THE JOURNAL OF PHYSICAL CHEMISTRY B

ARTICLE

pubs.acs.org/JPCB

Quantum Mechanical/Molecular Mechanical Structure, Enantioselectivity, and Spectroscopy of Hydroxyretinals and Insights into the Evolution of Color Vision in Small White Butterflies

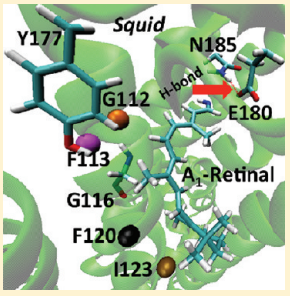
Sivakumar Sekharan,[†] Shozo Yokoyama,[‡] and Keiji Morokuma*[,†,§]

[†]Department of Chemistry and Cherry L. Emerson Center for Scientific Computation and [‡]Department of Biology, Emory University, Atlanta, Georgia 30322, United States

[§]Fukui Institute for Fundamental Chemistry, Kyoto University, 34-4 Takano Nishihiraki-cho, Sakyo, Kyoto 606-8103, Japan

Supporting Information

ABSTRACT: Since Vogt's discovery of A_3-retinal or 3-hydroxyretinal in insects in 1983 and Matsui's discovery of A_4-retinal or 4-hydroxyretinal in firefly squid in 1988, hydroxyretinal−protein interactions mediating vision have remained largely unexplored. In the present study, A_3- and A_4-retinals are theoretically incorporated into squid and bovine visual pigments by use of the hybrid quantum mechanics/molecular mechanics [SORCI+Q//B3LYP/6-31G(d):Amber96] method, and insights into structure, enantioselectivity, and spectroscopy are gathered and presented for the first time. Contrary to general perception, our findings rule out the formation of a hydrogen bond between the hydroxyl-bearing β-ionone ring portion of retinal and opsin. Compared to A_1-pigments, A_3- and A_4-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 scintillans*) opsin is very similar to that of the Japanese common squid (*Todarodes pacificus*) opsin; (ii) the molecular mechanism of spectral tuning in small white butterflies involve sites S116 and T185 and breaking of a hydrogen bond between sites E180 and T185; and finally (iii) A_3-retinal may have occurred during the conversion of A_1- to A_2-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.



1. INTRODUCTION

Visual pigments contain a protonated Schiff base of 11-*cis*-retinal or A_1-retinal encapsulated into an opsin apoprotein. For many years, A_1-retinal was assumed to be the light-sensitive chromophore mediating dim/light and/or color vision in the vertebrate and invertebrate eye. Later, some freshwater amphibians, fishes, and crustaceans were found to contain 3,4-dehydroretinal or A_2-retinal,[1−5] while insects (Diptera) were found to possess 3-hydroxyretinal or A_3-retinal.[6] However, not all insects were found to contain the A_3-retinal because, phylogenetically older groups of the insect family (Coleoptera, Orthoptera, and Hymenoptera) continue to utilize the A_1-retinal for vision.[7] Dragonflies (Odonata) and a subfamily of long-horned beetles were found to use both A_1- and A_3-retinals.[8−12] As a result, visual pigments containing A_1-retinal are called rhodopsins, those containing A_2-retinal are porphyropsins, and those containing A_3-retinal are xanthopsins.[13,14] Firefly squid (*Watasenia scintillans*), which exhibits intense bioluminescence from large photophores, was the only animal found to be utilizing three visual pigments[11,15−17] with three different chromophores for color vision, namely, A_1-, A_2-, and 4-hydroxyretinal or A_4-retinal (Scheme 1).

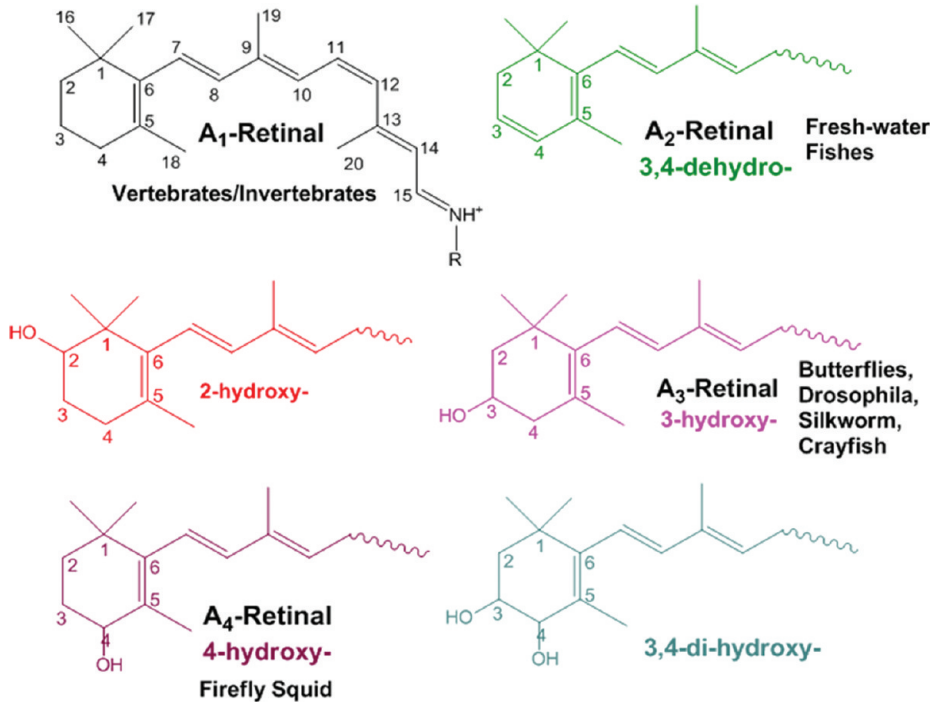
Interestingly, vertebrate pigments were found to contain only A_1- and A_2-retinals,[18] while invertebrate pigments were found to accommodate all four retinal congeners.[19] Experimental studies of absorption spectra and photosensitivity indicate that bovine opsin with A_3-retinal absorbs at 488 nm;[20] firefly squid opsin with A_1-, A_2- and A_4-retinal absorbs at 484, 500, and 470 nm, respectively;[15−17] and bacteriorhodopsin with an all-trans form of A_4-retinal analogue peaks at 540 nm.[21] Small white butterfly (*Pieris rapae*) opsins contain A_3-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 A_3 and A_4 pigments were found to exhibit blue-shifted λ_{max} compared to their A_1 and A_2 counterparts. This property has been attributed to changes in protein−chromophore interactions due to altered geometry of the hydroxyl-bearing retinal. Substitution of the hydroxyl group was assumed to increase the polarity and facilitate formation of a hydrogen bond between the β-ionone ring portion of the retinal and opsin.[20] Due to the absence of X-ray structure of visual pigments containing A_3- or A_4-retinal, such assumptions have so far remained untested. Note that, hydroxylretinals are known to form bleachable visual pigments[25] and are found to be present only in invertebrates. Apparently, theoretical incorporation of

Received: August 23, 2011
Revised: November 11, 2011
Published: November 15, 2011

ACS Publications © 2011 American Chemical Society

Scheme 1. Schematic Representation of A_1-, A_2-, A_3-, and A_4-Retinal Congeners and Some Representative Species Containing That Retinal



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 1) 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 5-fold: (i) to document the effect of hydroxyl group on the retinal geometry; (ii) to compare the stability of *R* versus *S* enantiomer and characterize the property of enantioselectivity; (iii) to study the wavelength regulation mechanism of A_3 and A_4 pigments; (iv) to use the A_3-retinal-incorporated squid opsin as a template and gather insights into the spectral tuning mechanism of blue- (*Pr*B, 450 nm) and violet-absorbing (*Pr*V, 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, quantum mechanics/molecular mechanics (QM/MM) optimized structures of A_1 squid and A_1 bovine visual pigments are taken from refs 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 by use of the hybrid 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) scheme[28] implemented in Gaussian09.[29] The A_1-retinals in squid and bovine opsins are replaced with A_3- and A_4-retinal derivatives and the structures are then reoptimized without any contraints (see the Supporting Information).

Table 1. Difference in Amino Acid Positions within 5.0 Å Environment from Any Atom of the Retinal in Squid, *P. rapae* Blue, and *P. rapae* Violet Visual Pigments

	amino acids that differ within 5.0 Å radius of the retinal						
visual pigments[a]	112	113	116	120	123	177	185
A_1-squid	G	F	G	F	I	Y	N
A_3-*Pr*B	A	T	S	I	A	F	T
A_3-*Pr*V	A	V	A	I	A	Y	T

[a] Visual pigments in squid contain A_1-retinal, while those in PrB and PrV contain A_3-retinal.

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 between squid and PrB pigments and three amino acids at sites 113 (T113 V), 116 (S116A), and 177 (F177Y) are different 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.

dx.doi.org/10.1021/jp208107r |*J. Phys. Chem. B* 2011, 115, 15380–15388

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 A_3 squid opsin is taken as the starting structure because visual pigments in butterfly contain A_3-retinal and not A_1-retinal. As a first step, seven mutations are introduced into A_3 squid opsin at sites 112, 113, 116, 120, 123, 177, and 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 by use of the ORCA 2.6.19 program package.[32] The vertical excitation energies as well as oscillator and rotatory strengths to the first (S_1) and second (S_2) 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 Supporting Information.

3. RESULTS AND DISCUSSION

3.1. Structure. The positions of seven aromatic residues (F208/F205, F212/F209, F261/F270, W265/W274 and Y268/Y277 in the transmemberane helices and Y178/Y177 and Y191/Y190 in the extracellular loop) near the β-ionone ring site are conserved in both bovine and squid opsins. QM/MM calculations show no direct contact in the form of hydrogen bonds between the hydroxyl-bearing ring portion of the retinal and opsin. It is possible that hydrophobic interactions and/or van der Waals forces between the β-ionone ring and opsin[35] 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 rather 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 A_3 and A_4 pigments, whereas in the case of dihydroxy derivatives, BLA increases by 0.011 Å in squid opsin but not in bovine opsin. The 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

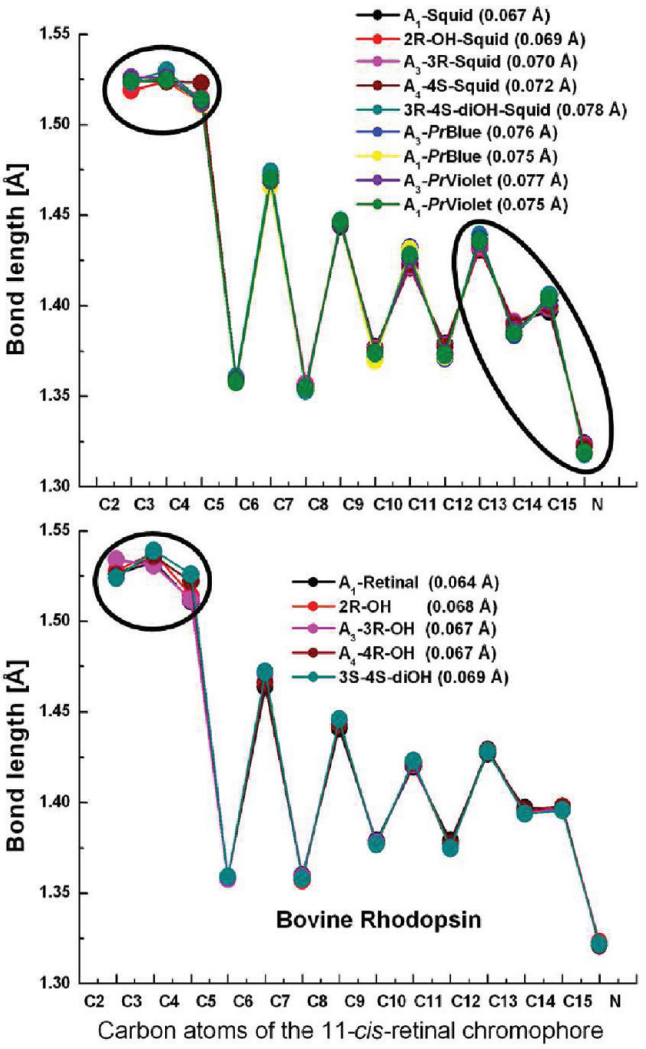


Figure 1. Comparison of bond lengths along the conjugated carbon chain of the optimized QM/MM geometries of A_1-, A_3-, A_4-, and dihydroxyretinal derivatives (depicted in Scheme 1) incorporated into (top) squid and (bottom) bovine visual pigments. Circled regions indicate parts of the retinal moiety 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.

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 2*R*- and 3*R*-hydroxy enantiomers are more stable than their 2*S* and 3*S* counterparts in both squid and bovine opsins. Differences arise at C4, where the 4*S* enantiomer is calculated to be more stable than the 4*R* isomer in squid opsin and the 4*R* enantiomer is calculated to be more stable than the 4*S*

isomer in bovine opsin. Although dihydroxy retinals are not found to occur in nature, we find the 3*R*,4*S* enantiomer to be more stable in squid opsin and the 3*S*,4*S* enantiomer to be more stable in bovine opsin. The findings are in agreement with the experimental observations for the presence of 4*R* enantiomer in bovine opsin[20] and 4*S* enantiomer in firefly squid opsin.[15−17] Apparently, with the exception of Cyclorrhapha (Diptera), which contains 3*S* enantiomer,[39] class Insecta (Odonata, Hemiptera, Coleoptera, Neuroptera, Lepidoptera, Nematocera, Brachycera, and Diptera) was generally found to contain 3*R* enantiomers.[8−12]

3.3. Spectroscopy. Effect of hydroxyl and/or polar groups on the absorption spectrum of visual pigments has already been studied. However, these studies were performed on cone pigments,

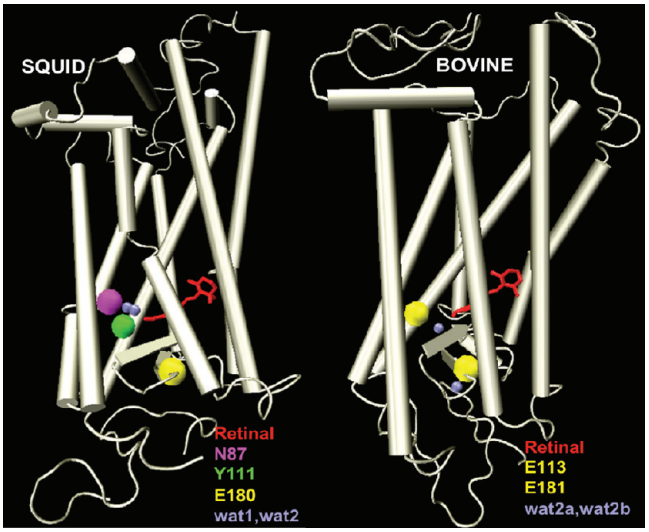


Figure 2. Retinal binding sites of squid (left) and bovine (right) visual pigments.

where hydroxyl-bearing amino acids and not hydroxyretinals 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 λ_{max} to be above 500 nm. Surprisingly, A_3 or A_4 pigments in proteins have consistently yielded λ_{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 A_1-retinal that absorbs at 604/616 nm in gas phase and 490/495 nm in protein environments (squid/bovine),[26,27,48] A_3 and A_4 pigments exhibit consistently a blue-shifted λ_{max}. As the small blue shift of ∼15 nm is already seen in the gas phase, we attribute the origin of slightly blue-shifted λ_{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 A_2- or 3,4-dehydroretinal, where a red shift of ∼20 nm was also attributed to the intrinsic property of the retinal, that is, 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 state (S_0) and excited state (S_1) due to excited-state charge density being shifted against the charge of the counterion is calculated to be ∼12.0 D in both squid and bovine opsins,[50] and this value remains unaffected even in the 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 energy (λ^{E180}, λ^{E113}) in the protein is almost equal to that obtained in the gas phase.[51−54] Therefore, spectral tuning mechanisms of A_3 and A_4 pigments are also steered by strong electrostatic interaction between the retinal and its counterion.[55−76] The calculated absorption wavelengths of 490, 510, and 474 nm for A_1-, A_2-, and A_4-retinals in the Japanese common squid (*T. pacificus*) opsin agree very well with the experimental measurements of 484, 500, and 470 nm in firefly squid (*W. scintillans*) opsin.[15−17] Apparently, the binding sites of both pigments must

Table 2. Calculated First Vertical Excited States, Absorption Wavelengths, and Oscillator Strengths of Retinal Congeners

	gas phase (QM-none)		protein (QM/MM)				
	λ, nm	f, au	λ, nm	f, au	$\Delta\mu$,[a] D	λ^{abs},[b] nm	λ (exptl),[c] nm
			Squid Visual Pigments				
A_1-retinal	604	0.9	490	1.1	11.7	591	488, 484
A_2-retinal[d]	707	0.8	510	1.1	10.7	722	500
2*R*-OH-retinal	588	1.1	478	1.2	11.1	593	
3*R*-OH-A_3-retinal	586	1.0	473	1.2	11.1	581	
4*S*-OH-A_4-retinal	593	1.0	474	1.2	10.9	589	470
3*R*,4*S*-diOH-retinal	562	1.1	438	1.3	10.8	551	
			Bovine Visual Pigments				
A_1-retinal	616	1.2	495	1.4	12.1	626	498
A_2-retinal[d]	720	1.1	534	1.3	12.3	743	520
2*R*-OH-retinal	564	1.3	461	1.4	11.8	583	
3*R*-OH-A_3-retinal	592	1.2	466	1.4	11.9	611	488
4*R*-OH-A_4-retinal	593	1.2	474	1.4	11.9	613	
3*S*,4*S*-diOH-retinal	547	1.3	454	1.5	11.9	572	

[a] Calculated (SORCI+Q/MM) difference between the ground-state (S_0) and excited-state (S_1) dipole moments ($\Delta\mu$) in protein are given in Debyes. [b] λ^{E180} (squid) and λ^{E113} (bovine) 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. [c] Experimental values are taken from refs 15−17, 20, 50. 58, and 60. [d] Calculated values for A_2-retinal are taken from ref 38. The experimental value for A_1-retinal in the Japanese common squid (*T. pacificus*) is 488 nm (taken from ref 50) and in the firefly squid (*W. scintillans*) is 484 nm (taken from ref 15).

dx.doi.org/10.1021/jp208107r |*J. Phys. Chem. B* 2011, 115, 15380–15388

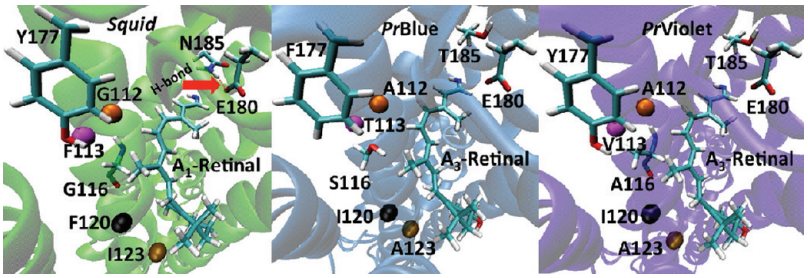


Figure 3. Retinal binding sites of squid, PrB, and PrV visual pigments. Residues at site 116, 177, 180, and 185 are shown in ball-and-stick format. Residues at site 112 (orange), 113 (magenta), 120 (black), and 123 (brown) are shown as colored circles. The residues are situated within 5.0 Å of any atom of the retinal. The red arrow indicates the presence of a hydrogen bond between N185 and E180 in squid.

be very similar to each other. The discrepancy between the calculated (466 nm) and experimental (488 nm) values for A_3-retinal in bovine opsin could be due to the fact that the experimental study was carried out with CHAPSO-solubilized bovine opsin,[20] 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, *P. 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.[24] showed that amino acid substitutions at sites 116 and 177 are crucial for spectral tuning in the PrB and PrV pigments. As already mentioned, within 5.0 Å environment of the retinal, seven sites in PrB opsin and three sites in PrV opsin differ in their amino acid 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 A_3-retinal, the experimental study was carried out with A_1-retinal,[24] and therefore, experimental λ_{max} values of PrB and PrV pigments with A_3-retinal have yet to be obtained. The fact that λ_{max} of an A_3 pigment will be slightly blue-shifted compared to that of an A_1 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 with an A_3-retinal.

To begin with, we use A_3 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 A_1-retinal. Unless and until mentioned explicitly, the following discussion on the topic of spectral tuning will correspond to λ_{max} of the A_3-retinal.

4.1. Structural Rearrangements. On one hand, replacement of A_1-retinal with A_3-retinal induces a slight increase in distance between the Cα atom of G116 and the C19 atom of the retinal (3.55 → 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 Cα atom of G116 and the C19 atom by 0.49 Å (3.57 → 4.06 Å) and between the -OH group of Tyr177 and the C19 atom by 0.84 Å (4.47 → 5.31 Å). Notice that a hydrogen bond temporarily formed between S116 and Y177 is lost due to Y177F mutation (see Figure 4).

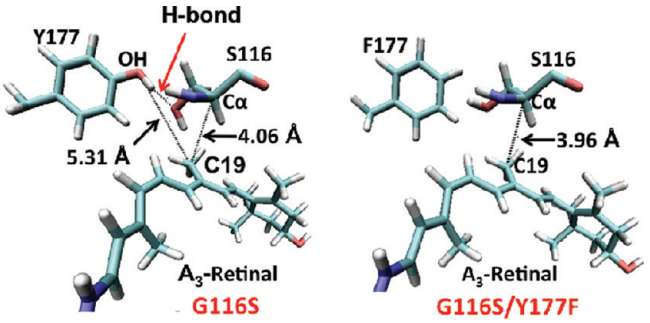


Figure 4. Structural changes due to G116S substitution (left) and Y177F substitution (right) in PrB pigment.

Substitution of tyrosine with phenylalanine at site 177 draws the Cα atom of S116 close to the C19 atom by 0.10 Å (4.06 → 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 at site 185, it induces a red shift of 6 nm at site 113.

4.2. Single Mutant Spectra. Performing single-mutant calculation helps in tracking the structural rearrangements and the 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 (Table 3). 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 to 453 nm. To examine whether serine can induce the blue shift from any site, we substituted serine at all 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 A_3-retinal and above 475 nm with A_1-retinal (Table 3). 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.

Table 3. Absorption Wavelengths and Oscillator and Rotatory Strengths of A_3- and A_1-Retinal in Protein Environments of Squid, PrB, and PrV Pigments That Contains One, Two, Three and Seven Mutations at a Time

	calcd A_3-retinal			calcd A_1-retinal			
	λ, nm	f, au	R, au	λ, nm	f, au	R, au	exptl λ_{max} (A_1-retinal),[a] nm
squid wild type	473	1.22	0.46	490	1.14	0.32	488
G112A	483	1.19	0.41	493	1.20	0.43	
F113T	479	1.21	0.46	492	1.19	0.48	
G116S	453	1.21	0.46	457	1.23	0.49	
F120I	479	1.29	0.39	491	1.20	0.39	
I123A	484	1.19	0.44	493	1.20	0.45	
Y177F	481	1.20	0.48	494	1.19	0.49	
N185T	452	1.23	0.35	467	1.19	0.28	
G112S	457	1.21	0.53	468	1.21	0.54	
F113S	473	1.21	0.48	487	1.20	0.48	
F120S	476	1.23	0.44	489	1.19	0.48	
I123S	483	1.24	0.44	487	1.19	0.48	
Y177S	470	1.21	0.54	488	1.19	0.44	
N185S	450	1.24	0.31	463	1.22	0.35	
G116S/G112A	455	1.20	0.51	477	1.23	0.40	
G116S/F113T	467	1.22	0.43	480	1.21	0.42	
G116S/F120I	465	1.21	0.41	482	1.22	0.38	
G116S/I123A	457	1.21	0.47	475	1.14	0.44	
G116S/Y177F	446	1.24	0.48	459	1.22	0.51	
G116S/N185T	446	1.24	0.46	456	1.23	0.40	
N185T/G112A	458	1.19	0.29	470	1.19	0.29	
N185T/F113T	466	1.19	0.31	467	1.20	0.27	
N185T/F120I	461	1.23	0.24	471	1.22	0.25	
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/F120I/ 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/T113 V/S116A/F120/ I123A/F177Y/N185T (P rV)	431	1.30	0.41	442	1.28	0.38	420

[a] Note that native PrB and PrV pigments contain only A_3-retinal, and experimental values for native A_3-PrB and A_3-PrV pigments have yet to be obtained.

Experimental studies have already shown G116S/Y177F mutations 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

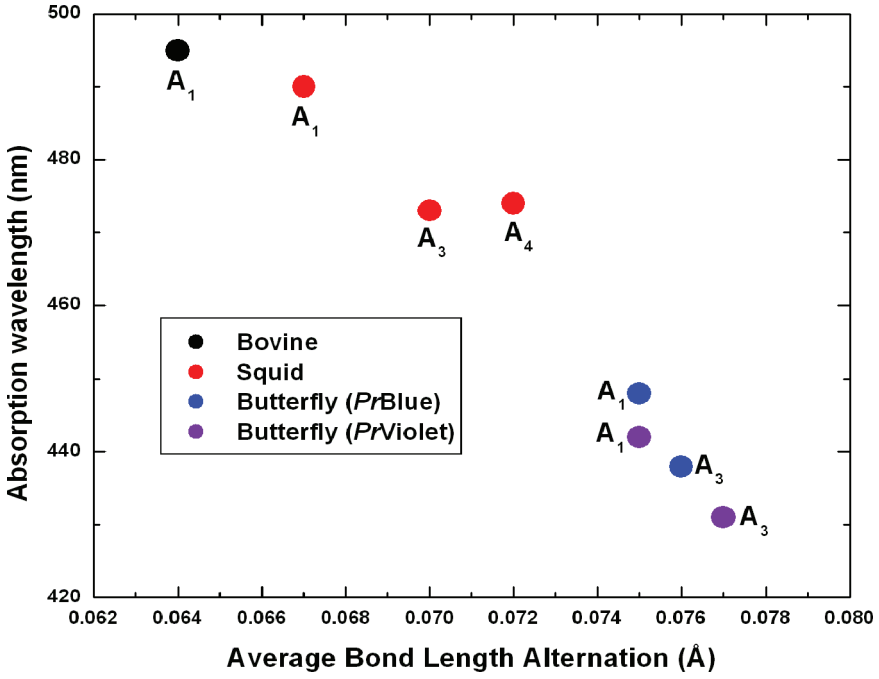


Figure 5. Correlation between the average BLA and absorption wavelength calculated for A_1, A_3, and A_4 pigments in bovine, squid, and butterfly.

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 (Table 3). This shows that G116S and N185T play a crucial role in blue-shifting 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 λ_{max} by almost 25 nm, while leaving out N185T (G116S/I123A/Y177F) also red-shifts λ_{max} but by ~15 nm (Table 3). 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 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 contain only an A_3-retinal and not an A_1-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, and 177) that differ between PrB and PrV pigments, mutation of threonine to valine at site 113 (T113V) and tyrosine to phenylalanine at site 177 (Y177F) induces a slight blue shift of ~3 nm individually. In contrast, mutation of serine to alanine at site 116 (S116A) induces a red shift of 10 nm in agreement with experimental findings.[24] Apparently, only half the blue shift (20 nm) induced by S116 in PrB is recovered with A116 in PrV. The calculated spectral shifts are nonadditive, as introduction of all three mutants decreases the λ_{max} by only 7 nm (Table 3).

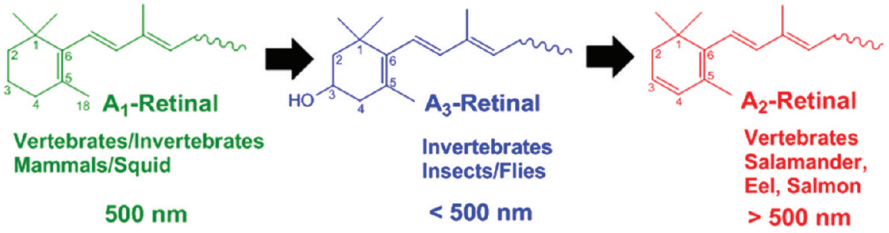
Although the calculations show relatively large deviations from experimental measurements for A_1-PrV pigment (442 versus 420 nm), there is a reasonable agreement when the A_3-PrV pigment value of 431 nm is taken into account. It is possible that the structures of PrB and PrV pigments are not identical 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.[77]

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 λ_{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 (Table 3). 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 into vertebrate (bovine) and invertebrate (squid) opsins. We show that the 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 λ_{max} due to increase in BLA of the hydroxyretinal.

Scheme 2. Evolutionary Pathway of A_3-Retinal in Insects



Firefly squid (*W. scintillans*), which contains three visual pigments with different chromophores, absorbs light at 470 nm by use of 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 (*T. pacificus*) opsin. Replacement of A_1- with A_4-retinal does not alter the architecture of the 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 *P. rapae* blue- (PrB) and *P. rapae* violet- (PrV) absorbing visual pigments. Experimental observations were based on the values obtained with A_1-retinal and not A_3-retinal; also, only two sites, 116 and 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 λ_{max} from 473 nm in squid opsin to 438 nm in PrB opsin and 431 nm in PrV opsin.

Due to the fact that no major difference was found between the binding sites of A_1-, A_3-, and A_4-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 A_2- over A_1-retinal, which absorbs light at longer wavelengths, has been restricted to freshwater fishes while migrating from salt to fresh water.[78] However, no such correlation has been established for the selection of A_3- over A_1-retinal in insects. Our results suggest that A_3-retinal may have occurred as a hydroxy intermediate during the conversion of A_1- to A_2-retinal via an enzymatic and/or metabolic pathway (Scheme 2), 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.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Additional text, two figures, and four tables with computational details, chromophore−counterion distances, distance of the Schiff-base nitrogen to key amino acid residues, geometric parameters of A_1-, A_3-, and A_4-retinal derivatives, calculated vertical ($S_0 \rightarrow S_2$) excited-state energies, complete ref 29, and Cartesian coordinates of all retinal models discussed in this study. This material is available free of charge via the Internet at http://pubs.acs.org

■ AUTHOR INFORMATION

Corresponding Author
*E-mail morokuma@emory.edu.

■ ACKNOWLEDGMENT

We thank Dr. Tetsuji Okada for bringing the topic of A_3-retinal to our attention and Dr. Kentaro Arikawa for the valuable discussions. The work at Emory is supported in part by a grant from the National Institutes of Health (R01EY016400) and at Kyoto by a JST Core Research for Evolutional Science and Technology (CREST) grant in the Area of High Performance Computing for Multiscale and Multiphysics Phenomena.

■ REFERENCES

(1) Wald, G. *J. Gen. Physiol.* **1936**, *19*, 351–371.
(2) Wald, G. *J. Gen. Physiol.* **1936**, *20*, 45–56.
(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.
(6) Vogt., K. *Z. Naturforsch., C: J. Biosci.* **1983**, *38*, 329–333.
(7) Vogt., K.; Kirschfeld., K. *Naturwissenschaften* **1984**, *71*, 211–213.
(8) Goldsmith, T. H.; Marks, B. C.; Bernard, G. D. *Vision Res.* **1986**, *26*, 1763–1769.
(9) Seki, T.; Fujishita, S.; Ito, M.; Matsuoka, N.; Kobayashi, C.; Tsukida, K. *Vision Res.* **1986**, *26*, 255–258.
(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.
(12) Gleadall, I. G.; Hariyama, T.; Tsukahara, Y. *J. Insect Physiol.* **1989**, *35*, 787–795.
(13) Suzuki, T.; Makino-Tasaka, M.; Miyata, S. *Vision Res.* **1985**, *25*, 149–154.
(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., J. C.; Seidou, M.; Narita, K.; Hamanaka, T.; Michinomae, M.; Sekiya, N.; Yoshihara, K. *Vision Res.* **1992**, *32*, 3–10.
(17) Michinomae, M.; Masuda, H.; Seidou, M; Kito, Y. *J. Exp. Biol.* **1994**, *193*, 1–12.
(18) Yokoyama, S.; Yokoyama R. In *Handbook of Biological Physics*, Vol. 3; Stavenga, D. G., Degrip, W. J., Pugh, E. N, Jr., Eds.; Elsevier: Amsterdam, 2000; Chapt. 6, pp 257−296.

(19) Gaertner. W. In *Handbook of Biological Physics*, Vol. 3; Stavenga, D. G.,Degrip, W. J.,Pugh, E. N., Jr.; Elsevier: Amsterdam, 2000; Chapt. 7, pp 297−388.
(20) Gaertner, W.; Ullrich, D.; Vogt, K. *Photochem. Photobiol.* **1991**, *54*, 1047–1055.
(21) Renk, G.; Grover, T.; Crouch, R.; Mao, B.; Ebrey, T. G. *Photochem. Photobiol.* **1981**, *33*, 489–494.
(22) Arikawa, K.; Inokuma, K.; Eguchi, E. *Naturwissenschaften* **1987**, *74*, 297–298.
(23) Arikawa, K.; Mizuno, S.; Scholten, D. G. W.; Kinoshita, M.; Seki, T.; Kitamoto, J.; Stavenga, D. G. *Vision Res.* **1999**, *39*, 1–8.
(24) Wakakuwa, M.; Terakita, A.; Koyanagi, M.; Stavenga, D. G.; Shichida, Y.; Arikawa, K. *PLoS One* **2010**, *5*, No. e15015.
(25) Renk, G.; Crouch, R. K. *Biochemistry* **1989**, *28*, 907–912.
(26) Sekharan, S.; Altun, A.; Morokuma, K. *Chem.—Eur. J.* **2010**, *16*, 1744–1749.
(27) Altun, A.; Yokoyama, S.; Morokuma, K. *J. Phys. Chem. B* **2008**, *112*, 6814–6827.
(28) Vreven, T.; Byun, K. S.; Komaromi, I.; Dapprich, S.; Montgomery, J. A., Jr.; Morokuma, K.; Frisch, M. J. *J. Chem. Theor. Comput.* **2006**, *2*, 815–826.
(29) Frisch, M. J., et al. Gaussian 09, Revision A.02; Gaussian, Inc.: Wallingford, CT, 2009.
(30) Murakami, M.; Kouyama, T. *Nature* **2008**, *453*, 363–367.
(31) Vought, B. W.; Salcedo., E.; Chadwell, L. V.; Britt, S. G.; Birge., R. R.; Knox., B. E. *Biochemistry* **2000**, *39*, 14128–14137.
(32) Neese, F. *ORCA, an ab-initio, DFT and semiempirical electronic structure package*, version 2.6, revision 19, 2007. Latest version available at http://www.thch.uni-bonn.de/tc/orca.
(33) Altun, A.; Yokoyama, S.; Morokuma, K. *J. Phys. Chem. B* **2008**, *112*, 16883–16890.
(34) Sekharan, S.; Morokuma, K. *J. Am. Chem. Soc.* **2011**, *133*, 4734–4737.
(35) Ahuja, S.; Hornak, V.; Yan, E. C.; Syrett, N.; Goncalves, J. A.; Hirshfeld, A.; Ziliox, M.; Sakmar, T. P.; Sheves, M.; Reeves, P. J.; Smith, S. O.; Eilers, M. *Nat. Struct. Mol. Biol.* **2009**, *16*, 168–175.
(36) Ota, T.; Furutani, Y.; Terakita, A.; Yoshinori, S.; Kandori, H. *Biochemistry* **2006**, *45*, 2845–2851.
(37) Sekharan, S.; Morokuma, K. *J. Phys. Chem. Lett.* **2010**, *1*, 668–672.
(38) Sekharan, S.; Altun, A.; Morokuma, K. *J. Am. Chem. Soc.* **2010**, *132*, 15856–15859.
(39) Seki, T.; Isono, K.; Ito, M.; Katsuta, Y. *Eur. J. Biochem.* **1994**, *226*, 691–696.
(40) Nathans, J.; Thomas, D.; Hogness, D. S. *Science* **1986**, *232*, 193–202.
(41) Yokoyama, R.; Yokoyama, S. *Proc. Nat. Acad. Sci. U.S.A.* **1990**, *87*, 9315–9318.
(42) Neitz, M.; Neitz, J.; Jacobs, G. H. *Science* **1991**, *252*, 971–974.
(43) Chan, T.; Lee, M.; Sakmar, T. P. *J. Biol. Chem.* **1992**, *267*, 9478–9480.
(44) Asenjo, A. B.; Rim, J.; Oprian, D. D. *Neuron* **1994**, *12*, 1131–1138.
(45) Kochendoerfer, G. G.; Wang, Z.; Oprian, D. D.; Mathies, R. A. *Biochemistry* **1997**, *36*, 6577–6587.
(46) Kochendoerfer, G. G.; Lin, S. W.; Sakmar, T. P.; Mathies, R. A. *Trends. Biochem. Sci.* **1999**, *24*, 300–305.
(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, T. L.; Ramos, L. S.; Galan, J. F.; Birge, R. R. *Biochemistry* **2008**, *47*, 4614–4620.
(50) Mathies, R. A.; Styrer, L. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 2169–2176.
(51) Andersen, L. H.; Nielsen, I. B.; Kristensen, M. B.; El Ghazaly, M. O. A.; Haacke, S.; Brondsted Nielsen, M.; Petersen, M. A. *J. Am. Chem. Soc.* **2005**, *127*, 12347–12350.
(52) Nielsen, I. B.; Lammich, L.; Andersen, L. H. *Phys. Rev. Lett.* **2006**, *96*, No. 018304.
(53) Sekharan, S.; Weingart, O.; Buss., V. *Biophys. J.* **2006**, *91*, L07–L09.
(54) Send, R.; Sundholm, D. *Phys. Chem. Chem. Phys.* **2007**, *9*, 2862–2867.
(55) Arnaboldi, M.; Motto, M. G.; 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, R. R.; Murray, L. P.; Pierce, B. M.; Akita, H.; Balogh-Nair, V.; Findsen, L. A.; Nakanishi., K. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 4117–4121.
(58) Einterz, C. M.; Lewis, J. W.; Kliger, D. S. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 3699–3703.
(59) Sakmar, T. P.; Franke, R. R.; Khorana, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 8309–8313.
(60) Zhukovsky, E. A.; Oprian, D. D. *Science* **1989**, *246*, 928–930.
(61) Nathans, J. *Biochemistry* **1990**, *29*, 9746–9752.
(62) Makino, C. L.; Kraft, T. W.; Mathies, R. A.; Lugtenburg, J.; Miley, M. E.; van der Steen, R.; Baylor, D. A. *J. Physiol.* **1990**, *424*, 545–560.
(63) Fahmy, K.; Jager, F.; Beck, M.; Zvyaga, T. A.; Sakmar, T. P.; Siebert, F. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10206–10210.
(64) Makino, C. L.; Groesbeek, M.; Lugtenburg, J.; Baylor, D. A. *Biophys. J.* **1999**, *77*, 1024–1035.
(65) Gascon, J. A.; Batista, V. S. *Biophys. J.* **2004**, *87*, 2931–2941.
(66) Wanko, M.; Hoffmann, M.; Strodel, P.; Kosolowski, A.; Thiel, W.; Neese, F.; Frauhenheim, T.; Elstner, M. *J. Phys. Chem. B* **2005**, *109*, 3606–3615.
(67) Gascon, J. A.; Sproviero, E. M.; Batista, V. S. *Acc. Chem. Res.* **2006**, *39*, 184–193.
(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.
(70) DeGrip, W. J.; Bovee-Geurts, P. H. M.; van der Hoef, I.; Lugtenburg, J. *J. Am. Chem. Soc.* **2007**, *129*, 13265–13269.
(71) Fujimoto, K.; Hayashi, S.; Hasegawa, J.; Nakatsuji, H. *J. Chem. Theory Comput.* **2007**, *3*, 605–618.
(72) Montero-Cabrera, M. A.; Rohrig, U.; Padron-Garcia, J. A.; Crespo-Otero, R.; Montero-Alejo, A. L.; Garcia de la Vega, J. M.; Chergui, M.; Rothlisberger, U. *J. Chem. Phys.* **2007**, *127*, No. 145102.
(73) Sekharan, S.; Buss, V. *J. Am. Chem. Soc.* **2008**, *130*, 17220–17221.
(74) Sekharan, S. *Photochem. Photobiol.* **2009**, *85*, 517–520.
(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.
(76) Frahmcke, J. S.; Wanko, M.; Phatak, P.; Mroginski, M. A.; Elstner, M. *J. Phys. Chem. B* **2010**, *114*, 11338–11352.
(77) Altun, A.; Morokuma, K.; Yokoyama, S. *ACS Chem. Biol.* **2011**, *6*, 775–780.
(78) Bridges, C. D. B. In *Handbook of Sensory Physiology VII/I*; Dartnall, H. J. A, Ed.; Springer: New York, 1972; Chapt. 11, pp 417−480.