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Shozo Yokoyama, *Emory University*
Takashi Tada, *Emory University*
Takahisa Yamato, *Nagoya University*

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Modulation of the Absorption Maximum of Rhodopsin by Amino Acids in the C-terminus†

Shozo Yokoyama[*,1], **Takashi Tada**[1], and **Takahisa Yamato**[2]

1 *Department of Biology, Emory University, Atlanta, GA*

2 *Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Japan*

Abstract

Vision begins when light is absorbed by visual pigments. It is commonly believed that the absorption spectra of visual pigments are modulated by interactions between the retinal and amino acids within or near 4.5 Å of the retinal in the transmembrane (TM) segments. However, this dogma has not been rigorously tested. In this study, we show that the retinal-opsin interactions extend well beyond the retinal binding pocket. We found that, although it is positioned outside of TM segments, the C-terminus of the rhodopsin in the rockfish longspine thornyhead (*Sebastolobus altivelis*) modulates its λ_{max} by interacting mainly with the last TM segment. Our results illustrate how amino acids in the C-terminus are likely to interact with the retinal. We anticipate our analyses to be a starting point for viewing the spectral tuning of visual pigments as interactions between the retinal and key amino acids that are distributed throughout the entire pigment.

INTRODUCTION

To adapt to diverse photic environments they encountered, organisms adjusted the absorption spectra of their visual pigments (1–3). In vertebrates, four gene duplication events have generated five pigment groups in the retina: (1) rhodopsin (RH1); (2) RH1-like (RH2); (3) short wavelength-sensitive type 1 (SWS1); (4) SWS type 2 (SWS2); and (5) middle and long wavelength-sensitive (M/LWS) pigments (3–6). When it is linked to a protonated Schiff base, the photosensitive molecule, 11-*cis*-retinal, absorbs light maximally (λ_{max}) at 440 nm (7). By interacting with various opsins, however, the retinal in different visual pigments has a λ_{max} between 360 and 570 nm; that is, currently known RH1, RH2, SWS1, SWS2 and M/LWS pigments have λ_{max}'s of 480–520, 460–510, 360–440, 410–460 and 510–570 nm, respectively (5,6,8). To date, amino acid changes at a total of 25 sites are known to be involved in the spectral tuning of these visual pigments and are located within or near the retinal binding pocket (4,6,8–21) (Fig. 1). Of the 25 critical sites, site 292 in transmembrane (TM) VII is unique because identical amino acid changes at this site cause a variable level of λ_{max}-shifts in different visual pigments. For example, A292S (substitution of serine for alanine at position 292) in the bovine RH1 pigment (11,22–24) and new SWS2 pigment (9) decrease the λ_{max} by 5–10 nm, while S292A in the coelacanth RH1 pigment (12), in human SWS1 pigment (25) and in M/LWS pigments of bottlenose dolphin (22) and mouse (11) increase it by 0–28 nm. Thus, the amino acid changes at site 292 cause the λ_{max}-shift by 0–30 nm, the extent of which is determined by interactions with other amino acids.

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*Corresponding author email: syokoya@emory.edu (Shozo Yokoyama).

From these and other mutagenesis results as well as the crystal structural analyses of the bovine rhodopsin (26–29), it is commonly believed that variable λ_{max}'s of visual pigments have been achieved by the retinal-opsin interactions within or near 4.5 Å of the retinal (9,10,26–30). This hypothesis has been difficult to test because it is not easy to evaluate precise interactions between amino acids which are located inside and outside of the retinal binding pocket. Here we compare the rhodopsins of bovine (*Bos taurus*) and rockfish longspine thornyhead (*Sebastolobus altivelis*) which have serine and alanine at site 292, respectively, and, as we will see below, the latter pigment has a λ_{max} of 483 nm which is one of the lowest for the RH1 pigments. These differences in the two pigments provide an excellent opportunity to explore the precise interactions among amino acids at site 292 and those at other sites. The difficulty in assessing the interactions between amino acids in the retinal binding pocket and others was overcome by subdividing the two pigments into four segments, one of which is site 292, and constructing chimeric pigments with all combinations of the four segments. Our results show that not only critical amino acids in the retinal binding pocket but also those in the C-terminus are likewise involved in the spectral tuning of rhodopsins by interacting with TM VII.

MATERIALS AND METHODS

The bovine and thornyhead rhodopsin genes

The bovine rhodopsin has been engineered previously (23). The thornyhead (*S. altivelis*) was caught off the West coast of the US by the National Oceanic and Atmospheric Administration (NOAA) and sent to us by Mr. Lyle Britt, Alaska Fisheries Service Center, Seattle, WA, USA. High molecular weight DNA was isolated from body tissue of the fish using a standard phenol–chloroform extraction procedure. The coding region of nucleotide sites 157–943 of the intron-less thornyhead rhodopsin gene was amplified using forward primer (5′-GGMTTCCCYRTYAACTTYCTSACNCT-3′) and reverse primer (5′ CCRCAGCACARNGTGGTGATCATGCAGT-3′). The polymerase chain reaction (PCR) products in the pBluescript SK(–) vector were sequenced by using a LI-COR automated DNA sequencer. To clone the remaining coding regions, inverse PCR was performed using the thornyhead gene-specific reverse primer (5′-AT-CATGGTCGTCGCCTTCCTGGTATGTTGG-3′) and forward primer (5′-AGCAGGATGTAGTTTAGAGGGGTCCG-3′), which are complementary to bases 205–230 and are identical to bases 766–795, respectively. These primers, facing outwards from the known DNA segments, were used to carry out PCR amplification with the thornyhead genomic DNA.

In vitro assays

Various opsins were expressed in COS1 cells by transient transfection, incubated with 11-*cis*-retinal in the dark, and the resulting rhodopsins were purified and their λ_{max}'s were measured in the dark using a spectrophotometer (23). Five spectral measures were obtained for the bovine, thornyhead and 14 chimeric pigments.

It should be cautioned that to purify the regenerated visual pigments in our *in vitro* assay, all pigments are designed to contain the amino acids at positions 334–348 in the C-terminus of bovine rhodopsin. Thus, compared with those that contain the C-terminus of bovine pigment, pigments that contain the C-terminus of thornyhead rhodopsin have the 15 extra amino acids. This extra sequence, however, is not expected to affect the evaluation of the λ_{max}'s of visual pigments because the λ_{max}'s evaluated using the *in vitro* assay and those by other methods such as electroretinogram flicker photometry and microspectrophotometry are generally in good agreement (23). We also evaluated the effect of having the extra 15 amino acids on the λ_{max}-shift by adding them to the C-termini of chimeric pigments TBB and TTB (see Results and Discussion). The λ_{max}'s of these two pigments with the repeated amino acid sequences were

492 ± 0 and 481.8 ± 0.4, respectively, and were identical to those of the unmodified corresponding chimeric pigments TBB and TTB.

Chimeras and point mutations

Chimeras between the bovine and thornyhead pigments were constructed by using restriction enzymes *Bsp*HI and *Bsa*BI, which digest the two genes between codons 277 and 278 and between codons 306 and 307, respectively (Fig. 2). Amino acid changes A292S and S292A were generated using Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

RESULTS AND DISCUSSION

The complete rhodopsin gene of the thornyhead has been cloned using PCR and inverse PCR methods and is sequenced. The amino acid sequence deduced from this newly found thornyhead gene consists of 352 residues and has 76% identity to that of the orthologous bovine rhodopsin (Fig. 2). The *in vitro* assay shows that the bovine and thornyhead rhodopsins have λ_{max}'s of 500 nm (11,22–24,31) and 483 nm, respectively (Fig. 3), and the latter value is lowest among the currently known λ_{max}'s of rhodopsins. Rhodopsins in a wide range of species, including the bovine rhodopsin, have λ_{max}'s of ~500 nm (5,8) and, furthermore, the engineered ancestral pigment of the thornyhead and closely related deep-sea fishes shows that it had a λ_{max} of ~500 nm (T. Tada and S. Yokoyama, unpublished data). Therefore, the thornyhead pigment must have modified its λ_{max} from ~500 to 483 nm to adapt to its photic environment at the water depth of 300–1500 m (32). The bovine and thornyhead rhodopsins contain amino acids A292 and S292, respectively, and the latter pigment has four extra amino acids in the C-terminus (Fig. 2).

Without knowing the amino acids that are involved in the spectral tuning of visual pigments, the construction of chimeric pigments is the most efficient way to study the effects of amino acid interactions on the λ_{max}-shift (11,13,14). To evaluate the interactions between amino acids at site 292 and those at other sites in the bovine and thornyhead rhodopsins, we subdivided them into residues 1–277, 278–306 excluding 292, 307–348 and residue 292. The first three segments contain TM I–VI, TM VII excluding site 292 and C-terminus, respectively (Fig. 2), and are designated simply as TM I–VI, TM VII* and C-terminus, where a star (*) indicates the exclusion of site 292 and C-terminus contains a short helix (VIII) between residues 311 and 323 (33). Based on the four segments of bovine (B) and thornyhead (T) rhodopsins, the respective pigments can be designated simply as BBB and TTT, where B and T in the middle denote the combined segments of the TM VII* and site 292 of bovine and thornyhead pigments, respectively.

We then denote the effects of replacing the TM I–VI, TM VII*, C-terminus and site 292 of bovine pigment by those of thornyhead pigment on the λ_{max}-shift by $\theta_{I–VI}$, θ_{VII*}, θ_{C} and θ_{A292S}, respectively. We also consider a total of 11 possible synergistic effects of the four segments on the λ_{max}-shift: 6 two-way, 4 three-way and 1 four-way interactions of the four segments. Therefore, in order to evaluate these 15 parameters, we have to evaluate the absorption spectra of the bovine and thornyhead pigments and all 14 chimeric pigments: TBB, $BT^{A}B$, BBT, $TT^{A}B$, TBT, $BT^{A}T$, $TT^{A}T$, $BB^{S}B$, $TB^{S}B$, BTB, $BB^{S}T$, TTB, $TB^{S}T$ and BTT, where T^{A} and B^{S} indicate that S292A and A292S have been generated in thornyhead and bovine pigments, respectively. Then, each observed λ_{max} can be expressed as a function of the λ_{max} of bovine pigment (Z) and these individual and synergistic effects of the four segments on the λ_{max}-shift (Fig. 4a). In this formulation, the meaning of the interaction between site 292 and the C-terminus may not be immediately clear because they are not connected directly. However, the two segments can still interact with each other simply because TM VII forms a bridge between them without participating in the spectral tuning of the thornyhead rhodopsin.

The success of this approach depends entirely on whether or not we can evaluate the absorption spectra of these 14 chimeric pigments. Fortunately, we could obtain highly reliable λ_{max}'s of these chimeric pigments, each having a standard error of <0.5 nm (Fig. 3).

The parameters estimated in this way reveal three major features of the spectral tuning in the thornyhead rhodopsin (Fig. 4b). First, the effects of TM I–VI and A292S are largest, decreasing the λ_{max} of bovine pigment by 8 and 10 nm, respectively. TM I–VI of the thornyhead pigment contains an amino acid replacement, D83 N, which can decrease the λ_{max} by 0–9 nm (9). On the other hand, when N83D is introduced into the thornyhead rhodopsin, the mutant pigment increases the λ_{max} by 4 nm (data not shown). Thus, D83N may explain a significant portion of $\theta_{I–VI}$. Second, site 292 does not interact directly with TM I–VI ($\theta_{I–VI\ xA292S}$ = 0 nm), but it interacts with TM VII* and C-terminus ($\theta_{VII*xA292S}$ =−3, $\theta_{CxA292S}$ =−0.8 and $\theta_{VII*xCxA292S}$ = 2.8 nm; all significantly different from zero with $P < 10^{-5}$). Third, together with site 292, TM I–VI contributes to the spectral tuning only through three- and four-way interactions ($\theta_{I–VIxVII*xA292S}$ = 0.8, $\theta_{I–VIxCxA292S}$ = 1.6, and $\theta_{I–VIxVII*CxA292S}$ =−2.2 nm; all significantly different from zero with $P < 10^{-3}$).

Note that the spectral difference between the bovine and thornyhead pigments is 17 nm, while $\theta_{I–VI}$ and θ_{A292S} are added up to −18 nm. Thus, it may seem that no amino acid interaction should be operating, but our results clearly show that this is not the case. This resembles the results of a series of chimeric pigments between the violet pigment in African clawed frog (*Xenopus laevis*) and its ancestral UV pigment. Their absorption spectra show that the spectral difference between the two pigments can be explained fully by the amino acid differences in TM I–III; at the same time, however, strong interactions between TM I–III and TM IV–VII also exist (15). The most surprising result is the involvement of the C-terminus in the spectral tuning; the C-terminus shifts the λ_{max} by ~3 and ~2 nm by interacting with TM VII and with the rest of the thornyhead pigment, respectively. This is totally unexpected not only because the previous extensive mutagenesis analyses did not uncover any such effects (34–36) but also because it is so distantly located from the retinal binding pocket (26–29). The two pigments contain a total of 18 different amino acids, including six indels, in their C-termini. Thus, some of these structural differences are involved in the spectral tuning of the thornyhead rhodopsin.

These results reject the conventional wisdom that the spectral tuning of visual pigments is based only on interactions between the retinal and amino acids in the retinal binding pocket. Instead, we conclude that in addition to critical amino acids in the neighborhood of the retinal, those in the C-terminus are likewise involved in the spectral tuning mostly by interacting with TM VII. Similar interactions between amino acids that cause major λ_{max}-shifts and those with much smaller individual effects have also been detected in other groups of visual pigments. For example, the interactions among amino acids at sites 164, 261 and 269 and four other sites explain the spectral shift from green pigment to red pigment in human (37,38). F86S and its interactions with amino acids at two other sites with minor effects have also been responsible for the functional differentiation of the elephant blue pigment (39).

It is suspected that the short helix VIII in the C-terminus not only allows attachment of the transducin trimer on the surface of a photoactivated rhodopsin but also affects rhodopsin regeneration (33). The remaining segment of the C-terminus may be necessary for the transport of rhodopsin from the site of synthesis to the rod outer segment (33). In addition, amino acids T94, E113, E181, S186 and two water molecules form the "hydrogen bonded network" and stabilize the charge distribution of the retinal and keep the Schiff base nitrogen atom protonated in the dark state (29) (Fig. 5). In order to evaluate the relative positions of these molecules in the thornyhead rhodopsin, its tertiary structure was modeled by applying Modeller v8.1 (40) to the bovine rhodopsin (PDB code 1U19; [29]) as the template. Five top models from the ensemble of 100 models were selected on the basis of best fit to the restraints and an assessment

of stereochemical quality was then performed using the PROCHECK program (41) to determine an overall best model. The result shows that not only are the four amino acids and two water molecules closely located to the Schiffbase nitrogen but also site 292 is very close to the "hydrogen bonded network" (Fig. 5). Thus, it is conceivable that critical amino acids in C-terminus and TM VII modulate the functions of the "hydrogen bonded network" through site 292.

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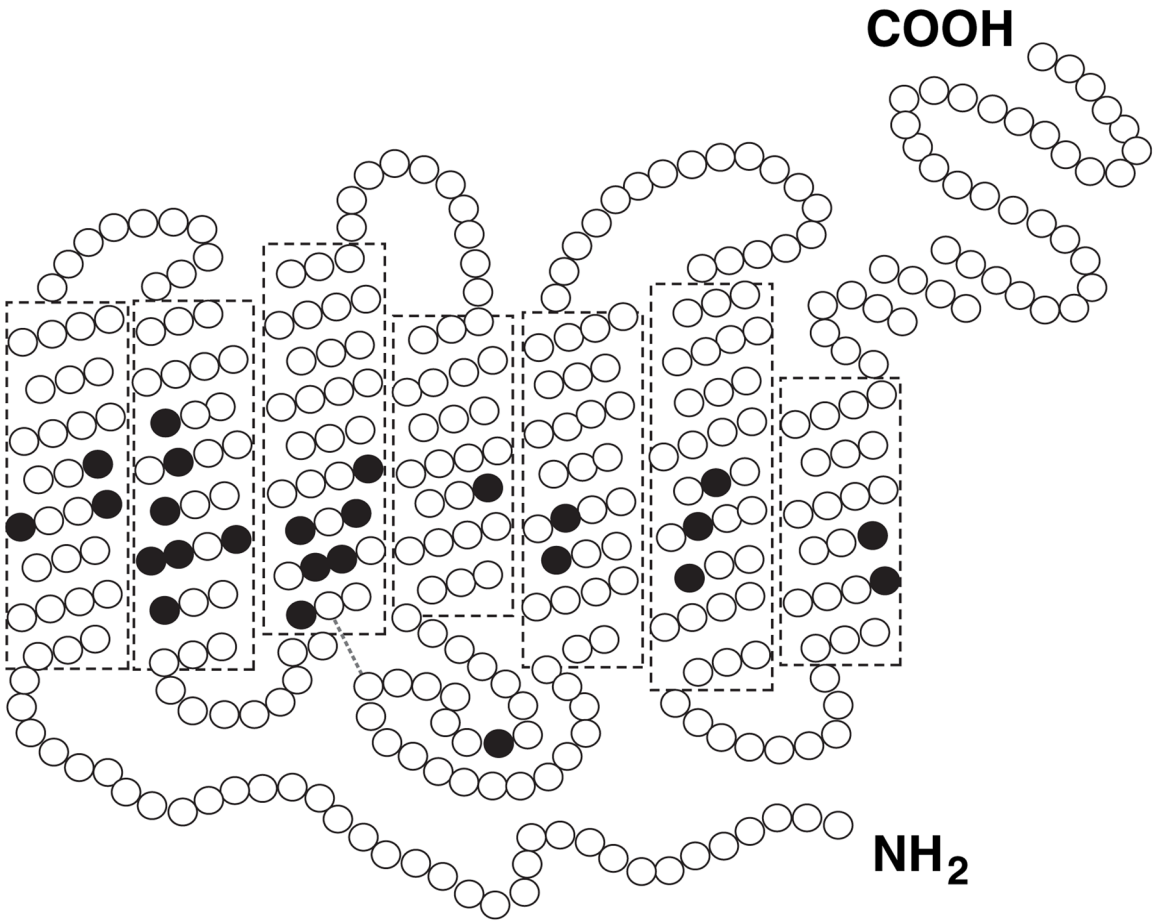


Figure 1.
Amino acid residues that are known to be involved in shifting the λ_{max}'s of five paralogous groups of visual pigments (black circles) (4–6,8–21). The topology of the bovine rhodopsin is based on Palczewski *et al.* (26).

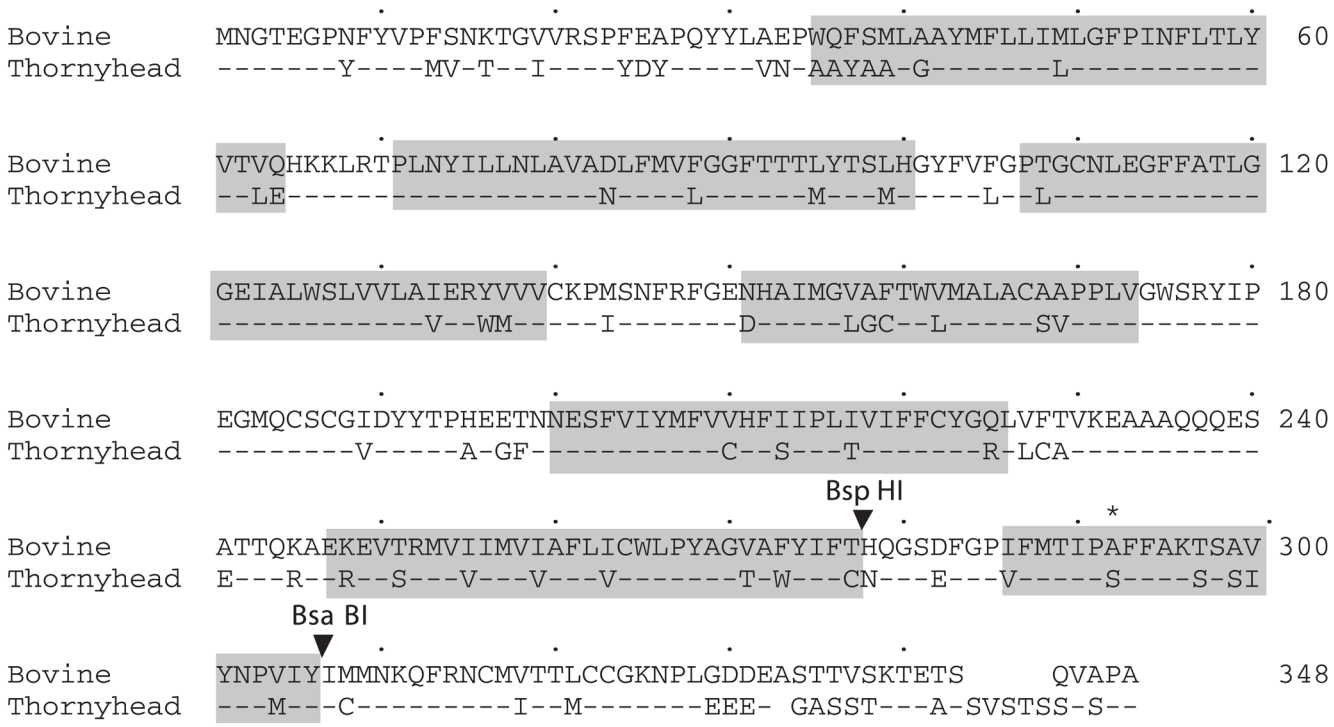


Figure 2.

The aligned amino acid sequences of bovine and thornyhead rhodopsins: –, amino acid identity between the two pigments; *, site 292. Seven TM segments are indicated by grey rectangles (26). The sequence data of the thornyhead RH1 opsin gene has been deposited with the EMBL/GenBank Data Libraries under accession no. DQ490124.

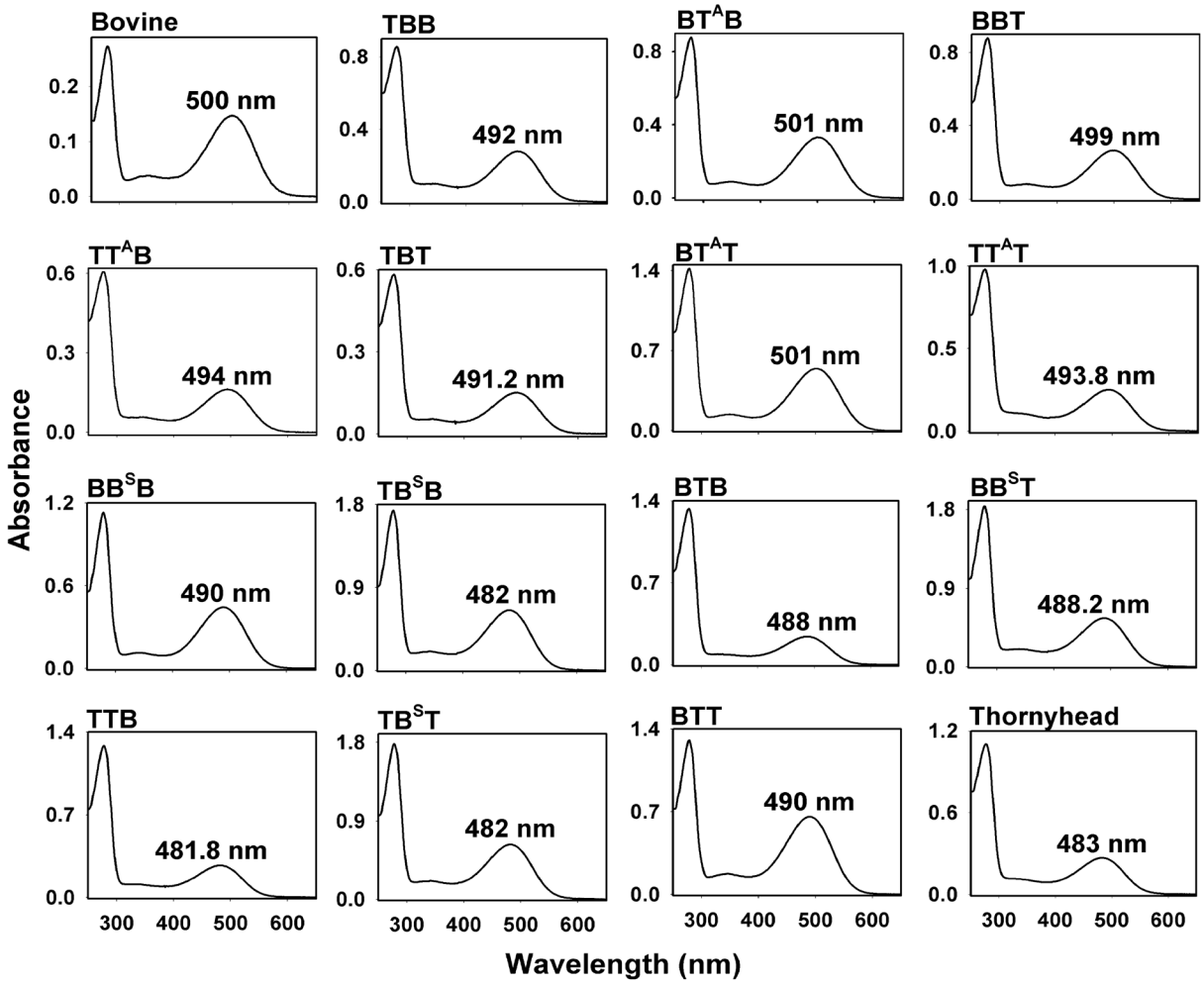
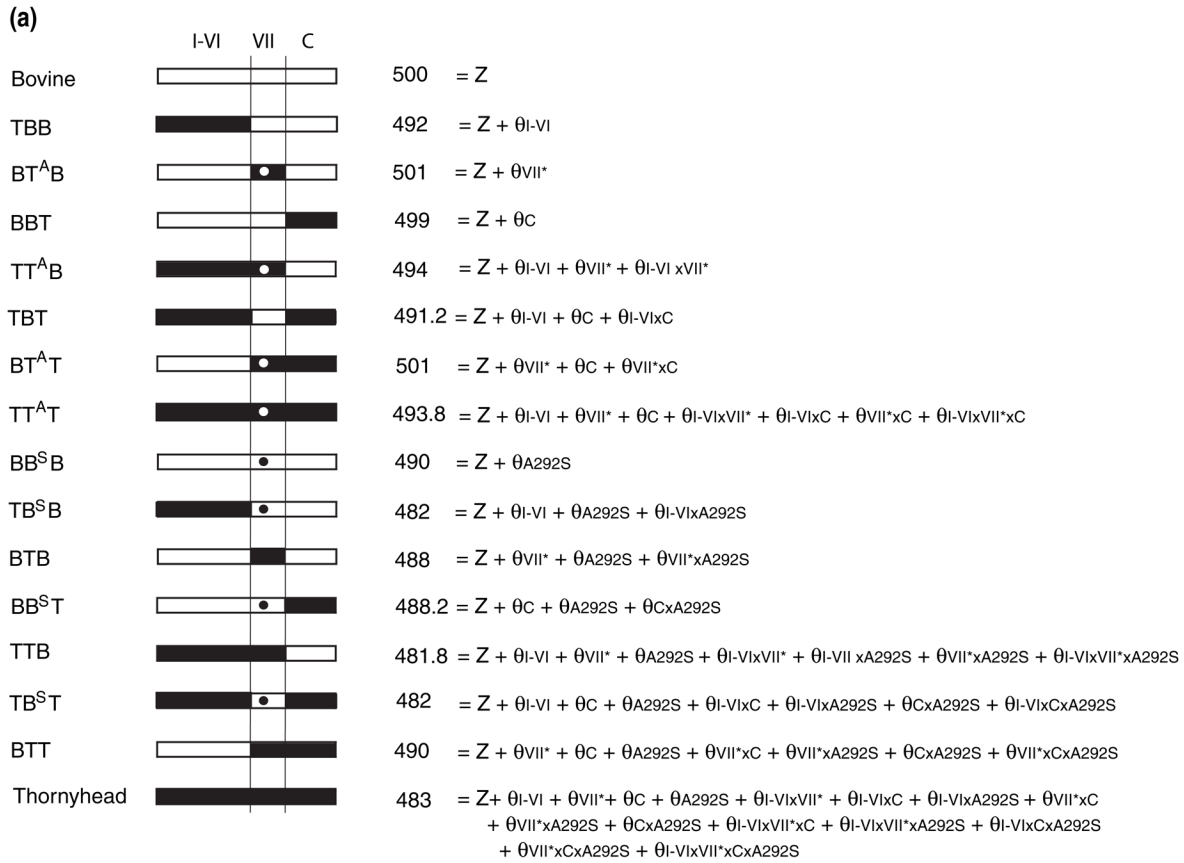


Figure 3.

The absorption spectra of bovine, thornyhead and 14 chimeric pigments measured in the dark. The standard errors of the mean of λ_{max}'s of pigments TBT, $TT^{A}T$, $BB^{S}T$, and TTB are 0.4 and those of the other 12 pigments are all zero (all $n = 5$).



(b)

Z = 500 ± 0.1	θI-VI = -8 ± 0.14	θVII* = 1 ± 0.14	θC = -1 ± 0.14
θA292S = -10 ± 0.1	θI-VIxVII* = 1 ± 0.2	θI-VIxC = 0.2 ± 0.2	θI-VIxA292S = 0 ± 0.2
θVII*xC = 1 ± 0.2	θVII*xA292S = -3 ± 0.2	θCxA292S = -0.8 ± 0.2	θI-VIxVII*xC = -0.4 ± 0.28
θI-VIxVII*xA292S = 0.8 ± 0.28	θI-VIxCxA292S = 1.6 ± 0.28	θVII*xCxA292S = 2.8 ± 0.28	θI-VIxVII*xCxA292S = -2.2 ± 0.4

Figure 4.

The λ_{max}'s of the rhodopsin and chimeric pigments. (a) The observed values and the corresponding expected values from multiple regression analysis (13). White and black circles indicate T^A and B^S, respectively. (b) The Z and θs estimated by solving the 16 equations.

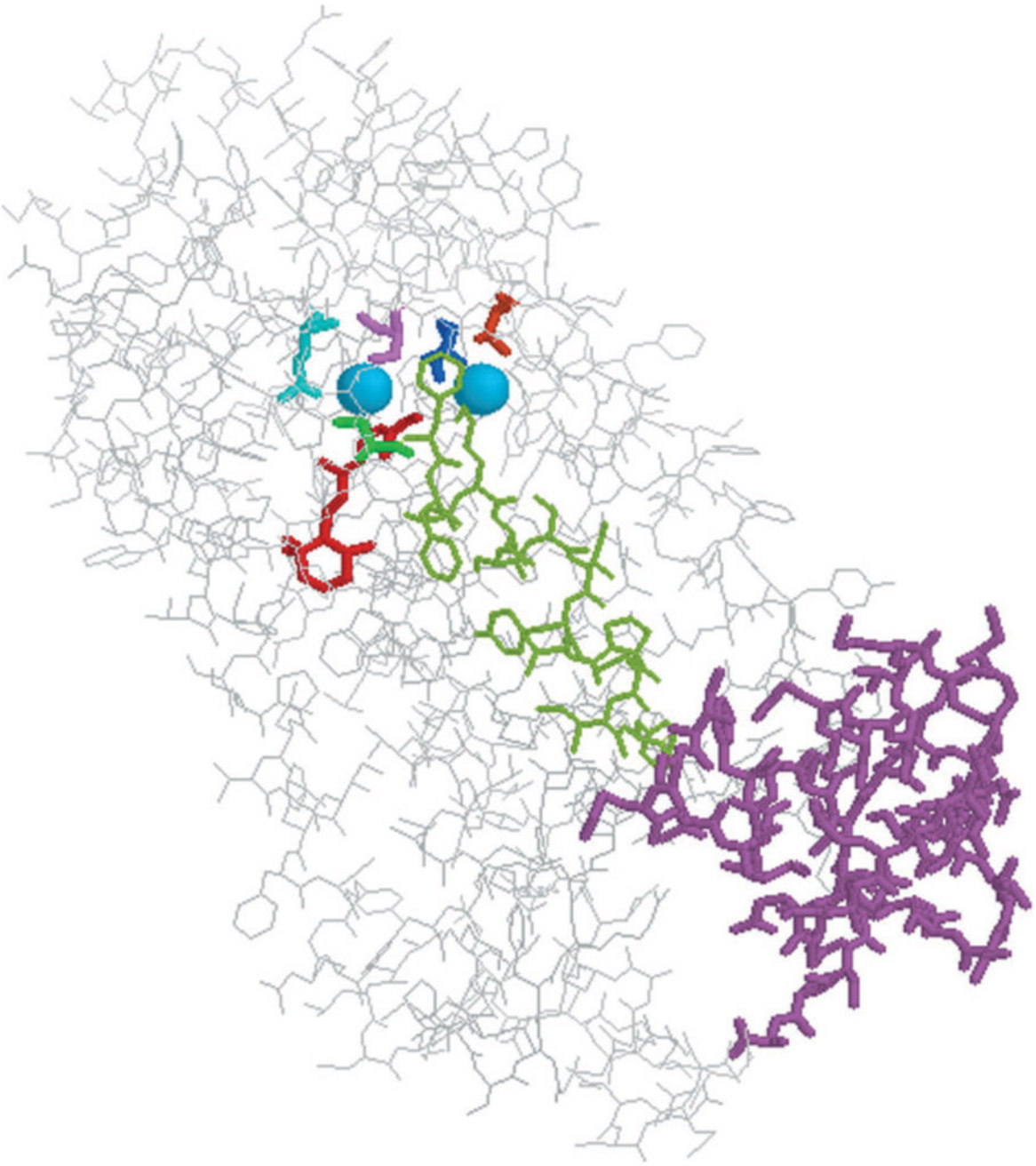


Figure 5.
A molecular model with the “hydrogen bonded network” in thornyhead rhodopsin, consisting of T94 (orange), E113 (blue), E181(cyan), S186 (violet), and two water molecules (light blue) (29); 11-*cis*-retinal (red); TM VII between amino acids 293 and 306 (light green); C-terminus (purple); S292 (green).