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QM/MM Structure, Enantioselectivity and Spectroscopy of HydroxyRetinals and Insights into the Evolution of Color Vision in Small White Butterflies

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Abstract

Since Vogt's discovery of A₃-retinal or 3-hydroxyretinal in insects in 1983 and Matsui's discovery of A₄-retinal or 4-hydroxyretinal in firefly squid in 1988, hydroxyretinal-protein interactions mediating vision remains largely unexplored. In the present study, A₃- and A₄-retinals are theoretically incorporated into squid and bovine visual pigments using the hybrid quantum mechanics/molecular mechanics (SORCI+Q//B3LYP/6-31G(d):Amber96) method and insights into the structure, enantioselectivity and spectroscopy are gathered and presented for the first time. Contrary to general perception, our findings rule out the formation of hydrogen bond between the hydroxyl-bearing β-ionone ring part of retinal and opsin. Compared to A₁-pigments, A₃- and A₄-pigments exhibit slightly blue-shifted absorption maxima due to increase in bond-length alternation of the hydroxyretinal. We suggest that, (i) The binding site of firefly squid (*Watasenia scintillians*) opsin is very similar to that of the Japanese common squid (*Todarodes pacificus*) opsin, (ii) Molecular mechanism of spectral tuning in the small white butterflies involve sites S116, T185 and breaking of hydrogen bond between sites E180 and T185; and finally, (iii) A₃-retinal may have occurred during the conversion of A₁- to A₂-retinal and insects may have acquired them, in order to absorb light in the blue-green wavelength region and to speed up the G-protein signaling cascade.

Keywords

Visual Pigment; Vertebrates; Invertebrates; Insects; Opsin; Quantum Mechanics/Molecular Mechanics

1. INTRODUCTION

Visual pigments contain a protonated Schiff base of 11-*cis*-retinal or A₁-retinal encapsulated into an opsin apoprotein. For many years, A₁-retinal was assumed to be the light-sensitive chromophore mediating dim/light and/or color vision in the vertebrate and invertebrate eye.

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Supporting Information Available. Cartesian coordinates of all the retinal models discussed in this study. This material is available free of charge via the Internet at <http://pubs.acs.org>

Later, some freshwater amphibians, fishes and crustaceans were found to contain 3,4-dehydroretinal or A₂-retinal,^{1–5} while insects (*Dipterans*) were found to possess 3-hydroxyretinal or A₃-retinal.⁶ However, not all insects were found to contain the A₃-retinal because, phylogenetically older groups of the insect family (*Coleoptera*, *Orthoptera* and *Hymenoptera*) continue to utilize the A₁-retinal for vision.⁷ Dragonflies (*Odonata*) and a sub-family of long horned beetles were found to use both A₁- and A₃-retinals.^{8–12} As a result, visual pigments containing A₁-retinal are called as rhodopsins, A₂-retinal as porphyropsins, A₃-retinal as xanthopsins.^{13,14} Firefly squid (*Watasenia scintillians*), which exhibits intense bioluminescence from large photophores, was found to be the only animal utilizing three visual pigments^{11,15–17} with three different chromophores for color vision, namely, A₁-, A₂- and 4-hydroxyretinal or A₄-retinal (Scheme I).

Interestingly, vertebrate pigments were found to contain only A₁- and A₂-retinals,¹⁸ while invertebrate pigments were found to accommodate all the four retinal congeners.¹⁹ Experimental studies of the absorption spectrum and photosensitivity indicate that, (i) Bovine opsin incorporated with A₃-retinal absorbs at 488 nm;²⁰ (iii) Firefly squid opsin with A₁-, A₂- and A₄-retinal absorbs at 484, 500 and 470 nm, respectively;^{15–17} and (iv) Bacteriorhodopsin with an all-*trans* form of A₄-retinal analogue peaks at 540 nm.²¹ (ii) Small white butterfly (*Pieris rapae*) opsins contain A₃-retinal and their eyes have six classes of photoreceptors peaking at 360 nm (UV), 420 nm (violet), 450 nm (blue), 563 nm (green), 620 nm (red) and deep-red (640 nm); respectively.^{22–24}

Irrespective of the identity of the species, both A₃- and A₄- pigments were found to exhibit blue-shifted λ_{max} compared to their A₁- and A₂-counterparts. This property has been attributed to changes in the protein-chromophore interactions due to altered geometry of the hydroxyl-bearing retinal. Substitution of hydroxyl group was assumed to increase the polarity and facilitate formation of a hydrogen bond between the β -ionone ring part of the retinal and opsin.²⁰ Due to the absence of X-ray structure of visual pigments containing A₃- or A₄-retinal, such assumptions have so far remained untested. Note that, hydroxylretinals are known to form bleachable visual pigments²⁵ and are found to be present only in invertebrates. Apparently, theoretical incorporation of hydroxyretinal derivatives into an already available structure of invertebrate pigment is a viable alternative.

Therefore, we theoretically incorporate 2-hydroxy-, 3-hydroxy-, 4-hydroxy- and 3,4-dihydroxyretinal derivatives (Scheme I) into squid and bovine opsins. Although 2-hydroxy and 3,4-dihydroxyretinal derivatives are not found in naturally occurring visual pigments, we chose to study them to evaluate the extent of geometric perturbation a mono- or dihydroxyl group may induce on the retinal geometry by operating from different stereo centers in the β -ionone ring. As a result, the goal of the study is five-fold: (i) to document the effect of hydroxyl group on the retinal geometry; (ii) to compare the stability of R vs. S enantiomer and characterize the property of enantioselectivity; (iii) to study the wavelength regulation mechanism of A₃- and A₄-pigments; (iv) to use the A₃-retinal incorporated squid opsin as a template and gather insights into the spectral tuning mechanism of blue- (*PrB*, 450 nm) and violet-absorbing (*PrV*, 420 nm) visual pigments in small white butterflies; and finally, (iv) to discuss the implications of our findings for understanding insect vision.

2. COMPUTATIONAL MODEL AND DETAILS

2.1. System Set-up

To begin with, QM/MM optimized structures of A₁-squid and A₁-bovine visual pigments are taken from references 26 and 27. In short, the retinal is treated quantum mechanically (QM) and the rest of protein environment is treated via molecular mechanics (MM) and the whole system is optimized without any constraints using the hybrid quantum mechanics/

molecular mechanics QM/MM (QM=B3LYP/6-31G(d); MM=AMBER96) method in ONIOM (Our own *N*-layer Integrated molecular Orbital plus molecular Mechanics) with electronic embedding (EE) scheme²⁸ implemented in Gaussian09.²⁹ The A₁-retinals in squid and bovine opsins are replaced with A₃- and A₄-retinal derivatives and the structures are then reoptimized without any constraints (see the *SI* text).

2.2. PrB and PrV Pigments

We aligned the amino acid residues of *PrB* and *PrV* pigments with that of the Japanese common squid rhodopsin (*Todarodes pacificus*),³⁰ and this approach is adopted from the experimental study by Wakakuwa et al.²⁴ Comparison of the amino acid sequence within 5.0 Å radius of any atom of the retinal (see Table 1) reveals that, seven amino acids at sites 112 (G112A), 113 (F113T), 116 (G116S), 120 (F120I), 123 (I123A), 177 (Y177F), and 185 (N185T) are different in their identity between squid and *PrB* pigments and three amino acids at sites 113 (T113V), 116 (S116A) and 177 (F177Y) are different in their identity between *PrB* and *PrV* pigments.

Although experimental studies have argued for the presence of a single counterion in invertebrate visual pigments,³¹ the exact location and identity of the invertebrate counterion remained elusive until the X-ray structure of squid rhodopsin became available. It was shown that a neutral Tyr111 residue in squid rhodopsin occupies the position corresponding to Glu113 counterion in bovine rhodopsin.³⁰ Later, QM/MM calculations on the X-ray structure clearly showed that Glu180 is the primary counterion to the protonated Schiff base in squid rhodopsin.²⁶ As the position of Tyr111 and Glu180 is conserved in both *PrV* and *PrB* pigments, we propose that Glu180 is the putative counterion in butterfly visual pigments.

The optimized structure of A₃-squid opsin is taken as the starting structure because visual pigments in butterfly contain A₃-retinal and not A₁-retinal. As a first step, seven mutations are introduced into A₃-squid opsin at sites 112, 113, 116, 120, 123, 177, 185 and the resulting structure reoptimized without any constraints is referred to as the *PrB* pigment. To the optimized *PrB* structure, three mutations are introduced at sites 113, 116 and 177 and the resulting structure reoptimized without any constraints is referred to as the *PrV* pigment. *Ab initio* multireference QM/MM calculations on all the optimized geometries were performed at the spectroscopy oriented configuration interaction (SORCI+Q) level with 6-31G(d) basis set on top of three-root (6e, 6o) complete active space self-consistent field (CASSCF) wave functions using the ORCA 2.6.19 program package.³² The vertical excitation energies as well as oscillator and rotatory strengths to the first (S₁) and second (S₂) excited states were calculated for all of the structures discussed in this study. From calculations on related retinal systems, accuracy of the computational setup for calculating the vertical excitation energies was estimated to be within ±15 nm.^{26,27,33,34} For further details see the *SI* text.

3. RESULTS and DISCUSSION

3.1. Structure

The position of seven aromatic residues (F208/F205, F212/F209, F261/F270, W265/W274 and Y268/Y277 in the transmembrane helices, and Y178/Y177 and Y191/Y190 in the extracellular loop) near the β-ionone ring site is conserved in both bovine and squid opsins. QM/MM calculations show no direct contact in the form of hydrogen bond between the hydroxyl bearing ring part of the retinal and opsin. It is possible that the hydrophobic interactions and/or van der Waals forces between the β-ionone ring and opsin³⁵ may assist in fitting the different enantiomers into different pigments.

Influence of the hydroxyl group on the retinal geometry is minimal and mostly restricted to its immediate environment (from C2 to C5) as shown in Figure 1 and this property is evident in the calculated average bond length alternation (BLA). Apparently a hydroxyl group, disconnected from the conjugated chain, influences the BLA by stabilizing the partially delocalized positive charge on the alternate resonance structures via an inductive and/or electrostatic effect. In particular, a gradual increase in BLA is seen when the hydroxyl group is introduced at C4 than at C2 or C3. This could be due to the domination of inductive effect over electrostatic effect when the hydroxyl group nears the conjugated chain.

In the case of monohydroxy derivatives, BLA increases by ~ 0.005 Å in both A₃- and A₄-pigments, whereas in the case dihydroxy derivatives BLA increases by 0.011 Å in squid opsin but not in bovine opsin. This can be attributed to the difference in orientation of the Schiff base relative to its counterion in the pigments. In squid opsin, N87, Y111, E180 and two water molecules are present near the Schiff base (SB) region (Figure 2, left) and E180 serves as a non-H-bonded counterion to the retinal. In bovine opsin, two glutamate residues E113, E181 and two water molecules (wat2a, wat2b) are present in this region (Figure 2, right) and E113 serves as a H-bonded counterion and anchors the proton on SB nitrogen atom. As a consequence, the invertebrate retinal moiety responds to more environmental perturbation than its vertebrate counterpart.^{26,27,36–38}

3.2. Enantioselectivity

Substitution of a hydroxyl group at C2, C3 or C4 position allows exploitation of chiral centers in the β -ionone ring and existence of an R or S enantiomer in visual pigments. Comparison of ONIOM-EE energies shows that at C2 and C3 positions, the 2R- and 3R-hydroxy enantiomers are more stable than their 2S and 3S counterparts in both squid and bovine opsins. Differences arise at C4, where the 4S enantiomer is calculated to be more stable than 4R isomer in squid opsin and 4R enantiomer to be more stable than 4S isomer in bovine opsin. Although dihydroxy retinals are not found to occur in nature, we find the 3R, 4S-enantiomer to be more stable in squid opsin and 3S,4S-enantiomer to be more stable in bovine opsin. The findings are in agreement with the experimental observations for the presence of 4R-enantiomer in bovine opsin²⁰ and 4S-enantiomer in firefly squid opsin.^{15–17} Apparently, with the exception of Cyclorrhapha (Diptera) that contains 3S-enantiomer,³⁹ class insecta (*Odonata*, *Hemiptera*, *Coleoptera*, *Neuroptera*, *Lepidoptera*, *Nematocera*, *Brachycera*, *Diptera*) was generally found to contain 3R-enantiomer.^{8–12}

3.3. Spectroscopy

Effect of hydroxyl and/or polar groups on the absorption spectrum of visual pigments has already been studied before. However, these studies were performed on cone pigments, where hydroxyl bearing amino acids and not hydroxy retinals were found to mediate green (530 nm) and red (560 nm) vision.^{40–47} Therefore, for pigments already containing a hydroxyl bearing retinal, one may expect $\lambda_{\max}$ to be above 500 nm. Surprisingly, A₃- or A₄-pigments in proteins have consistently yielded $\lambda_{\max}$ less than 500 nm, as shown in Table 2. To identify the origin of this blue shift we have calculated vertical excited state energies both in gas phase and in protein environments (see Table 2). Compared to the A₁-retinal that absorbs at 604/616 nm in gas phase and 490/495 nm in protein environments (squid/bovine),^{26,27,48} A₃- and A₄-pigments exhibit consistently a blue-shifted $\lambda_{\max}$. As the small blue shift of ~ 15 nm is already seen in the gas phase, we attribute the origin of slightly blue-shifted $\lambda_{\max}$ to the increase in BLA of the hydroxyretinal rather than to any specific residue-protein interaction in pigments. This is in agreement with earlier studies on A₂- or 3,4-dehydroretinal, where a red shift of ~ 20 nm was also attributed to the intrinsic property of the retinal *i.e.* to the presence of an additional C3=C4 double bond in the β -ionone ring.^{38,49} The change in dipole moment ($\Delta\mu$) between the ground- (S_0) and excited-states (S_1) due to

excited state charge density being shifted against the charge of the counterion is calculated to be ~ 12.0 D both in squid and bovine opsins⁵⁰ and this value remains unaffected even in presence of hydroxyl group in the β -ionone ring. As we turn off charges of the counterion (E180 in squid opsin and E113 in bovine opsin), the calculated vertical excitation energies (λ^{E180} , λ^{E113}) in the protein is almost equal to that obtained in the gas phase.^{51–54}

Therefore, spectral tuning mechanisms of A₃- and A₄-pigments are also steered by strong electrostatic interaction between the retinal and its counterion.^{55–78} The calculated absorption wavelength of 490, 510 and 474 nm of A₁-, A₂- and A₄-retinals in the Japanese common squid (*Todarodes pacificus*) opsin agree very well with the experimental measurements of 484, 500 and 470 nm in firefly squid (*Watasenia scintillians*) opsin.^{15–17} Apparently, the binding site of both the pigments must be very similar to each other. The discrepancy between the calculated (466 nm) and experimental (488 nm) values for A₃-retinal in bovine opsin could be due to the fact that the experimental study was carried out using a CHAPSO-solubilized bovine opsin²⁰ and variation in the standard error was not given. Therefore, it is not easy to compare the calculated value (with an error limit of ± 15 nm) with the single experimental value.

4. Spectral Tuning in Small White Butterflies

Small white butterflies, *Pieris rapae*, contain six classes of photoreceptors. We chose to study only two among the six classes of photoreceptors, because site-directed mutagenesis experimental measurements are available for only the *PrB* and *PrV* photoreceptors. Wakakuwa et al. showed that amino acid substitutions at sites 116 and 177 are crucial for spectral tuning in the *PrB* and *PrV* pigments.²⁴ As already mentioned before, within 5.0 Å environment of the retinal, seven sites in *PrB* opsin and three sites in *PrV* opsin differ in their identity compared to the squid opsin. However, due to difference in their polarity, only sites 116 and 177 were mutated in the experimental study. Furthermore, due to lower reconstitution efficiency of A₃-retinal the experimental study was carried out with A₁-retinal²⁴ and therefore, experimental $\lambda_{\max}$ values of *PrB* and *PrV* pigments with A₃-retinal is yet to be obtained. The fact that, $\lambda_{\max}$ of an A₃-pigment will be slightly blue-shifted than that of an A₁-pigment was also not taken into account in the experimental study. In light of theoretical evidence presented thus far, we consider it is important to re-examine the spectral tuning mechanism of *PrB* and *PrV* pigments using an A₃-retinal.

To begin with, we use A₃-squid opsin as the starting structure and introduce seven mutations to model *PrB* opsin and three mutations to model *PrV* opsin. To compare the calculated results with the available experimental data, the calculations are also repeated with A₁-retinal. Unless and until mentioned explicitly, the following discussion on the topic of spectral tuning will correspond to $\lambda_{\max}$ of the A₃-retinal.

4.1. Structural Rearrangements

On one hand, replacement of A₁-retinal with A₃-retinal induces slight increase in distance between the C α atom of G116 and the C19 atom of the retinal (3.55 $\rightarrow$ 3.57 Å). On the other hand, distance between the -OH group of Tyr177 and the C19 atom undergoes a slight decrease from 4.50 to 4.47 Å. Presence of serine at site 116 further increases the distance between the C α atom of G116 and the C19 atom by 0.49 Å (3.57 $\rightarrow$ 4.06 Å) and between the -OH group of Tyr177 and the C19 atom by 0.84 Å (4.47 $\rightarrow$ 5.31 Å). Notice that, a hydrogen bond temporarily formed between S116 and Y177 is lost due to Y177F mutation (see Figure 4). Substitution of tyrosine with phenylalanine at site 177 draws the C α atom of S116 close to the C19 atom by 0.10 Å (4.06 $\rightarrow$ 3.96 Å). During the formation of *PrB* pigment, mutation of asparagine with threonine at site 185 (N185T) breaks an already existing hydrogen bond between N185 and E180 (see Figure 3). Although threonine has been found to induce a blue shift of 21 nm from site 185, it induces a red shift of 6 nm from site 113.

4.2. Single Mutant Spectra

Performing single mutant calculation helps in tracking the structural rearrangements, sign and magnitude of the spectral shift induced by that particular mutant. In this case, substitution of alanine at sites 112 and 123 induces a red shift of ~10 nm, while substitution of isoleucine and phenylalanine at sites 120 and 177 induces a red shift of ~7 nm. Substitution of serine at site 116 and threonine at site 185 induces a large blue shift of ~20 nm. Effect of these polar residues is so strong that they individually shift the λ_{max} from 473 nm to 453 nm. To examine if serine can induce the blue shift from any site, we substituted serine at all the remaining six sites one at a time and reoptimized the structures. The analysis reveal that serine at site 185 can also induce a strong blue shift and move the λ_{max} to 450 nm. In contrast, serine at sites 112, 113, 120, 123 or 177 does not induce any significant blue shift. Therefore, we suggest that serine plays an important role in the spectral tuning process and can operate from site 116 or site 185.

4.3. Double Mutant Spectra

To further substantiate the effect of S116, double mutant calculations are performed with S116 as the common mutant throughout the calculations. The calculated λ_{max} for G116S/G112A, G116S/F113T, G116S/F120I and G116S/I123A mutants are found to be close to 460 nm with A₃-retinal and above 475 nm with A₁-retinal. Therefore, these mutant combinations are unlikely to be responsible for the spectral tuning process. Surprisingly, we find G116S/Y177F and G116S/N185T mutants to yield values close to 453 nm. Experimental studies have already shown G116S/Y177F mutation to be responsible for the spectral tuning mechanism. However, the discovery of N185T as a possible source for spectral tuning is new and deserves further examination. To understand the effect of T185, double mutant calculations are performed with T185 as the common mutant. Excluding the N185T/I123A model that absorbs at 446 nm, all other models yielded values close to 460 nm. Because I123A and Y177F mutants have already been shown to individually shift the λ_{max} above 480 nm, it is possible that these sites become more effective only in the presence of S116 and T185.

4.4. Triple Mutant Spectra

To seek further evidence for the role of I123A and Y177F, we constructed a set of triple mutant models with sites, 116, 123, 177 and 185. Replacing Y177F with I123A does not seem to affect the calculated λ_{max} as both G116S/Y177F/N185T and G116S/I123A/N185T models were found to absorb around 435 nm. This shows that G116S and N185T play a crucial role in blue-shifting the λ_{max} to less than 440 nm. To double-check this finding, we constructed models with and without G116S and N185T. Leaving out G116S (I123A/Y177F/N185T) red shifts the λ_{max} by almost 25 nm, while leaving out N185T (G116S/I123A/Y177F) also red shifts the λ_{max} but, by ~15 nm. One advantage of performing the triple mutant analysis is that, it allows us to disentangle the importance of G116S over N185T, which was not possible with the single/double mutant analysis.

4.5. PrB Pigment

Having shown that sites 116 and 185 strongly mediate the spectral tuning pathway, we take all the seven mutants into consideration and construct a more realistic model of the PrB binding site. This model absorbs at 438 nm, which is only 8 nm less than the double mutant G116S/N185T model and almost equal to the triple mutant models of G116S/Y177F/N185T or G116S/I123A/N185T. Therefore, we predict that the true λ_{max} of PrB pigment must be around 440 nm because the naturally occurring pigment will only contain an A₃-retinal and not an A₁-retinal. In partial agreement with the experimental study, we suggest that sites 116

and 185 steer the spectral tuning mechanism in *PrB* pigment and sites 123 and 177 may play a secondary role in the presence of S116 and T185.

4.6. *PrV* Pigment

Of the three sites (113, 116, 177) that differ between *PrB* and *PrV* pigments, mutation of threonine with valine at site 113 (T113V) and tyrosine with phenylalanine at site 177 (Y177F) induces a slight blue shift of ~3 nm individually. In contrast, mutation of serine with alanine at site 116 (S116A) induces a red shift of 10 nm in agreement with experimental findings.²⁴ Apparently, only half of the blue shift (20 nm) induced by S116 in *PrB* is recovered with A116 in *PrV*. The calculated spectral shifts are non-additive as introduction of all three mutants decreases the $\lambda_{\max}$ by only 7 nm.

Although the calculations show relatively large deviations from experimental measurements for *A*₁-*PrV* pigment (442 nm vs. 420 nm), there is a reasonable agreement when *A*₃-*PrV* pigment value of 431 nm is taken into account. It is possible that the structure of *PrB* and *PrV* pigments are not identical to that of the squid pigment. To obtain an accurate picture, further experimental and theoretical studies that include the *ancestor* pigments are needed because it is becoming increasingly clear that by ignoring the actual evolutionary process, neither the molecular basis of spectral tuning nor the evolutionary mechanisms of visual pigments can be elucidated in detail.⁷⁹

Apparently, there is an interesting correlation between the average BLA and absorption wavelength calculated for *A*₁-, *A*₃- and *A*₄-retinals in bovine, squid and butterfly pigments, with the increase in BLA contributing to the decrease in $\lambda_{\max}$ (Figure 5). Also compared to the squid pigment, the calculated oscillator strength (*f*) increases from 1.22 to 1.24 in *PrB* and to 1.30 in *PrV* pigments. In contrast, the rotatory strength (*R*) decreases from 0.46 in squid to 0.45 in *PrB* and to 0.41 in *PrV*. The decremental blue shift separating *A*₃-squid (473 nm) from *A*₃-*PrB* (438 nm) and *A*₃-*PrB* from *A*₃-*PrV* (431 nm) can be related to the small change in the dipole moment ($\Delta\mu$); 11.1 D in squid to 10.9 D in *PrB* and to 10.7 D in *PrV* pigments.

5. Summary and Perspectives

To sum up, we have presented a hybrid QM/MM evaluation of the structure, enantioselectivity and spectroscopy of *A*₃- and *A*₄-retinal incorporated vertebrate (bovine) and invertebrate (squid) opsins. We show that, effect of hydroxyl groups on the retinal geometry is minimal and restricted to its immediate vicinity. Contrary to general perception, the hydroxyl-bearing ring part of the retinal does not form a H-bond with opsin. Compared to *A*₁-pigments, *A*₃- and *A*₄-pigments exhibit a blue-shifted $\lambda_{\max}$ due to increase in BLA of the hydroxyretinal.

Firefly squid (*Watasenia scintillians*), which contains three visual pigments with different chromophores, absorbs light at 470 nm using the *A*₄-retinal. To evaluate the possible binding site of the *A*₄-pigment, we theoretically replaced *A*₁- with *A*₄-retinal in the QM/MM structure of Japanese common squid (*Todarodes pacificus*) opsin. Replacement of *A*₁- with *A*₄-retinal does not alter the architecture of retinal binding site and the calculated value of 474 nm for *A*₄-retinal was also found to be in excellent agreement with the experimental value of 470 nm. We conclude that the binding site of firefly squid opsin is similar to that of Japanese common squid opsin.

By using the *A*₃-squid opsin as the starting point, we have also studied the spectral tuning mechanism of *Pieris rapae* Blue (*PrB*) and *Pieris rapae* Violet (*PrV*) absorbing visual pigments. Experimental observations were based on the values obtained using *A*₁- and not

A₃-retinal and also only two sites 116, 177 were considered for site-directed mutations. Therefore, we chose to re-examine the molecular basis for spectral tuning in small white butterflies. We substituted all the amino acids within 5.0 Å radius of the retinal that differ between squid, *PrB* and *PrV* pigments. To our knowledge, this is also the first detailed computational study of site-directed mutations in an invertebrate visual pigment. In contrast to the experimental findings, we find sites 116 and 185 and breaking of H-bond between sites 185 and 180 due to substitution of asparagine with threonine to be critical for the spectral tuning mechanism in small white butterflies. Increase in BLA of the hydroxyretinal and presence of S116 and T185 induce strong blue shifts and move the λ_{max} from 473 nm in squid opsin to 438 nm in *PrB* opsin and to 431 nm in *PrV* opsin.

Due to the fact that no major difference was found between the binding sites of A₁-, A₃- and A₄-retinals, one may wonder why an insect would select hydroxyretinal as its visual chromophore. Generally, selection of retinal in animals and/or human is directly proportional to the constraints of both visual and environmental requirements of their habitat. For example, the choice of A₂- over A₁-retinal has been restricted to fresh-water fishes, which absorbs light at longer wavelengths while migrating from salt to fresh-water.⁸⁰ However, no such correlation has been established for the selection of A₃-over A₁-retinal in insects. Our results suggest that A₃-retinal may have occurred as an hydroxy-intermediate during the conversion of A₁- to A₂-retinal via an enzymatic and/or metabolic pathway (Scheme II) and butterflies may have acquired them in order to absorb light in the blue-green wavelength region and to speed up the G-protein signaling cascade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

1. Wald G. J. Gen. Physiol. 1936; 19:351–371. [PubMed: 19872932]
2. Wald G. J. Gen. Physiol. 1936; 20:45–56. [PubMed: 19872983]
3. Wald G. Nature. 1937; 139:1017–1018.
4. Wald G. Nature. 1937; 140:545–546.
5. Wald G. J. Gen. Physiol. 1939; 20:775–794. [PubMed: 19873132]
6. Vogt K. Naturforsch. 1983; 38c:329–333.
7. Vogt K, Kirschfeld K. Naturwiss. 1984; 71:211–213.
8. Goldsmith TH, Marks BC, Bernard GD. Vision Res.. 1986; 26:1763–1769. [PubMed: 3617517]
9. Seki T, Fujishita S, Ito M, Matsuoka N, Kobayashi C, Tsukida K. Vision. Res. 1986; 26:255–258. [PubMed: 3087059]
10. Tanimura T, Isono K, Tsukahara Y. Photochem. Photobiol. 1986; 43:225–228.
11. Matsui S, Seidou M, Uchiyama I, Sekiya N, Hiraki K, Yoshihara K, Kito Y. Biochim. Biophys. Acta. 1988; 966:370–374. [PubMed: 3416013]
12. Gleadall IG, Hariyama T, Tsukahara Y. J. Insect. Physiol. 1989; 35:787–795.
13. Suzuki T, Makino-Tasaka M, Miyata S. Vis. Res. 1985; 25:149–154. [PubMed: 3160161]
14. Nakanishi K. Pure & Appl. Chem. 1991; 63:161–170.

15. Seidou M, Sugahara M, Uchiyama H, Hiraki K, Hamanaka T, Michinomae M, Yoshihara K, Kito Y. *J. Comp. Physiol. A*. 1990; 166:769–773.
16. Kito Y, Partridge JC, Seidou M, Narita K, Hamanaka T, Michinomae M, Sekiya N, Yoshihara K. *Vision Res*. 1992; 32:3–10. [PubMed: 1386953]
17. Michinomae M, Masuda H, Seidou M, Kito Y. *J. Exp. Biol*. 1994; 193:1–12. [PubMed: 9317205]
18. Yokoyama, S.; Yokoyama, R. *Handbook of Biological Physics*. Stavenga, DG.; Degrip, WJ.; Pugh, EN., Jr, editors. Vol. Volume 3. 2000. p. 257-296.Chap. 6
19. Gaertner, W. *Handbook of Biological Physics*. Stavenga, DG.; Degrip, WJ.; Pugh, EN., Jr, editors. Vol. Volume 3. 2000. p. 297-388.Chap. 7
20. Gaertner W, Ullrich D, Vogt K. *Photochem. Photobiol*. 1991; 54:1047–1055. [PubMed: 1837929]
21. Renk G, Grover T, Crouch R, Mao B, Ebrey TG. *Photochem. Photobiol*. 1981; 33:489–494.
22. Arikawa K, Inokuma K, Eguchi E. *Naturwiss*. 1987; 74:297–298.
23. Arikawa K, Mizuno S, Scholten DGW, Kinoshita M, Seki T, Kitamoto J, Stavenga DG. *Vision Res*. 1999; 39:1–8. [PubMed: 10211390]
24. Wakakuwa M, Terakita A, Koyanagi M, Stavenga DG, Shichida Y, Arikawa K. *PLoS one*. 2010; 5:e15015. [PubMed: 21124838]
25. Renk G, Crouch RK. *Biochemistry*. 1989; 28:907–912. [PubMed: 2540811]
26. Sekharan S, Altun A, Morokuma K. *Chem. Eur. J*. 2010; 16:1744–1749. [PubMed: 20066712]
27. Altun A, Yokoyama S, Morokuma K. *J. Phys. Chem. B*. 2008; 112:6814–6827. [PubMed: 18473437]
28. Vreven T, Byun KS, Komaromi I, Dapprich S, Montgomery JA Jr, Morokuma K, Frisch MJ. *J. Chem. Theor. Comput*. 2006; 2:815–826.
29. Frisch, MJ., et al. *Gaussian 09, Revision A. 02*. Wallingford, CT: Gaussian, Inc.; 2009.
30. Murakami M, Kouyama T. *Nature*. 2008; 453:363–367. [PubMed: 18480818]
31. Vought BW, Salcedo E, Chadwell LV, Britt SG, Birge RR, Knox BE. *Biochemistry*. 2000; 39:14128–14137. [PubMed: 11087361]
32. Neese, F. *ORCA-an ab-initio, DFT and semiempirical electronic structure package*. 2007. version 2.6, revision 19; Latest version available <http://www.thch.uni-bonn.de/tc/orca>
33. Altun A, Yokoyama S, Morokuma K. *J. Phys. Chem. B*. 2008; 112:16883–16889. [PubMed: 19367945]
34. Sekharan S, Morokuma K. *J. Am. Chem. Soc*. 2011; 133:4734–4737. [PubMed: 21391708]
35. Ahuja S, Hornak V, Yan EC, Syrett N, Goncalves JA, Hirshfeld A, Ziliox M, Sakmar TP, Sheves M, Reeves PJ, Smith SO, Eilers M. *Nat. Struct. Mol. Biol*. 2009; 16:168–175. [PubMed: 19182802]
36. Ota T, Furutani Y, Terakita A, Yoshinori S, Kandori H. *Biochemistry*. 2006; 45:2845–2851. [PubMed: 16503639]
37. Sekharan S, Morokuma K. *J. Phys. Chem. Lett*. 2010; 1:668–672. [PubMed: 20396622]
38. Sekharan S, Altun A, Morokuma K. *J. Am. Chem. Soc*. 2010; 132:15856–15859. [PubMed: 20964383]
39. Seki T, Isono K, Ito M, Katsuta Y. *Eur. J. Biochem*. 1994; 226:691–696. [PubMed: 8001586]
40. Nathans J, Thomas D, Hogness DS. *Science*. 1986; 232:193–202. [PubMed: 2937147]
41. Yokoyama R, Yokoyama S. *Proc. Nat. Acad. Sci. U. S. A*. 1990; 87:9315–9318.
42. Neitz M, Neitz J, Jacobs GH. *Science*. 1991; 252:971–974. [PubMed: 1903559]
43. Chan T, Lee M, Sakmar TP. *J. Biol. Chem*. 1992; 267:9478–9480. [PubMed: 1577792]
44. Asenjo AB, Rim J, Oprian DD. *Neuron*. 1994; 12:1131–1138. [PubMed: 8185948]
45. Kochendoerfer GG, Wang Z, Oprian DD, Mathies RA. *Biochemistry*. 1997; 36:6577–6587. [PubMed: 9184137]
46. Kochendoerfer GG, Lin SW, Sakmar TP, Mathies RA. *Trends. Biochem. Sci*. 1999; 24:300–305. [PubMed: 10431173]
47. Katayama K, Furutani Y, Imai H, Kandori H. *Angew. Chem. Int. Ed*. 2010; 49:891–894.
48. Shichida S, Tokunaga F, Yoshizawa T. *Biochim. Biophys. Acta. Bioenerg*. 1978; 504:413–430.

49. Amora TL, Ramos LS, Galan JF, Birge RR. *Biochemistry*. 2008; 47:4614–4620. [PubMed: 18370404]
50. Mathies RA, Styrer L. *Proc. Natl. Acad. Sci U.S.A.* 1976; 73:2169–2176. [PubMed: 1065867]
51. Andersen LH, Nielsen IB, Kristensen MB, El Ghazaly MOA, Haacke S, Brondsted Nielsen M, Petersen MA. *J. Am. Chem. Soc.* 2005; 127:12347–12350. [PubMed: 16131214]
52. Nielsen IB, Lammich L, Andersen LH. *Phys. Rev. Lett.* 2006; 96 018304–4.
53. Sekharan S, Weingart O, Buss V. *Biophys. J.* 2006; 91:L07–L09. [PubMed: 16648170]
54. Send R, Sundholm D. *Phys. Chem. Chem. Phys.* 2007; 9:2862–2867. [PubMed: 17538731]
55. Arnaboldi M, Motto MG, Tsujimoto K, Balogh-Nair V, Nakanishi K. *J. Am. Chem. Soc.* 1979; 101:7082–7084.
56. Sheves M, Nakanishi K. *J. Am. Chem. Soc.* 1983; 105:4033–4039.
57. Birge RR, Murray LP, Pierce BM, Akita H, Balogh-Nair V, Finsden LA, Nakanishi K. *Proc. Natl. Acad. Sci. U.S.A.* 1985; 82:4117–4121. [PubMed: 2987964]
58. Einterz CM, Lewis JW, Kliger DS. *Proc. Natl. Acad. Sci. U.S.A.* 1987; 84:3699–3703. [PubMed: 3473477]
59. Sakmar TP, Franke RR, Khorana HG. *Proc. Natl. Acad. Sci. USA.* 1989; 86:8309–8313. [PubMed: 2573063]
60. Zhukovsky EA, Oprian DD. *Science*. 1989; 246:928–930. [PubMed: 2573154]
61. Nathans J. *Biochemistry*. 1990; 29:9746–9752. [PubMed: 1980212]
62. Makino CL, Kraft TW, Mathies RA, Lugtenburg J, Miley ME, van der Steen R, Baylor DA. *J. Physiol.* 1990; 424:545–560. [PubMed: 2391661]
63. Fahmy K, Jager F, Beck M, Zvyaga TA, Sakmar TP, Siebert F. *Proc. Nat. Acad. Sci. U. S. A.* 1993; 90:10206–10210.
64. Makino CL, Groesbeek M, Lugtenburg J, Baylor DA. *Biophys. J.* 1999; 77:1024–1035. [PubMed: 10423447]
65. Gascon JA, Batista VS. *Biophys. J.* 2004; 87:2931–2941. [PubMed: 15339806]
66. Wanko M, Hoffmann M, Strodel P, Kosolowski A, Thiel W, Neese F, Frauenheim T, Elstner M. *J. Phys. Chem. B.* 2005; 109:3606–3615. [PubMed: 16851399]
67. Gascon JA, Sproviero EM, Batista VS. *Acc. Chem. Res.* 2006; 39:184–193. [PubMed: 16548507]
68. Sekharan S, Sugihara M, Buss V. *Angew. Chem. Int. Ed.* 2007; 46:269–271.
69. Sekharan S, Sugihara M, Weingart O, Okada T, Buss V. *J. Am. Chem. Soc.* 2007; 129:1052–1054. [PubMed: 17263385]
70. DeGrip WJ, Bovee-Geurts PHM, van der Hoef I, Lugtenburg J. *J. Am. Chem. Soc.* 2007; 129:13265–13269. [PubMed: 17918843]
71. Fujimoto K, Hayashi S, Hasegawa J, Nakatsuji H. *J. Chem. Theory Comput.* 2007; 3:605–618.
72. Montero-Cabrera MA, Rohrig U, Padron-Garcia JA, Crespo-Otero R, Montero-Alejo AL, Garcia de la Vega JM, Chergui M, Rothlisberger U. *J. Chem. Phys.* 2007; 127:145102–145107. [PubMed: 17935446]
73. Sekharan S, Buss V. *J. Am. Chem. Soc.* 2008; 130:17220–17221. [PubMed: 19035639]
74. Sekharan S. *Photochem. Photobiol.* 2009; 85:517–520. [PubMed: 19192210]
75. Tomasello G, Olaso-Gonzalez G, Altoe P, Stenta M, Serrano-Andres L, Merchan M, Orlandi G, Bottoni A, Garavelli M. *J. Am. Chem. Soc.* 2009; 131:5172–5186. [PubMed: 19309158]
76. Frahmcke JS, Wanko M, Phatak P, Mroginiski MA, Elstner M. *J. Phys. Chem. B.* 2010; 114:11338–11352. [PubMed: 20698519]
77. Altun A, Morokuma K, Yokoyama S. *ACS. Chem. Biol.* 2011; 6:775–780. [PubMed: 21650174]
80. Bridges, CDB. *Handbook of Sensory Physiology VII/I*. Dartnall, HJA., editor. Springer; 1972. p. 417-480. Chap. 11

Figure 1.

Comparison of bond lengths along the conjugated carbon chain of the optimized QM/MM geometries of A₁-, A₃- and A₄- and dihydroxy-retinal derivatives (depicted in Scheme I) incorporated into squid (top) and bovine (bottom) visual pigments. Circled regions indicate parts of the retinal moiety that have undergone substantial perturbation due to the presence of hydroxyl groups. Average bond length alternation (BLA), which is defined as the average of the bond lengths of single bonds minus that of double bonds, in this case from C5—C6 to C15—N moiety, is given in parentheses. For bond angles and dihedral angle deviations, see the Supporting Information.

Figure 2.
Retinal binding sites of squid (left) and bovine (right) visual pigments.

Figure 3.

Retinal binding sites of Squid, *PrB* and *PrV* visual pigments. Residues at site 116, 177, 180, 185 are given in ball and stick. Residues at site 112 (orange), 113 (magenta), 120 (black) and 123 (brown) are given in colored circles. The residues are situated within 5.0 Å from any atom of the retinal. Arrow (in red) indicates the presence of hydrogen bond between N185 and E180 in Squid.

Figure 4. Structural changes due to G to S substitution at site 116 (left) and Y to F substitution at site 177 (left) in the *PrB* pigment.

Figure 5. Correlation between the average BLA and absorption wavelength calculated for A₁- and A₃- and A₄-pigments in bovine, squid and butterfly pigments.

Scheme I.

Schematic representation of the A₁- or A₂- or A₃- or A₄-retinal congeners and some representative species containing that retinal are listed.

Scheme II.
Evolutionary pathway of A₃-retinal in insects.

Table 1

Difference in the amino acid positions within 5.0 Å environment from any atom of the retinal in Squid, *Pieris rapae* Blue (*PrB*) and *Pieris rapae* Violet (*PrV*) visual pigments.

Visual Pigments	Amino acids that differ within 5.0 Å radius of the retinal						
	112	113	116	120	123	177	185
A ₁ -Squid	G	F	G	F	I	Y	N
A ₃ - <i>PrB</i>	A	T	S	I	A	F	T
A ₃ - <i>PrV</i>	A	V	A	I	A	Y	T

Visual pigments in Squid contain A₁-retinal, while those in *PrB* and *PrV* contain A₃-retinal.

Table 2

The calculated (SORCI+Q)/MM first vertical excited state ($S_0 \rightarrow S_1$) absorption wavelengths (λ) in nm, oscillator (f) and rotatory (R) strengths in au of the retinal congeners in gas-phase (QM-none) and in protein environments (QM/MM) of squid and bovine visual pigments. Difference between the ground- (S_0) and excited-state (S_1) dipole moments ($\Delta\mu$) in protein are given in Debye. λ^{E180} and λ^{E113} correspond to absorption wavelengths of the retinal in protein environments where, negative charge of the counterion (E180 in squid opsin, E113 in bovine opsin) is turned off. Experimental values are taken from references 15, 16, 17, 20, 50, 58, 60.

Squid	Gas phase			Protein		
	λ	f	λ	f	$\Delta\mu$	Exp
A ₁ -Retinal	604	0.9	490	1.1	11.7	591 488, 484
A ₂ -Retinal [‡]	707	0.8	510	1.1	10.7	722 500
2R-OH-Retinal	588	1.1	478	1.2	11.1	593 -
3R-OH-A ₃ -Retinal	586	1.0	473	1.2	11.1	581 -
4S-OH-A ₄ -Retinal	593	1.0	474	1.2	10.9	589 470
3R-4S-diOH-Retinal	562	1.1	438	1.3	10.8	551 -
Bovine						
	Gas phase			Protein		
	λ	f	λ	f	$\Delta\mu$	Exp
A ₁ -Retinal	616	1.2	495	1.40	12.1	626 498
A ₂ -Retinal [‡]	720	1.1	534	1.3	12.3	743 520
2R-OH-Retinal	564	1.3	461	1.42	11.8	583 -
3R-OH-A ₃ -Retinal	592	1.2	466	1.44	11.9	611 488
4R-OH-A ₄ -Retinal	593	1.2	474	1.40	11.9	613 -
3S-4S-diOH-Retinal	547	1.3	454	1.46	11.9	572 -

[‡] Calculated values for A₂-retinal are taken from reference 38. The experimental value for A₁-retinal in the Japanese Common Squid (*Todarodes Pacificus*) is 488 nm (taken from reference 50) and in the Firefly Squid (*Watasenia scintillans*) is 484 nm (taken from reference 15).

Table 3

The calculated (SORCI+Q)/MM first vertical excited state ($S_0 \rightarrow S_1$) absorption wavelengths (λ) in nm, oscillator (f) and rotatory (R) strengths in au of the A_3 - and A_1 -retinal in protein environments (QM/MM) of squid, PrB and PrV pigments that contains one, two, three and seven mutations at a time. Note that the native PrB and PrV pigments contain only A_3 -retinal and experimental values for the native A_3 - PrB and A_3 - PrV pigments are yet to be obtained.

Visual Pigments	Calculated						Experiment
	A ₃ -Retinal			A ₁ -Retinal			
	λ	f	R	λ	f	R	
Squid (Wildtype)	473	1.22	0.46	490	1.14	0.32	488
	483	1.19	0.41	493	1.20	0.43	-
	479	1.21	0.46	492	1.19	0.48	-
G112A							
F113T							
G116S							
F120I							
I123A							
Y177F							
N185T							
G112S							
F113S							
F120S							
I123S							
Y177S							
N185S							
G116S/G112A							
G116S/F113T							
G116S/F120I							
G116S/I123A							
G116S/Y177F							
G116S/N185T							
N185T/G112A							
N185T/F113T							
N185T/F120I							

Visual Pigments	Calculated						Experiment
	A ₃ -Retinal			A ₁ -Retinal			
	λ	f	R	λ	f	R	
N185T/I123A	446	1.24	0.46	456	1.23	0.40	-
N185T/Y177F	464	1.20	0.26	466	1.21	0.33	-
G116S/Y177F/N185T	437	1.20	0.49	445	1.21	0.49	-
G116S/I123A/N185T	436	1.25	0.37	457	1.24	0.43	-
I123A/Y177F/N185T	457	1.20	0.23	464	1.25	0.32	-
G116S/I123A/Y177F	448	1.24	0.47	449	1.22	0.48	-
G112A/F113T/G116S/F120/I123A/Y177F/N185T (PrB)	438	1.24	0.45	448	1.23	0.41	450
T113V	435	1.25	0.47	445	1.23	0.46	-
S116A	448	1.24	0.46	452	1.30	0.40	437
F177Y	436	1.24	0.48	446	1.23	0.43	446
G112A/T113V/S116A/F120/I123A/F177Y/N185T (PrV)	431	1.30	0.41	442	1.28	0.38	420