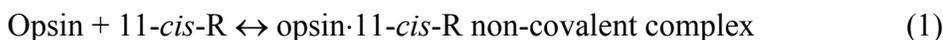
Step 2: Slow formation of Schiff base between the retinal -CHO and the ε-amino group of the

lysine

$$\text{Opsin·11-}cis\text{-R non-covalent complex} \rightarrow \text{rhodopsin} \qquad (2)$$

Almost a quarter of a century later the existence of the β-ionone ring-binding site in a

hydrophobic pocket in rhodopsin has been confirmed by structural studies[8, 9]. Furthermore,

the occupation of the β-ionone binding pocket with a proper ligand including all-*trans*-R was

shown to activate visual transduction[10, 11], implying the physiological role of the β-ionone

binding pocket in visual excitation.

In the past decades, it was well established that retinal proteins including visual

pigments rhodopsin and iodopsin represent a prototypical protein family that led to an

evolutional development of GPCR family proteins that evoke cellular signaling essential in the

maintenance of life[12]. In contrast to visual pigments in which an inverse agonist ligand

always occupies the binding site in a resting state, GPCRs dynamically bind their ligands to

initiate cellular signals. In this regard the visual pigment regeneration is a step equivalent to

that of ligand-binding step in other GPCR proteins. Therefore, comprehensive understanding of

the regeneration mechanism of visual pigments would assist us to elucidate the activation of

GPCRs by various ligands in detail.

2. Hypothesis and prediction: ***The non-covalent complex formation between 11-cis-R and***

opsin accelerates the following Schiff base formation in rhodopsin regeneration

Our hypothesis is based on the fact that opsin forms a visual pigment with only a subgroup of 16 isomers of retinal with a certain range of longitudinal lengths[13], suggesting that the non-covalent complex formation shown in Equation (1) restricts the Schiff base formation so that it proceeds only if the proximity and the orientation of the atomic groups involved in the Schiff base formation are optimized.

Assuming fixed bond angles and bond distances based on known crystallographic data of retinal, we evaluated the longitudinal lengths of 16 potential isomers of retinal (Fig. 1). (INSERT FIGURE 1 HERE) Although this is a rough estimation, the relative ratios of the longitudinal lengths between each isomer appear to be well represented. In Fig. 1A, all 16 isomers are drawn on a hexagonal grid and known retinal isomers that form pigments are indicated. In Fig. 1B, all the mono-*cis* and all-*trans* isomers are drawn overlapped, illustrating that the longitudinal lengths of 7-*cis*, 9-*cis*, and 11-*cis*, but not those of 13-*cis* and all-*trans*, would fall in a proper range, enabling the following Schiff base formation. An erroneous prediction that the 7,9-dicis isomers may not form a pigment because it belongs to the 13-*cis* isomeric group[13], but in fact it does[14], has been interpreted based on the crystal structure of methyl 7,9-dicis retinoate because the longitudinal length of 7,9-*dicis* isomer in the crystal is shortened by the twisted polyene chain at carbons C7-C10[15].

With availability of structural information of rhodopsin and its intermediates the first approximation on the longitudinal length of retinal chromophore in various configurations can now be evaluated based on structural data. Thus we calculated the distance between C6 from which the polyene chain extends to C15 at which the retinylidene Schiff base is formed with the ε-amino group of lysine based on PDB data; 1U19 (Rhodopsin)[16], 2PED

(Isorhodopsin)[17], 2G87 (Bathorhodopsin)[18], and 3PX0 (Metarhodopsin II)[19], which is

shown in Table 1.

(INSERT TABLE 1 HERE)

The results in Table 1 support our early conjecture[13] that the longitudinal restriction

of retinal binding site of rhodopsin determines the potency of retinal isomers to form pigments,

or "figments" as Allen Kropf designated at the 1st Gordon Research Conference on "Physico-

Chemical Aspects of the Visual Transduction Process" held in New Hampshire in 1978.

Namely, the C6-C15 distances in rhodopsin and isorhodopsin are 10.0 Å and 9.9 Å,

respectively. The longitudinal polyene chain dynamically expands to 10.3 Å and 11.0 Å in

bathorhodopsin and metarhodopsin II, respectively, after excitation is triggered. The

reevaluation of the longitudinal length of retinal isomers based on their crystal structures

strongly support our early assumption that rhodopsin regeneration follows a transient non-

covalent formation of opsin·11-*cis*-R non-covalent complex between its cyclohexene ring and a

hydrophobic cavity of opsin.

3. Assay system of rhodopsin regeneration

We assumed that rhodopsin regeneration follows a transient non-covalent formation of

opsin·11-*cis*-R complex between its cyclohexene ring and a hydrophobic cavity of opsin. In

order to prove our hypothesis we measured the second order rate constants (k_2) of rhodopsin

regeneration from 11-*cis*-R and opsin and compared the results with a model retinylidene

Schiff base formation. Bovine opsin was prepared by conventional protocol[20]. Because

digitonin slows the rhodopsin regeneration at concentrations higher than its critical micelle

concentration (ca 0.1%), the opsin extract was diluted by a factor of 41 into the reaction buffer

at each pH in order to reduce the digitonin concentration to 0.012%. Reaction mixture (total

volume, 2.06 mL) contained 1 μM opsin and 7 μM 11-*cis*-R as final concentrations. The reaction was started by adding 11-*cis*-R dissolved in 10 μL of ethanol. Ethanol at this concentration did not affect rhodopsin regeneration. After the addition of 11-*cis*-R, the absorption spectra were measured[21] (Fig. 2): Spectrum 1, after the completion of regeneration reaction. Spectrum 2, after the addition of 10 mM NH_2OH. Spectrum 3, after complete bleaching of rhodopsin by orange light. Note that the difference spectra between 1 and 2 represent the random Schiff base between 11-*cis*-R and unidentified amines.

(INSERT FIGURE 2 HERE)

The results shown in Fig. 2 indicate that rhodopsin regenerates nearly 100% throughout the pH range examined (pH 5-10). The results of the experiments illustrated in Fig. 2 are plotted in Fig. 3. The plot at 530 nm (closed circles) shows that rhodopsin regenerates 100% independent of the pH of the reaction mixture. However, if one plots the absorbance increase at 480 nm (which is close to the absorption maximum of rhodopsin 498 nm thereby representing rhodopsin formation well), the regeneration kinetics would not be observed properly because the Schiff base formation between 11-*cis*-R and unidentified amines contribute significantly at this wavelength. Note that absorption "a" represents the real amount of regenerated rhodopsin, whereas absorption "b" represents the contribution of the unidentified random Schiff base at 480 nm. This is illustrated in Fig. 3 by plotting the absorbance ratio b/a, which shows that the random Schiff base at 480 nm interferes severely with the true kinetics of rhodopsin regeneration, whereas the absorbance measurement at 530 nm escapes errors.

(INSERT FIGURE 3 HERE)

4. The second order rate constant k_2 of rhodopsin regeneration is independent of pH

Using the assay system described in Fig. 2, the second order rate constant, k_2, of rhodopsin regeneration was measured under different pH conditions. Combined representation of equations (1) and (2) can be justified because the intermediary formation of opsin·11-*cis*-R non-covalent complex represented by equation (1) is likely to occur significantly faster than the formation of rhodopsin represented by equation (2). Thus the overall rhodopsin regeneration can be analyzed by equation (3) shown below where k_2 is an apparent second order rate constant involving [opsin] and [11-*cis*-R].

$$\text{Opsin} + 11\text{-}cis\text{-R} \xrightarrow{k_2} \text{Rhodopsin} \qquad\qquad (3)$$

The observed k_2 values shown in Fig. 4 indicate that the retinal Schiff base formation in rhodopsin occurs at a constant rate independent of pH of the reaction mixture. The pH independence further suggests that water solvents in the reaction mixture do not have access to the milieu of the retinal-binding pocket of rhodopsin during the Schiff base formation. We suspected that, because of the complex formation between opsin and 11-*cis*-R, the following covalent formation of Schiff base is likely to be accelerated. Therefore, we compared the Schiff base formation in rhodopsin and that in solution.

(INSERT FIGURE 4 HERE)

5. Second order rate constant k_2 of a model retinylidene Schiff base in solution

We measured Schiff base formation between all-*trans*-R and N-α-acetyl-L-lysine in a model system and compared that of rhodopsin regeneration[21, 22]. In Fig. 5 the spectra of all-*trans* retinal and its Schiff base formed with N-α-acetyl-L-lysine (Fig. 5A) and its formation kinetics (Fig. 5B) are shown. The experimental details are described in the figure caption. From these results we calculated the second order rate constant k_2 of a model retinylidene

Schiff base in comparison to that corresponding to k_2 in rhodopsin regeneration: The mean and standard deviation were calculated to be 6100 ± 300 mol^{-1} s^{-1} (25 °C, pH 5–10) with the confidence of p < 0.01 calculated from Fig. 4):

A model retinylidene Schiff base; $k_2 = 5.7 \times 10^{-4}$ M^{-1} s^{-1} (25 °C in 22% ethanol/water, pH8.2) and

Rhodopsin; $k_2 = 6100$ M^{-1} s^{-1} (25 °C in 0.1% digitonin/water, over the range of pH5-10).

(INSERT FIGURE 5 HERE)

These results indicate that opsin catalyzes/accelerates the formation of retinylidene Schiff base in rhodopsin approximately 10^7 times compared to that in solution. The acceleration of k_2 is likely due to the non-covalent complex formation, which enables a proper proximity and alignment of the aldehyde -CHO group of retinal to the ε-amino group of active lysine. Elaborated model experiments in which the formation of retinylidene Schiff base in various micelle systems were reported by Cooper *et al.*[23], the results of which illustrate a slow formation of model Schiff base consistent with our results.

6. Discussion

Additional studies on the inhibitory action of cyclohexenyl analogs upon the regeneration reaction of rhodopsin in vitro

Additional evidence to support the existence of *β*-ionone ring binding site in opsin had been added later by two other groups[24, 25], further supporting the original hypothesis by Matsumoto and Yoshizawa[1].

Regeneration of rhodopsin and iodopsin in vitro and in vivo

Regeneration of chicken iodopsin *in vitro* occurs more than 500 times faster than that of rhodopsin[26]. More recently, Shichida's group investigated why such a huge difference in regeneration rate exists in the context of the three dimensional structure of rhodopsin[27, 28]. According to their account the glutamic acid-122 in rhodopsin contributes to the deceleration of its regeneration reaction. Thus, if the polar glutamic acid residue at 122 were eliminated, rhodopsin regeneration would be accelerated to the level of that of iodopsin. This notion was supported by an experiment in which the glutamic acid-122 of chicken rhodopsin was mutated into glutamine (E122Q), resulting in significant acceleration of regeneration reaction. The suppression of k_2 in wild-type rhodopsin with glutamic acid-122 and the release of suppression, *i.e.*, acceleration, by the E122Q mutation could be due to a diminished population of the opsin·11-*cis*-R non-covalent complex shown in equations (1) and (2). A further examination of the E122Q mutant rhodopsin would clarify the mechanism. In the *in vitro* experiments in which artificial pigments are studied, reasons why certain groups of retinal analogs and isomers can form a pigment, although at substantially slow rates and yields, should be reevaluated in the context that the pigment formation follows the two distinct steps. For example, a retinal analog lacking the methyl group at the C5 position forms a pigment at an approximately 100 times slower rate[29]. The reason why the removal of a methyl group from the *β*-ionone ring could be understood if the two distinct steps involved in rhodopsin regeneration are evaluated separately. Retinal analog studies suggest that as long as the *β*-ionone ring binding-site can accommodate the chemical structure and the formation of non-covalent retinal-opsin complex is formed in favor of the following Schiff base formation, a retinal analog can form a pigment; for example, open-ring analogs[30, 31] and α-analogs[32].

In vivo, whether the difference of regeneration rates between iodopsin and rhodopsin is responsible for determining the visual threshold as observed by Hecht *et al.*[3] needs careful

evaluation because the supply of 11-*cis*-R for regeneration differs between cones and rods *in vivo*. Both mechanisms, *i.e.*, acceleration of regeneration controlled by the supply of available 11-*cis*-R and acceleration attributed to the intrinsic property of the retinal-binding site structure, are not mutually exclusive.

Role of β-ionone ring-binding site in excitation process

In contrast to vertebrate visual pigments, bacteriorhodopsin (bR) does not require occupancy of a cyclohexene (β-ionone ring) binding site[33]. However, there is a clear preference of a 6-s-*trans* conformer over a 6-s-cis conformer in bacteriorhodopsin[34], suggesting that there is some ring recognition or constraint rather than a nonselective cavity. Nonetheless, bR regeneration is likely to start from binding of retinal into a crevice from the polyene chain (-CHO) side, not from the cyclohexene (β-ionone) end[35]. The structural difference between visual pigments and bacteriorhodopsin, *i.e.*, the former has a β-ionone ring-binding site, whereas the latter does not, could be reflected in the fast kinetic phases of both retinal proteins during photoisomerization. In order to explain molecular basis for fast kinetics of 11-*cis* to all-*trans* isomerization of rhodopsin, two models "bicycle-pedal model" [36] and "hula-twist model"[37] have been proposed. These models are based on the idea that the both ends, *i.e.*, protonated Schiff base and the β-ionone ring of retinal chromophore, are tethered in the retinal-binding pocket. The restriction of the retinal chromophore in the hydrophobic pocket appears to enable highly efficient and sensitive photo-isomerization in rhodopsin. In fact, the 11-*cis* to all-*trans* isomerization of rhodopsin occurs at an extremely high speed, within ~200 femtoseconds, at room temperature[38]. In contrast, the photoisomerization in bacteriorhodopsin (in this case, all-*trans* to 13-*cis* conversion) takes place at a slightly slower rate in the range of 0.5 picoseconds, i.e., 500 femtoseconds[39]. We interpret the difference in

the speed of conjugated polyene isomerization to arise from a lack or loose tethering of chromophore at the both ends in the case of bacteriorhodopsin. Logunov *et al.* [40] reported a 2.5-4 psec transition in absorption changes of protonated retinal Schiff base in 13-*cis* or all-*trans* conformation, which is consistent with the fluorescent transition data of protonated 11-*cis* retinal isomer in methanol reported by Kandori *et al.* [41] and represents the isomerization process in organic solvents. Thus the protein moiety of bacteriorhodopsin catalyzes the photoisomerization by the factor of at least 10 compared to that in solution. In the case of protonated 11-*cis* isomer Kandori et al. observed two fluorescent decay components [41]; the fast femtosecond (90-600 fsec) and the slow picosecond (2-3 ps) components. Kandori *et al.*[41] suggested that the fast femtosecond component is due to inhomogeneous distribution of the protonated retinal Schiff base in the ground state. However, Logunov *et al.*[40] argued that there are other possibilities for the interpretation of the fast femtosecond component. Nonetheless, these results are consistent with our idea that the tethering of retinal molecules in rhodopsins affects the physicochemical properties of retinal photoisomerization and catalyzes it toward acceleration.

Elegant works[18, 42] by Nakamichi and Okada in which difference electron density maps of crystals were collected between rhodopsin, bathorhodopsin and lumirhodopsin clearly snapshot the role of the β-ionone ring-binding pocket of rhodopsin in the early excitation process: In the transition from rhodopsin to bathorhodopsin the difference electron density maps imply that the both ends of the 11-*cis*-R are tightly tethered[18], apparently causing a hindrance within the bathorhodopsin chromophore, and this structural hindrance will eventually be relaxed thermally in the following lumirhodopsin stage by the departure of the cyclohexene ring from the original binding location in rhodopsin and bathorhodopsin[42].

The retinal-binding pocket consists of the β-ionone ring-binding pocket buried deep in the cavity surrounded by a bundle of hydrophobic α-helices and the molecular milieu near the retinylidene Schiff base. The molecular environment of the retinal-binding pocket exhibits significant variation between different visual pigment species and bacteriorhodopsin, rendering unique physicochemical properties such as pKa of the protonation of Schiff base and its dissociation constant. For example, while the retinylidene Schiff base of bovine rhodopsin is completely shielded from the titration[43], those of octopus rhodopsin (pKa =10.4) and Gecko cone pigment (pKa =9.9), can be titrated[44]. The retinylidene Schiff base in iodopsin is exposed to solvent milieu and its chromophore is exchangeable with 9-*cis* retinal added in the solution[45]. The dissociation of retinylidene Schiff base appears to be a general property of cone pigments because it was also reported in Gecko P521 pigment[46].   The dissociable nature of retinylidene Schiff base in iodopsin renders the accessibility to chloride ions added to the solvent in vitro[47]. The dissociable property of cone pigments caused by the different molecular milieu in the retinal-binding pocket may contribute to fundamental difference in response properties between rods and cones[48]: cones are 25- to 100-fold less sensitive to light than rods and their photoresponses are several fold faster in kinetics, which is attributed to such difference in the dissociable property of retinylidene Schiff base of cone pigments.

Role of the β-ionone ring-binding site in the adaptation process; the relevance to visual threshold data

Kefalov *et al*.[11] reported the differentiated roles of the non-covalent binding of 11-*cis*-retinal to opsin in the dark between rods and cones of salamander. They conclude: "In rods, 11-*cis*-retinal produces a transient activation of the phototransduction cascade that precedes sensitivity recovery, thus slowing dark adaptation. In cones, 11-*cis*-retinal

immediately deactivates phototransduction. Thus, the initial (non-covalent) binding of the same ligand to two very similar G protein receptors, the rod and cone opsins, activates one and deactivates the other, contributing to the remarkable difference in the rates of rod and cone dark adaptation." Whether or not the difference in the adaptive properties between rod pigment and cone pigment observed in salamander photoreceptors is applicable to all vertebrate retinae remains to be determined; for example, two distinct sets of parameters, for example, one that originates in the β-ionone ring-binding site structure and the other in the downstream transduction cascade, could affect the adaptation process. One such example has been reported[49]. Also, with the same retinal-binding pocket, a case in which a different retinal analog modified in the ring structure with 4-OH substitution affected the adaptation parameters was reported[50]. Nonetheless, the behaviors of salamander rod-cone system, if applicable to humans, would contribute significantly to quicker recovery of photopic (cone) vision threshold that Hecht *et al*.[3] reported in 1935. Future investigations should be based on structural data on overall retinal binding pocket consisting of the *β*-ionone ring-binding site buried deep and the molecular milieu surrounding the protonated retinylidene Schiff base. The differences of the molecular parameters among visual pigment species should result in distinct physiological responsiveness between rods and cones.

In summary, the existence of a *β*-ionone ring binding site in a prototypical GPCR receptor rhodopsin that was predicted in 1975 has been confirmed by structural works reported early this century. Dark regeneration of rhodopsin from 11-*cis*-R and opsin is likely to involve a non-covalent complex. The existence of the non-covalent complex likely accelerates the Schiff base formation via the proximity and proper alignment of the retinal -CHO and the ε-amino group of lysine-296. Participation of the non-covalent complex in photoreceptor transduction is shown by the series of works by Kefalov *et al*.[10, 11]. Curiously, the non-

covalent occupation of 11-*cis* R in the *β*-ionone ring-binding pocket exerts opposite effects on the photoreceptor transduction cascade, slowing the dark adaptation in rods while accelerating in cones. The acceleration of retinal Schiff base formation via the non-covalent 11-*cis*-R·opsin complex *in vitro* and the activation/inactivation of photoreceptor transduction cascade by the occupation of *β*-ionone ring binding site in the complex *in situ* illustrate a beautiful synergistic action in the dark adaptation that has been achieved in the long evolutionary history of vertebrate photoreceptor transduction. Moreover, the *β*-ionone ring-binding pocket appears to contribute in the excitation process by tethering the both ends of the elongated conjugated polyene side chain of retinal chromophore thereby enabling extremely rapid photoisomerization *in vivo*. As more structural data become available on retinoid proteins including visual pigments, inquiries on the role of retinal binding site in physicochemical events, biochemical reactions and their physiological manifestations could be understood thoroughly in the future.

Acknowledgment

Figure Legends

Fig. 1: A. Active retinal isomers, which can form photosensitive pigments in combination with opsin, are marked with a superscript circle (°), and inactive ones with a cross (+). Other isomers have never been examined. All the active retinal isomers except 7,9-*dicis* retinal can be classified into two groups, 10.1 Å (11-*cis* and 7-*cis* groups) and 9.78 Å (9-*cis* group) in

terms of the longitudinal length *l*. Thus the reactive group has the same or a smaller value in *l* compared to that of the active 11-*cis* group. In contrast, inactive isomers have much greater *l* values: all-*trans* (12.4 Å) and 13-*cis* (11.0 Å) retinal. C15 of each isomer group is designated P1 (all-*trans*), P2 (13-*cis*), P3 (11-*cis*), P4 (9-*cis*), and P5 (7-*cis*), respectively. The number in the parenthesis indicates the relative longitudinal length of each isomer group normalized to 11-*cis* retinal group. **B.** All the mono-*cis* and all-*trans* isomers are superimposed for comparison. Reproduced from reference 14 with the publisher's permission.

Fig. 2: Rhodopsin regeneration under different pH conditions. The absorbance of both rhodopsin (designated "a") and a random Schiff base (designated "b") contribute to the absorbance at 480 nm. Rhodopsin regeneration was monitored at 530 nm (shown by arrow) at which wavelength the contribution of a random Schiff base is negligible. Spectrum 1, after the completion of regeneration reaction. To achieve the completion we waited at least 60 min before we proceeded to the measurement of spectrum 2. Spectrum 2, after the addition of 10 mM NH_2OH. Spectrum 3, after complete bleaching of rhodopsin by orange light. Reproduced from reference 22 with the publisher's permission.

Fig. 3: pH dependence of the formation of random Schiff base and the regenerated amount of rhodopsin. The ratio b/a indicates the contribution of the random Schiff base and rhodopsin, respectively at 480 nm. Note that b/a diminishes to zero as pH increases to the alkaline region because the random Schiff base becomes deprotonated, the pK value of which was 8.65. Reproduced from reference 22 with the publisher's permission.

Fig. 4: k_2 is independent of pH. The mean and standard deviation are 6100 ± 300 M^{-1} s^{-1} (25°C, pH 5-10), p<0.01. Reproduced from reference 22 with the publisher's permission.

Fig. 5:   Determination of k_2 for the retinylidene Schiff base in a model system in a reaction between all-*trans* retinal and N-α-acetyl-L-lysine in aqueous solution. A. Spectra of the components are illustrated. Curve 1, 13 μM all-*trans* retinal in 78 mM borate buffer (pH 8.2)/22% ethanol in a total volume of 2.3 ml. Curve 2, 120 min after adding 0.2 ml of 1M N-α-acetyl-L-lysine, the spectrum of the solution in equilibrium was measured. Curve 3, in order to protonate the retinylidene Schiff base a drop (ca 50 μl) of concentrated HCl was added and the spectrum was recorded. B. Dependence of the retinylidene Schiff base formation at three different concentrations of N-α-acetyl-L-lysine all of which were excess over the concentration of all-trans retinal by the factor of larger than 10^3. Under the conditions the reaction followed pseudo-first order kinetics. The retinylidene Schiff base formation was measured by monitoring the absorbance increase at 500 nm, which is likely to involve formation of both of its unprotonated and protonated forms. The y-axis represents the percentage of remaining all-*trans* retinal after being converted to retinylidene Schiff base normalized to the equilibrium state; $y=100(A_{eq}-A_t)/(A_{eq}-A_0)$ where A_{eq} represents the absorbance at 500 nm when the reaction became equilibrated, A_t represents the absorbance at 500 nm at time = t, and A_0 represents the absorbance at 500 nm at t = 0. The pseudo-first order rate constant of retinylidene N-alpha-acetyl-L-lysine formation (k_{pseudo}) (under the N-alpha-acetyl-L-lysine excess) depended on the concentration of N-alpha-acetyl-L-lysine as shown in B. The regeneration rate of the model Schiff base k_2 was calculated from the tangent of the line when we plotted k_{pseudo} against [N-alpha-acetyl-L-lysine] (not shown).

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Fig. 1

A

B

Fig. 2

Fig. 3

Fig. 4

phosphate (–▲–), MES (–○–), HEPES (–●–) and TAPS (–□–)

Fig. 5

A.

B.

[N-α-acetyl-L-lysine]
●—●: 2.0 x10⁻² M
X—X: 2.5 x10⁻² M
○—○: 5.0 x10⁻² M
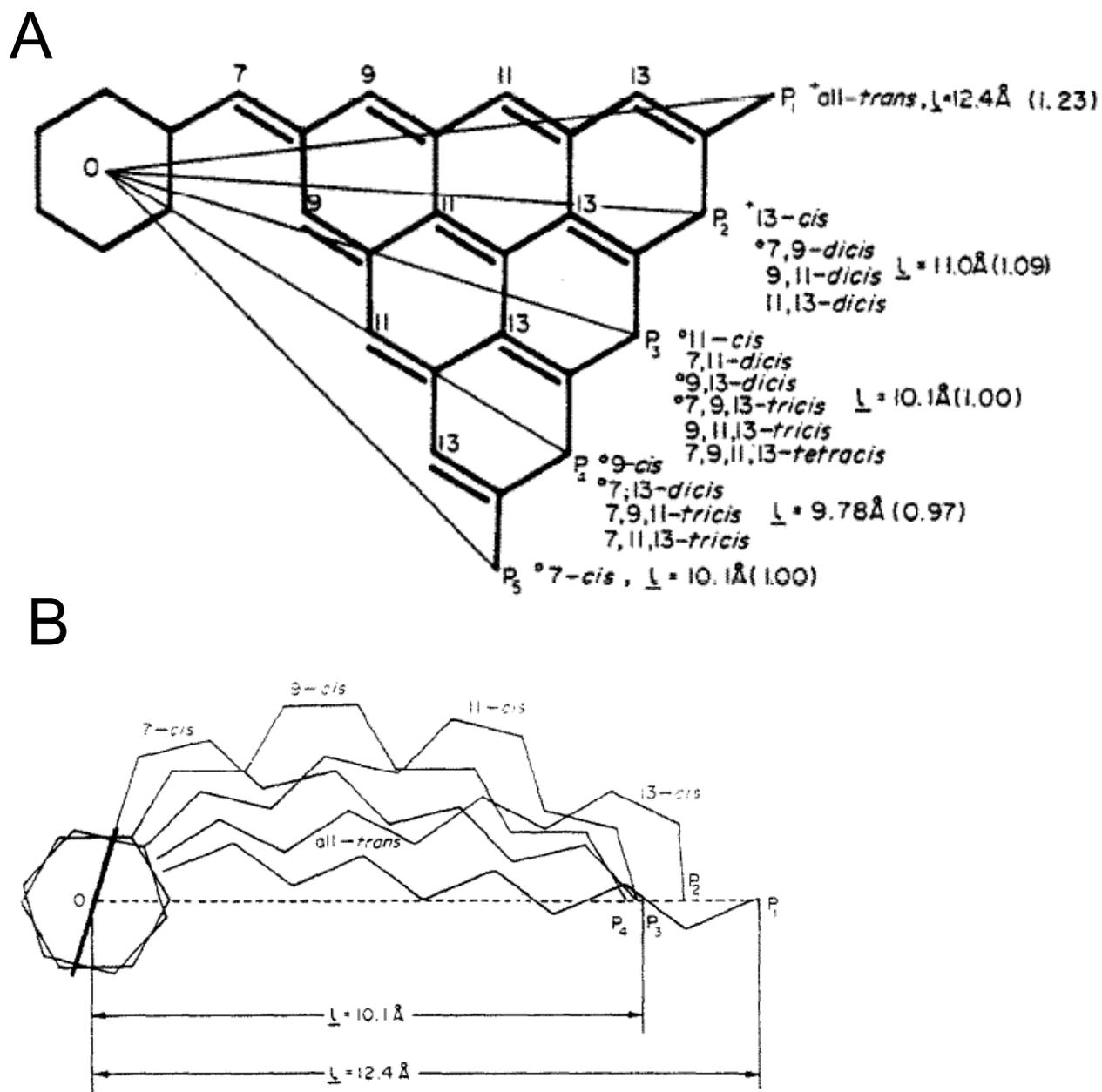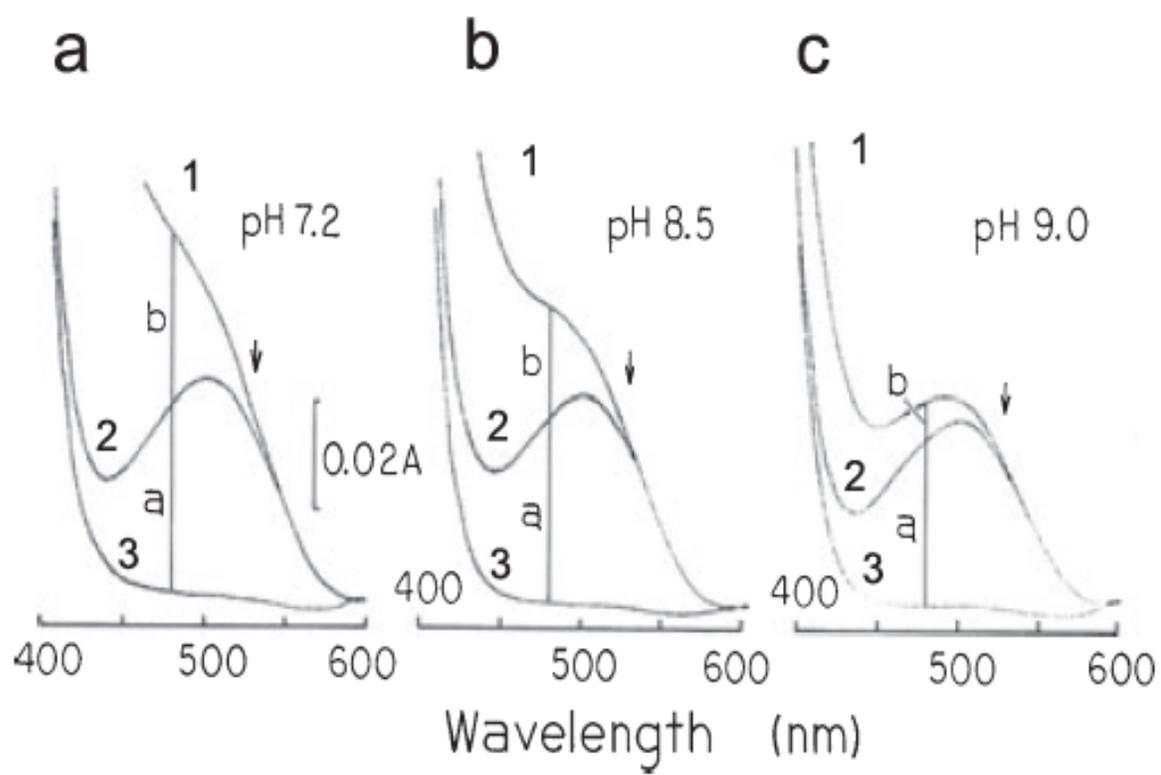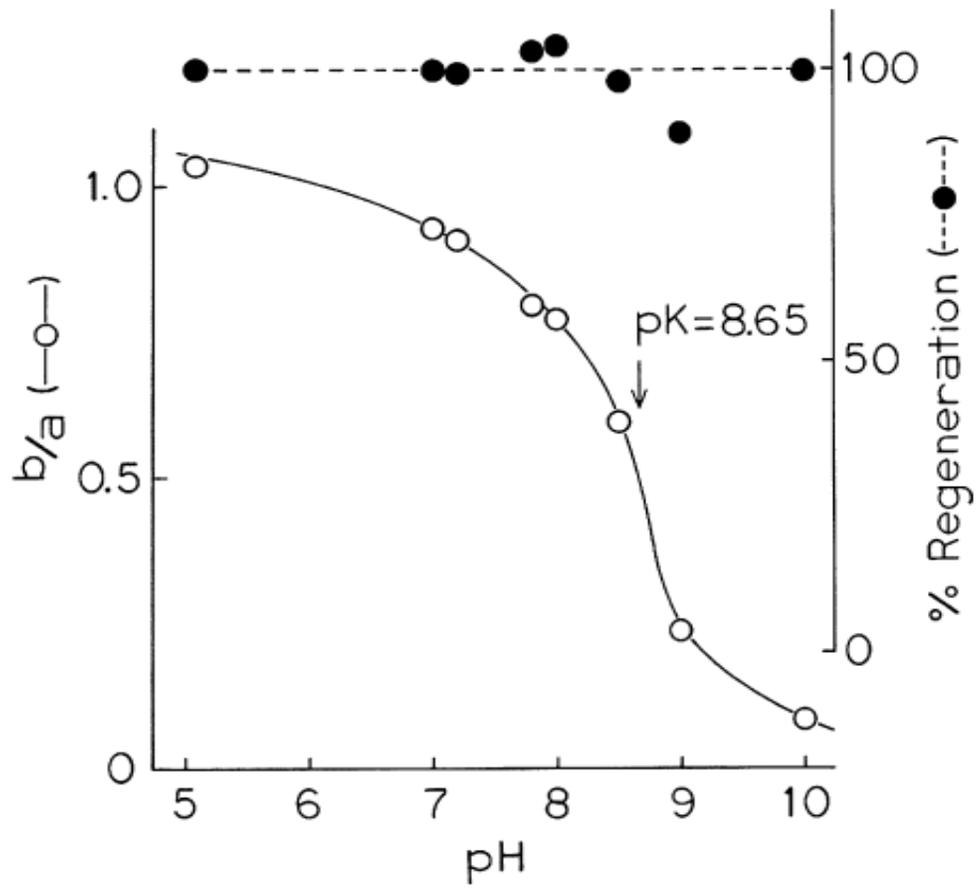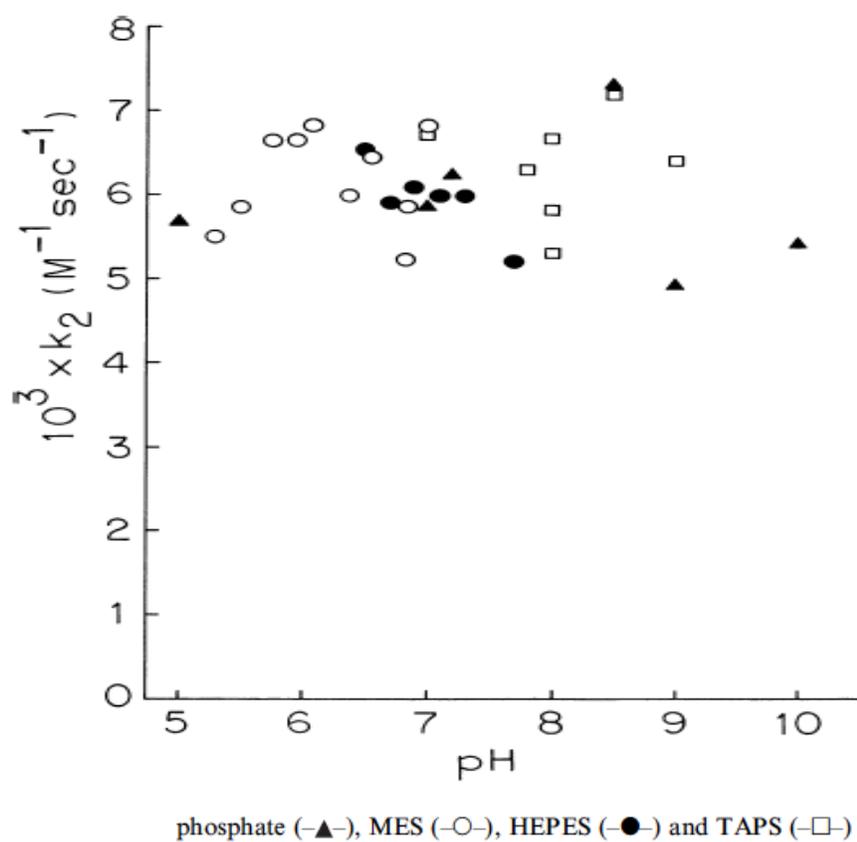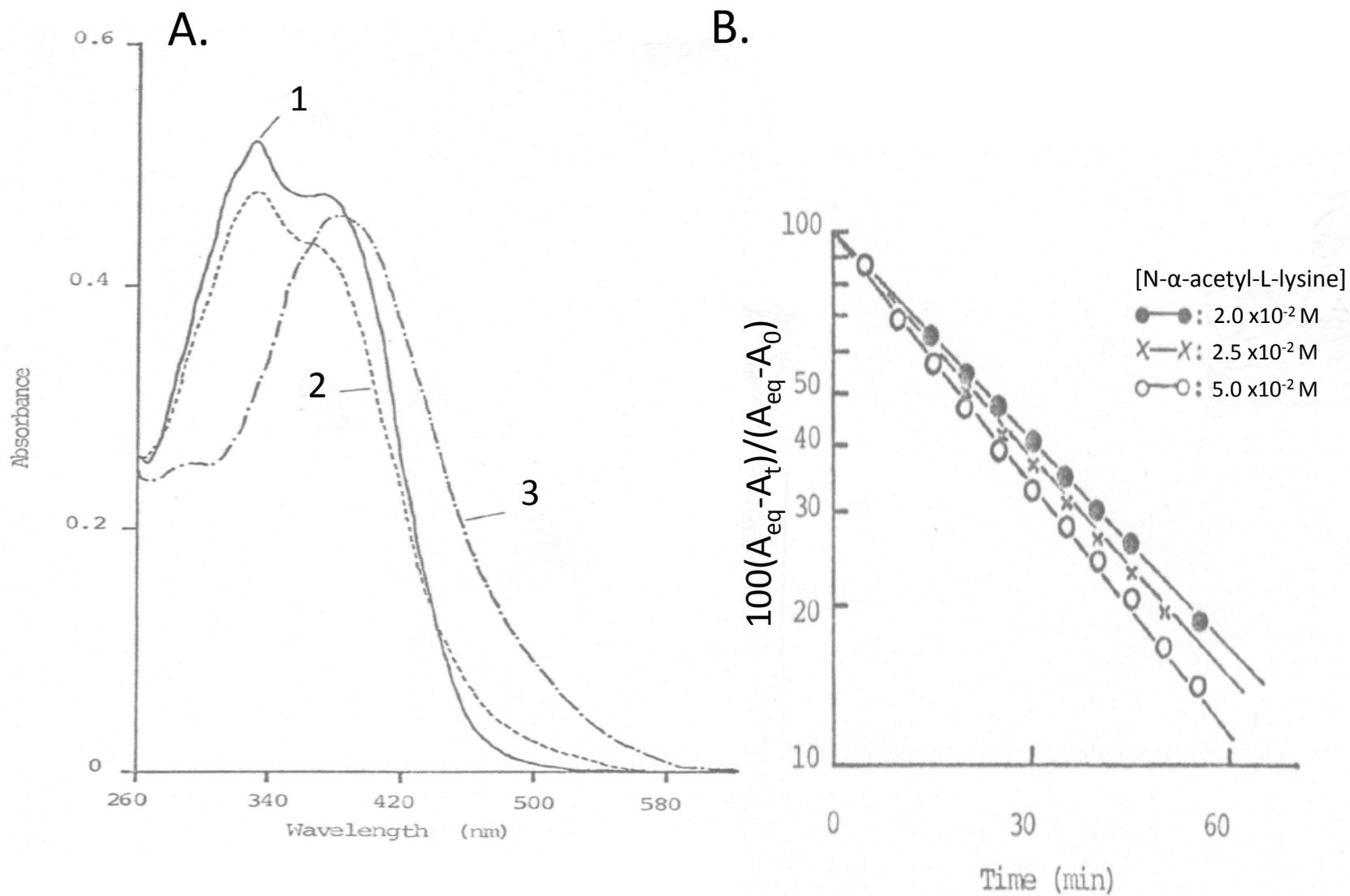
Table 1   The C6-C15 distance of bovine rhodopsin and intermediates calculated based on structural data by using PyMOL.

	Configuration	Property	PDB file	C6-C15 Distance in Å
Rhodopsin	11-*cis*	Stable	1U19	10.0
Isorhodopsin	9-*cis*	Stable	2PED	9.9
Bathorhodosin	all-*trans*	Transient	2G87	10.3
Metarhodopsin II	all-*trans*	Transient	3PX0	11.0

Rhodopsin regenerates through a non-covalent 11-*cis* retinal•opsin complex followed by a covalent formation of retinylidene Schiff base, rendering the β-ionone ring binding pocket a distinct physiological role.

Opsin   11-*cis* retinal   Non-covalent complex

k_1

k_{-1}

k_2

Rhodopsin

= β-ionone ring binding pocket

k_2 is likely to be the rate limiting step of rhodopsin regeneration because of slow Schiff base formation. The figures are modified from Scheerer et al. (2008) and from Imai et al. (2005).