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Conserved Residues in the Extracellular Loops of Short-Wavelength Cone Visual Pigments

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Abstract

The role of the extracellular loop region of a short-wavelength sensitive pigment, *Xenopus* violet cone opsin, is investigated via computational modeling, mutagenesis, and spectroscopy. The computational models predict a complex H-bonding network which stabilizes and connects the EC2-EC3 loop and the N-terminus. Mutations which are predicted to disrupt the H-bonding network are shown to produce visual pigments that do not stably bind chromophore and exhibit properties of misfolded protein. The potential role of a disulfide bond between two conserved Cys residues, Cys¹⁰⁵ in TM3 and Cys¹⁸² in EC2, is necessary for proper folding and trafficking in VCOP. Lastly, certain residues in the EC2 loop are predicted to stabilize the formation of two anti-parallel β strands joined by a hairpin turn, which interact with the chromophore via H-bonding or Van der Waals interactions. Mutations to conserved residues result in a decrease in chromophore binding. These results demonstrate that the extracellular loops are crucial for the formation of this cone visual pigment. Moreover, there are significant differences in structure and function of this region in VCOP compared to rhodopsin.

Keywords

visual pigments; visual transduction; rhodopsin; blue cones; opsin

The visual pigments are a class of guanine protein-coupled receptors (GPCRs) that initiate phototransduction in retinal photoreceptors. Each protein consists of seven transmembrane α -helices connected by surface exposed loops and an 11-*cis* retinal chromophore (1-4). Following absorption of light, the retinal chromophore, covalently bound to a conserved lysine residue in transmembrane helix 7 (TM7), isomerizes from 11-*cis* to the all-*trans* conformation to trigger a series of conformational changes in the tertiary structure of the protein that leads to the formation of the active state, R* or *meta II*. The corresponding conformational changes in the covalently bound opsin polypeptide initiate a secondary messenger cascade within the photoreceptor that results in the changes in plasma membrane potential and synaptic transmission (5-10).

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Both rod and cone visual pigments contain conserved residues located in the extracellular loop regions which interact with proteins involved in phototransduction. The cytoplasmic domain (CP), formed by four loops tethered to the lipid bilayer with a flexible carboxyl terminus, contain several palmitoylated Cys residues as well as multiple Ser and Thr residues which serve as targets for GRK phosphorylation (11). In the case of bovine rhodopsin, a significant portion of the CP domain is involved in the light-dependent interactions with transducin (12-14), GRK1 (15) and arrestin (16). The high degree of homology in the CP loop regions of the cone opsins with rhodopsin indicates that cone opsins will exhibit many of the same structure-function relationships as rhodopsin.

Recent studies have indicated that the extracellular (EC) loop regions of GPCRs have an equally important role in proper protein folding and trafficking (1, 4, 17). Mutations in the EC2 loop of bovine rhodopsin, which penetrates the chromophore binding crevice and stabilizes the overall helical bundle, have either 1) induced a shift in the wavelength of absorption maximum, 2) destabilized retinal binding, 3) caused a defect in N-linked glycosylation and protein trafficking and/or 4) cause retinitis pigmentosa (18-25). In the medium and long wavelength sensitive (M/LWS) cone opsins, an anion binding site is formed partly by the EC2 loop and regulates spectral and photobleaching properties (26). NMR analysis indicates the EC2 loop of rhodopsin forms a cap over the chromophore, which exhibits structural changes upon light activation. Furthermore, the β 4 strand in EC2 interacts with TM4, TM5, and TM6 during photoactivation (27). Not only do the EC loops stabilize the protein and facilitate the proper photobleaching but they function to stabilize ligand binding in other receptors as well (28, 29).

Elucidation of EC loop function remains a primary objective toward a complete mechanistic understanding of GPCR activation. Although the crystal structure of bovine rhodopsin is known with high resolution, the flexibility in the EC loops suggests the crystal structure is just a snapshot of one of several possible conformations. Monte Carlo simulated temperature annealing combined with a scaled collective variables technique has been employed to search the conformation space for loop structures (30), revealing the crystal structure could be one conformation in the native ensemble. Geometric sampling and energy minimization have also been used to specifically study the loop regions in both the R and R* state of rhodopsin (31-33). Collectively, these studies exemplify the means by which theoretical computation can be combined with emerging experimental data to study the function of EC residues.

Although a major effort has been made toward the study of rhodopsin EC loop residues, cone opsins warrant equally intense investigation because of their important role in daylight vision, significant differences in the spectral, photoactivation and chromophore binding compared to with rhodopsin and potential clinical problems associated with human mutations. *Xenopus* violet cone opsin (VCOP) has been an important model cone opsin for biochemical and biophysical studies, for a number of reasons. The complete photobleaching pathway of the pigment and protonation state of the Schiff base in the dark state have been determined (34, 35). Additionally, the pigment has shown an ability to activate transducin and the apoprotein is able to regenerate 11-*cis* retinal more efficiently than other cone opsins (36). Furthermore, the retinylidene Schiff base counterion has been identified and the role of this residue in regulating the photobleaching cascade has been investigated. Using a combination of mutagenesis, spectroscopy, molecular dynamics simulations, semi-empirical modeling and homology modeling this treatment elucidates the role of the EC2 and EC3 loops in cone opsins and brings insight into the role of the EC loop regions on protein folding.

Materials and Methods

Homology Modeling of VCOP

The crystal structure of bovine rhodopsin (chain A from PDB 1U19) (1) was used as the template for modeling the three dimensional structure for VCOP. The sequence alignment between rhodopsin, VCOP, and VCOP homology models were generated using Modeller (37). Hydrogen atoms, a protonated Schiff base linkage between Lys²⁹¹ and 11-*cis* retinal and a disulphide linkage between Cys¹⁰⁵ and Cys¹⁸² were added using the visualization program VMD (38). Thirty-six internal hydration water molecules were introduced using both a comparison between the rhodopsin crystal structure and those predicted by the program DOWSER (39). The DOWSER program detected twenty internal hydration sites for VCOP, five of which closely match with the crystal structure water molecules (ID 2000, 2020, 2021, 2024, and 2028). The complete VCOP homology model with internal hydration water molecules was minimized for 4000 steps using the program NAMD (40). A lipid bilayer was included in the model to simulate the unsaturated phospholipids of the outer segment of the rod photoreceptor (41). The protein was embedded in a POPC (palmitoyl-oleoyl-phosphatidyl choline) lipid box ($90 \times 90 \text{ \AA}^2$) using VMD. Lipid molecules overlapping with the protein were removed and the final model contained 169 POPC molecules. A short molecular dynamics (120 ps) was performed on the protein-membrane system to relax the protein-lipid interface. The entire system was then solvated with water molecules (TIP3) using VMD. The fully solvated system was $90 \times 90 \times 100 \text{ \AA}^3$ in size and contains 13,542 water molecules. To neutralize the system, 38 sodium and chloride ions were added and manually redistributed based on the surface potential map generated by GRASP (42).

Molecular Dynamics Simulations

All molecular dynamics simulations were carried out with full Particle-Mesh Ewald (PME) calculation for electrostatic interactions. To fully relax the system, the simulations were carried out systematically as follows: 25 ps with the protein fixed, 25 ps with the entire protein harmonically constrained, 25 ps with the peptide backbone harmonically constrained, 25 ps with alpha carbons harmonically constrained and finally 25 ps with only retinal constrained. An NPT ensemble (constant pressure and temperature) simulation was then performed for 2 ns. The calculations were done using an SGI® Altix® 350 server (12 Intel Itanium 2 CPUs) and the time required for 1 ns simulation was 36 hours. Thermal noise reduction was performed by sliding a window of 9 frames on the trajectory file using a modified VMD script developed but unpublished by J. Saam (43). To compare different models, structural alignment of proteins was performed using the STAMP algorithms (44) built within VMD (solvent water molecules, POPC molecules and ions were removed prior to comparison). Hydrogen bonds ($<2.5 \text{ \AA}$) were identified using “HBOND” in DS ViewerPro. All minimizations and molecular dynamics simulations were performed using the program NAMD. The CHARMM27 forcefield was used for proteins and lipids and TIP3P for water molecules. All titratable groups were assigned protonation states according to the CHARMM forcefield. (38). Asp¹⁰⁸ was assumed to be unprotonated (45).

Semi-empirical approximation to Enthalpy of Formation

The enthalpy of formation for each mutant and for the wild type (WT) VCOP protein was estimated by using routines from the MOPAC software package (46). The model built for the aforementioned CHARMM molecular dynamics simulations was employed as a starting structure for all calculations. Mutations to the WT model were made via tools included in the Swiss PDB program (47) and a steepest decent minimization algorithm was employed for geometry optimization. The PM6 Hamiltonian (48) energy was evaluated via standard self-consistent field minimization methods and the enthalpy of formation was calculated as

described in reference (46). Structural analysis of the energy-minimized protein geometry was accomplished via programs coded in extended basic, using the MathScriptor compiler (R.R. Birge, unpublished and available upon request). Computations were performed on a Microway Opteron computer cluster with ten 32GB AMD Quad Core Opteron 2350 processors running Fedora Core 8. Each minimization required 3-6 days computation time.

Sequence comparisons

The consensus sequence for the regions including the EC2 loop (amino acids 177-199 in bovine rhodopsin), C-segment of TM6, the EC3 loop and the N-segment of TM7 (amino acids 266-296 in bovine rhodopsin) were obtained for each of the five vertebrate opsin families from the G-protein coupled receptor database (49). Alignments were prepared using Clustal X methods as implemented in the MegAlign program (DNASStar).

Preparation and Expression of VCOP Mutants

The 1D4 epitope-tagged VCOP plasmids used for mutagenesis and expression have been described previously (45). The 1D4 epitope is not present in wild type VCOP, but has been added in place of the wild type carboxyl terminus. All amino acid changes were made by restriction fragment replacement: single and double D279Q and L280P using NcoI-SacI, R281Q using BglII-SacI, Y269F and the chimeric constructs using EaeI-NcoI. The sequences of the resulting mutant plasmids were verified. The resulting plasmids had the cDNA containing the codons coding for the first 328 amino acids of the mutant VCOP fused in frame with the codons coding for the last 14 amino acids of bovine rhodopsin, which serves as an epitope tag (50). The mutants were expressed in COS1 cells by transient transfection, purified by immunoaffinity chromatography and analyzed as previously described (34, 36). Pigments were eluted in buffer Y1 [50 mM HEPES, 140 mM NaCl, and 3 mM MgCl₂ (pH 6.6)] with 0.1% DM and 20% glycerol.

UV-vis spectroscopy

Conventional UV-vis spectroscopy was done using a Beckman DU-640 at a temperature of 22°C (36). Light-dark difference spectra were obtained by collecting four spectra in the dark, bleaching the samples with white light from a projector lamp (300 Watts, EXR-5, Wiko, Inc.) for 1 minute and subsequently recording four spectra. The total radiant energy of the white light was measured to be 8 mW using an optometer (Graseby Optronics). Subtraction of light scattering using a λ^{-4} correction factor was done for all spectra using the FitSpectra program (R.R.B, unpublished and available upon request). Light-dark difference spectra were calculated using four averaged spectra using SigmaPlot (Jandel Scientific, CA).

Western Blots and Immunohistochemistry

Purified protein samples were diluted into buffer containing 4% SDS, resolved on 10% polyacrylamide gels, transferred to nylon membranes and detected with the anti-rhodopsin monoclonal antibody, 1D4. Samples were maintained below room temperature prior to solubilization with SDS and electrophoresis. Bound antibody was detected by chemiluminescence (Amersham ECL kit). For immunohistochemistry, transfected COS cells were grown on gelatin-coated cover slips for 48 h, fixed with -20 °C methanol followed by acetone. Opsin was detected using 1D4 antibody and a FITC-conjugated secondary antibody. Endoplasmic reticulum was stained with ER marker antibody against Calnexin (Antibody ab10286, Abcam, www.abcam.com) with detection using Cy5-conjugated secondary antibody. In cells transfected with wild type VCOP, cross-reactivity with antibody was observed predominantly in plasma membrane with some staining of organelles. In cells transfected with some mutants intracellular staining was predominantly found.

Results and Discussion

Alignment of the EC2 loop consensus sequences for each of the sub-families of visual pigments (Fig. 1), compared to the EC2 loop consensus sequence of rhodopsin-like type 1 pigments (Rh1), indicated rhodopsin-like type 2 pigments (Rh2) had 83% sequence homology, short wavelength sensitive type 1 pigments (SWS1) ~60%, short wavelength sensitive type 2 (SWS2) ~65%, and medium to long wavelength sensitive pigments (M/LWS) ~39%. Seven conserved residues within the EC2 loop amongst all the opsin families are noted: Arg¹⁷⁷, Pro¹⁸⁰, Gly¹⁸², Ser¹⁸⁶, Cys¹⁸⁷, Gly¹⁸⁸, and Asp¹⁹⁰, using the sequence numbers of rhodopsin, the protein for which the structural roles of these conserved residues have been previously reported (2, 4, 51). Conversely to the EC2 loop, the EC3 loop shares no conserved residues and displays little primary sequence identity between the different opsin families. The EC3 loop in our VCOP model spans only 7 residues, extending out of TM6 at Arg²⁷⁴, pivoting at Gly²⁷⁷, and returning to TM7 at Leu²⁸⁰.

Experimental Analysis of *Xenopus* Violet Cone Opsin Mutants

In order to evaluate the potential role of particular amino acid residues in the structure-function of VCOP, we characterized protein expressed in a well-established heterologous system based upon transfection of COS1 cells (*see Methods*). Mutant proteins purified from transfected COS1 were analyzed by denaturing polyacrylamide electrophoresis to determine the post-translational glycosylation status. Bovine rhodopsin and VCOP cells exhibit a strong immunoreactive band with an apparent molecular weight of ~34-35 kD and an associated broad smear of immunoreactive protein up to ~45 kD (Fig. 2). For both rhodopsin and VCOP, these bands are heterogeneously glycosylated with a mixture of complex carbohydrates which can be partially removed by digestion with PNGaseF (36, 39, 52). In addition, VCOP produces more dimer (~75 kD) than rhodopsin in these SDS gels. The mutants studied here exhibited a range of patterns in the electrophoretic analysis. Representative mutants are shown in Figure 2. Some mutants (e.g. E176D or S181A) had similar patterns as wild type VCOP, while others (C105A, C182A, D279Q or R281Q) showed an absence of the broad smear. Mutants in the latter class apparently have a deficiency in post-translational glycosylation that is possibly caused by a protein processing defect associated with protein misfolding in the endoplasmic reticulum or Golgi. These results show that a number of the EC2 and EC3 mutants play a significant role in the biosynthesis of VCOP.

All VCOP mutants were purified by immunoaffinity chromatography in dodecyl maltoside following addition of 11-cis retinal to cells and the resulting protein was analyzed by UV-Visible spectroscopy. We observed three different mutant phenotypes (Table 1) as judged by chromophore binding: indistinguishable from WT VCOP, reduced chromophore and no detectable chromophore bound. Those mutants with chromophore bound to some level are shown (Fig. 3) and their spectral properties are summarized in Table 1. In addition to the amount of bound chromophore, several of the mutants showed a slightly altered λ max compared to the value for WT VCOP ~427 nm. Cone pigments differ from rhodopsins in their reactivity to hydroxylamine in the dark and this is thought to reflect different accessibilities of the solvent to the Schiff base local environment. Rhodopsins are essentially non-reactive in the dark, while cone opsins rapidly lose chromophore in the dark. We measured the rate of chromophore hydrolysis in the presence of hydroxylamine for the EC3 mutants that generated a good yield of pigment. Under conditions of excess hydroxylamine and at room temperature, for all of the mutants tested the half-times of chromophore decay were between 5-9 min compared to WT VCOP which had a half time of 7.5 min (*data not shown*). Additional discussion of the mutant spectroscopic results is given below.

Computationally predicted hydrogen-bonding networks

To aid in understanding the role of EC2 and EC3 residues in the structure and function of VCOP in the absence of a high resolution structure, computational modeling was utilized (*see Methods*). The equilibrated VCOP model is stable and offers a suitable means for inferring the function of key residues. The relaxation of the model helical bundle showed an expansion of the system volume by 262 Å³, compared to the rhodopsin crystal structure, to a total volume of 33,972 Å³. Very little movement in the residues forming the binding pocket was observed during molecular dynamics (MD) equilibration and the retinal, despite exhibiting minor stretching and bending of the polyene chain during the MD, showed no loss of planarity or isomerization. The Asp¹⁰⁸ residue, the primary counterion to the protonated Schiff base, interacts with the charged moiety within a distance of 1.8 Å. Furthermore, 13 of the 36 crystal water molecules remained in position, all of which bridge amino acids. The backbone trace for the N-terminus of the model deviated by only 3.8 Å from the rhodopsin crystal structure, though a significant difference in the orientation of the amino acids was observed. VCOP EC2 loop is predicted to contain two antiparallel β strands, labeled β3 (Arg¹⁷²-Glu¹⁷⁶) and β4 (Ser¹⁸¹-Asp¹⁸⁵), connected by a hairpin linker ¹⁷⁷GLQC¹⁸⁰ facilitated by Gly¹⁷⁷ identical in size and structure to rhodopsin's ¹⁷⁷GMQC¹⁸⁰ (Fig. 4). However, the β4 strand of VCOP shows no intramolecular interaction with the chromophore, as in rhodopsin. Thus, the computational modeling predicts a structural element that is significantly different from that of bovine rhodopsin, namely the absence of contacts between EC2 and 11-*cis* retinal. This may have an important consequence for the stability of retinal in the binding pocket and highlight a key difference between rod and cone pigments.

The VCOP model predicts the EC2 loop has hydrogen bonding to the EC3 loop, via Asp²⁷⁹ and Leu²⁸⁰ which itself is bonded to Asn¹² in the N-terminus. In rhodopsin, the EC3 loop is also predicted to form a H-bonding network with the N-terminus but not with the EC2 loop. The VCOP network involves Asn¹², Gly¹⁷⁷, Asp²⁷⁹, Leu²⁸⁰, and 3 water molecules. This H-bonding pattern orients the EC2 and EC3 loops over the retinal, introducing a H-bond between Ser¹⁸¹ and Asp¹⁰⁸, dictating the protein-chromophore interaction. The VCOP model predicts that, contrary to the X-ray crystal structure of bovine rhodopsin, the EC2 loop is indirectly H-bonded to the N-terminus via EC3 loop amino acids Asp²⁷⁹ and Leu²⁸⁰, highlighting a novel function for the short EC3 loop. Arg²⁸¹ does not interact with any residue, but is exposed to the solvent which determines the proper axial orientation for TM7 in the membrane. The importance of these residues in protein folding and stabilizing the binding of 11-*cis* retinal was evident in replacements involving either Asp²⁷⁹ or Arg²⁸¹ individually or in combination. PM6 energy minimization predicted neutralizing either residue in the charged pair individually was enthalpically unfavored, whereas the double mutation was slightly favored (*see Table 1*). Exchanging the positions of the two charged residues in the double mutant D279R/R281D yielded a misfolded protein as well, indicating residues Asp²⁷⁹ and Arg²⁸¹ are not simply involved in forming a single intramolecular salt bridge. These results demonstrate the necessity for this conserved charged pair for proper protein folding in VCOP and support the extended hydrogen bonding network predicted by the computational modeling. Leu²⁸⁰ is predicted to be another vital residue in the EC3-DLR motif. The experimental results on L280P, L280G, and L280A support this function. The L280P and L280G models (Fig. 4) suggest that the mutant at position 280 causes Thr²⁸⁴ in TM7 to directly interact with EC2 loop residues Leu¹⁷⁸ and Glu¹⁷⁶, respectively. The L280P model predicts a disruption in the H-bonding interaction between the N-terminus and the EC loops. As shown in Fig. 5, Pro²⁸⁰ is predicted to have a main chain-side chain interaction with Thr²⁸⁴, extended to main chain-main chain interactions with Arg²⁸¹ in the EC3 loop as well as side chain-side chain interactions with Glu¹⁷⁶, none of which were present in the WT VCOP model (Fig. 4). The aberrant H-bonding network in L280P causes a water molecule

previously H-bonded to Asp¹⁰⁸ to H-bond to Ser¹⁸¹, potentially destabilizing the primary counterion of the retinal. Furthermore, Pro²⁸⁰ interacts primarily with the transmembrane domain whereas Leu²⁸⁰ interacted with the extracellular domain. An RMSD analysis of the position of the atoms in the EC3 loop, throughout the 2ns MD simulation, yielded ~2Å less movement for the L280P mutant compared to WT, indicating the mutant contains more rigidity in the EC3 loop. Calculations indicate the mutation is enthalpically unfavored.

The L280G model contains a similar H-bonding network to the L280P mutant H-bonding network. Similar to L280P, the introduction of Gly at position 280 provides enough flexibility for the EC3 loop to interact more strongly with the helical bundle and weaker with the N-terminus compared to WT. The L280G mutant has a main chain-side chain interaction with Thr²⁸⁴, in TM7, and a main chain-main chain interaction with Asp²⁷⁹ (Fig. 5). The water molecules bridging H-bonds from Glu176, as observed in both L280P and WT models, have dissociated out of the protein and into the bulk simulation box. This mutation was computed to be enthalpically unfavored.

Further computations and experiments supported the importance of the EC3 loop. A double mutant, L280G/T284A, failed to improve the level of retinal binding and induced a ~13nm red-shift in the $\lambda_{\max}$ compared to WT. The double mutant model predicts a major disturbance in the interactions between the EC2 and EC3 loop (Fig. 5), leading to a random-coil like structure instead of the β strands in the EC2 loop (Fig. 5) and neutralization of the side chains of Asp²⁷⁹ and Arg²⁸¹ (Fig. 5). In summary, direct changes to the H-bonding network residues in the EC loops increase the flexibility of the region and possibly destabilize the chromophore binding crevice, thus reducing interactions with the 11-*cis* retinal. The computational models highlight the vital function of residues Asp²⁷⁹, Leu²⁸⁰, and Arg²⁸¹ and suggest the reason mutations to these residues decrease chromophore binding.

Disulfide linkage between Cys¹⁰⁵ and Cys¹⁸²

The VCOP model was constructed with a disulfide bond between Cys¹⁰⁵ and Cys¹⁸², highly conserved residues in all visual pigments. Mutations to these two residues produced misfolded protein rather than a specific destabilized retinal binding. Supporting evidence for this disulfide bond was obtained by UV-Vis spectra: C105A and C182A mutants had no detectable chromophore in purified samples, while the C180A mutant bound chromophore, albeit at a reduced level compared to wild type with a 10 nm blue-shifted $\lambda_{\max}$ (Fig. 3). Computationally, the C105A mutation was predicted to be enthalpically favored, the C180A mutation was enthalpically null, and the C182A mutation was enthalpically unfavored. The double mutant C180A/C182A, also predicted to be enthalpically unfavored, did not bind chromophore. These experiments strongly suggest that, in the WT VCOP protein, Cys¹⁰⁵ and Cys¹⁸² are covalently interconnected via a disulfide linkage. These results further suggest that the disulfide bond is essential for proper folding and post-translational processing in the endoplasmic reticulum. In bovine rhodopsin comparable Cys to Ala mutants fold and bind chromophore but with reduced stability of *meta II* (23). The formation of an abnormal disulfide bond between Cys¹⁸⁵ and Cys¹⁸⁷ has been identified in a misfolded rhodopsin (53).

The VCOP model predicts H-bonding network within the EC2 (Fig. 6), that includes residues Cys¹⁰⁵, Cys¹⁸⁰, Ser¹⁸¹, and Cys¹⁸² and the backbone oxygen of Gly¹⁷⁷ H-bonded to the H of water 2035. The H-bonding network within EC2 is then directed over the chromophore by Cys¹⁸² which is presumably assigned a disulfide linkage to Cys¹⁰⁵ in TM3. The computational models correctly predict the cysteine residues form a disulphide linkage. Experimental data on the stability of VCOP mutant proteins supports these predictions.

Binding site residues Glu¹⁷⁶ and Ser¹⁸¹

Two residues, Glu¹⁷⁶ and Ser¹⁸¹, are highly conserved in visual pigments. In contrast to Glu¹⁸¹ in bovine rhodopsin, the VCOP model suggests that the equivalent residue Glu¹⁷⁶ does not have any direct intramolecular interaction with the polyene chain of 11-*cis* retinal. However, Glu¹⁷⁶ is predicted to H-bond to Ser¹⁸¹ strongly to stabilize the β 3- β 4 hairpin, to Tyr²⁶³ and through side chains to align the β 4 strand into the binding crevice (Figs 5, 7). All four mutations of Glu¹⁷⁶ (to D, Q, H, and S) prevented stable chromophore binding. All single mutations of Glu¹⁷⁶ are predicted to be enthalpically unfavored despite little change in the overall structure of the protein. Mutation of Ser¹⁸¹ also had severe effects, although S181T and S181C did bind retinal at a very reduced level. Double mutants (E176D/S181T and E176S/S181E) could not compensate for the deficiency in retinal binding in either single mutant. These results suggest there are specific interactions of Ser¹⁸¹ with other residues that do not tolerate even small changes, such as those found in Thr or Cys. Surprisingly, single mutants at E176 and S181 had glycosylation patterns similar to WT VCOP and even some localization in the plasma membrane. These data suggest that the mutations to E176 and S181 induce a specific defect in the binding pocket rather than a global misfolding of the protein. In summary, Glu¹⁷⁶ and Ser¹⁸¹ in VCOP are both essential for proper protein folding and stabilization of the chromophore binding site.

A chimera model of the protein was produced by mutating the entire EC2 loop of VCOP to match residues from rhodopsin. No chromophore was detected in either the rhodopsin EC2 loop chimera or the chimera Y186W mutant. These chimera mutants suggest that a stable retinal binding pocket requires very specific interacting residues in VCOP (Fig. 6). The EC2 loop chimera models predict a disruption of the β strands, yielding a more random coil-like structure for the EC2 residues. Differences in the intramolecular interactions between helices TM4 and TM5 and a peptide consisting of the last five amino acids in the EC2 loop (¹⁸⁹VGTKYR¹⁹⁴ in WT VCOP and ¹⁹⁴PHEETN¹⁹⁹ in bovine rhodopsin) were noted as well. The most significant difference is the H-bonding pattern along the β strands. The WT VCOP model suggests that the backbone of Arg¹⁷², Met¹⁷⁴, and Glu¹⁷⁶ H-bond with that of Asp¹⁸⁵, Gly¹⁸³, and Ser¹⁸¹ respectively, with an average distance between β strands of 2.06Å. Comparatively, the EC2 loop chimera model predicts no H-bond formation in the analogous residues, yielding a widening of up to 3.39Å. PM6 semi-empirical minimization of the protein generated data which reinforced the predictions of the CHARMM models. The distance between β strands was predicted to increase by 2.5Å as well as a 4.2 degree increase in the angle between the β strands. The total RMS shift of the center of mass of selected key residues predicted was 3.8Å. These β strand disruptions destabilize the EC2 loop interactions with the 11-*cis* retinal in the chimera model and consequently prevent the Y186W mutant from binding retinal despite the increase in favorable chromophore interaction with the mutation. In summary, computational models predict the nature in which Glu¹⁷⁶ and Ser¹⁸¹ stabilize the binding of the chromophore, as well as the sensitivity of the EC2 loop to mutation.

Conserved Residues in the EC2 Loop

Four conserved residues among the SWS1 pigments Leu¹⁷⁸, Gly¹⁸³, Pro¹⁸⁴, and Trp¹⁸⁶ in the EC2 loop have important functional properties. All four mutations of Leu¹⁷⁸ (to M, A, H, and D) decreased retinal binding significantly or completely, and were predicted to be enthalpically unfavored. Leu¹⁷⁸ is predicted to coordinate the turn connecting the two EC2 β strands. Shortening the β 3 strand by a E176G mutation together with a reintroduction of Glu at position 178 in the double mutant E176G/L178E did not bind the chromophore. PM6 minimization indicated the double mutant was enthalpically unfavored and a 2 degree widening of the hairpin turn between β strands was predicted. At position 178, the structural

requirements for proper folding are truly only exhibited by Leu and Met and not by Ala, His, or Asp. The mutant models all predict disruptions of the two β strands.

In addition to Leu¹⁷⁸, conserved residues Pro¹⁸⁴ and Gly¹⁸³ are shown to facilitate a turn so that the EC2 loop goes out of the binding crevice quickly. Substitution of Pro¹⁸⁴ with Ile led to a lower binding of retinal (50%) in the mutant protein and slight change in the $\lambda_{\max}$ (426nm). Semi-empirical minimization suggests the mutation is enthalpically unfavored and that the angle between $\beta 3$ and $\beta 4$ strands increases by 12 degrees. The reduced level of retinal binding is due to the EC2 loop moving above the chromophore binding crevice, which reorients the two β strands over 11-*cis* retinal. This could either affect the interaction between the chromophore and protein or it could prevent or destabilize the formation of the putative disulphide bond between Cys¹⁰⁵ and Cys¹⁸².

The fourth conserved residue in the SWS1 pigments is a Trp at position 186 in VCOP. The indole ring of this residue, together with the phenolic ring of Tyr²⁶³, may have van der Waals interactions with the retinylidene polyene chain. The mutant W186Y had barely detectable chromophore which likely could be the result of a weakened attractive interaction with the chromophore because the phenolic ring is significantly smaller than an indole ring. Although little structural change compared to WT was predicted in the PM6 minimization of the protein W186Y, the mutation is predicted to be enthalpically unfavored.

Conclusions

The importance of the extracellular (EC) loops in the proper folding of a short wavelength sensitive 1 pigment has been elucidated via computational modeling, mutagenesis, and spectroscopic experiments. Despite the seemingly high flexibility in the EC loops as a whole, several EC loop residues have been identified as critical for either the stabilization of the chromophore binding site or for a more global folding, e.g. stabilization of the helical bundle. Specifically, four novel features of cone opsin proteins have been determined.

First, a H-bonding network that connects the EC2 loop, EC3 loop, and the N-terminus of the protein has been predicted via computational modeling. Although the EC3 loop of bovine rhodopsin is H-bonded to the N-terminus, there are no H-bonds connecting the EC2 and EC3 loops. The network in VCOP dictates the importance of the charged pair, Asp²⁷⁹ and Arg²⁸¹, which is only conserved amongst the SWS1 pigments. These residues function to stabilize the helical bundle and also orient a third conserved residue, Leu²⁸⁰, which facilitates the H-bonding between the EC2 and EC3 loops. Mutations to the SWS1 conserved ²⁷⁹DLR²⁸¹ motif have been shown to yield a misfolded protein.

Second, a disulfide bond between a residue in TM3, Cys¹⁰⁵, and a residue in EC2, Cys¹⁸², has been assigned based on homology models with bovine rhodopsin. The nearby Cys¹⁸⁰ presents the possibility that the disulfide bond may be misassigned, however mutagenesis experiments allow us to conclude that the correct assignment is a covalent linkage between Cys¹⁰⁵ and Cys¹⁸² via a disulfide bond. Furthermore, Western blot and immunohistochemistry analysis indicate that the disulfide bond in VCOP is required for proper protein folding whereas in bovine rhodopsin tolerates substitution of the disulfide bond (18).

Third, mutations of three residues in the β strands of the EC2 loop (Leu¹⁷⁸, Pro¹⁸⁴, and Trp¹⁸⁶) conserved in cone pigments caused disruption in β strand formation or formation of a hairpin turn. Proper hairpin formation was facilitated by either Leu or Met in position 178, however improper formation resulted with Ala, His or Asp residues in position 178. Pro¹⁸⁴ directs the EC2 loop toward the binding site pocket. The decreased retinal binding observed for the P184I mutant due to a directional change in the EC2 loop, which results in

destabilizing favorable interactions of binding site residues with the chromophore, such as Van der Waals attraction with the indole side chain of Trp¹⁸⁶. The direct correlation between a favorable Van der Waals interaction of Trp¹⁸⁶ and the chromophore is shown by a decrease in retinal binding in the W186Y mutant.

Lastly, mutations of two residues in the binding pocket of VCOP, Glu¹⁷⁶ and Ser¹⁸¹, have been explored and found to be important to the stability of chromophore binding. The analogous residues in bovine rhodopsin, Glu¹⁸¹ and Ser¹⁸⁶, can tolerate many substitutions with proper folding and small shifts in $\lambda_{\max}$. However, in VCOP, the side chain length and charge on residue 176 must be conserved for proper folding. Small structural changes to the Ser residue at position 181, such as those found in S181C and S181T, also failed to bind retinal. Western blot and immunohistochemistry analysis indicate the mutations in VCOP induce a specific defect in the binding pocket rather than protein misfolding. Additional work is necessary to further characterize these findings.

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Abbreviations

SWS1	Short-wavelength sensitive-1 pigment
SWS2	Short-wavelength sensitive-2 pigment
Rh1	rhodopsin
Rh2	rhodopsin-like pigment
LWS	long-wavelength sensitive pigment
VCOP	<i>Xenopus laevis</i> violet cone opsin
EC	extracellular
TM	transmembrane
CP	cytoplasmic

Figure 1.

A) Secondary structural model of *Xenopus* violet cone opsin (VCOP). Predicted alpha helical regions, based upon homology modeling, are boxed and the seven transmembrane helices (TM) are labeled. Dark arrows indicate predicted β -strands. The conserved lysine residue (K291) in TM7 to which 11-*cis* retinal is covalently bound and the aspartic acid residue (D108) which serves as the counterion to the protonated Schiff base are highlighted with black circles. Post translation modification at N15 (glycosylation) is indicated by CHO and palmitoylation by C317 by a jagged line. B) Comparison of the consensus EC2 loop sequences for the five vertebrate visual pigment families is shown. The composite consensus sequences were generated using a majority rule. The composite consensus sequence for the visual pigments is listed first (Consensus), followed by rhodopsin-like type 1 (RH1),

rhodopsin-like type 2 (RH2), short wavelength sensitive set 1 (SWS1), short wavelength sensitive set 2 (SWS2), long/medium wavelength sensitive (LWS). The bovine opsin and VCOP EC2 loop sequences are shown for comparison. Majority conserved residues are boxed. A highly conserved cysteine residue (C187 in bovine rhodopsin) is highlighted in black. C) Consensus sequences for the EC3 loop of visual pigments are shown. The consensus sequences for each family of visual pigment were generated by majority rule. There were no amino acids conserved in all 5 visual pigments. D279 and R281 from VCOP are indicated by arrows.

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Figure 2.

Western blot of visual pigments heterologously expressed in COS1 cells. Equal amount of purified visual pigment were resolved on SDS PAGE and detected using a monoclonal antibody that recognizes the C-terminus of all constructs. Bovine rhodopsin (Rhodopsin) and normal (Wild type) VCOP migrate with multiple species including a monomer at ~ 34 kD and a smear of heterogeneously glycosylated monomers highlighted with a bracket. VCOP also exhibits a significant dimer that runs ~85 kD. VCOP single amino acid substitution mutants have a diversity of patterns. Mutants E176D and S181A have the same pattern as wild type VCOP while mutants C105A, C182A, D279Q, and R281Q all exhibit a significant reduction in the broadly glycosylated protein between the monomer and dimer species. The molecular weight standards are 25, 35, 50, 75 and 100 kD.

Figure 3.

UV/Vis spectroscopy of single amino acid substitution mutants in EC2 and EC3 loops. Following addition of 11-*cis* retinal, mutant proteins were purified in dodecyl maltoside and UV/Vis spectra were obtained (black lines). For comparison, a spectrum of wild type VCOP is presented in each panel (grey lines). Absorption at 280nm arises from aromatic amino acids of protein, while absorption above ~300 nm arises from bound chromophore. The inset shows the chromophore region of the spectrum expanded to show pigment bound at reduced levels compared to wild type. Each spectrum was normalized to have a relative OD of 1.0 at 280 nm to facilitate comparisons between different protein samples. There was a wide range of pigment formation, from nearly wild type levels (e.g. L280A) to barely detectable (e.g. W186Y). The fraction of protein that binds chromophore can be quantified by the ratio of the absorbance at 425 nm to that of the protein at 280 nm (see Table 1).

Figure 4.

A, Predicted hydrogen bonding network derived from homology modeling in the extracellular domain of VCOP. Critical residues (Asn¹², Gly¹⁷⁷, Asp²⁷⁹, Leu²⁸⁰) were distributed between EC2, EC3 and N-terminus, and involved three structural water molecules. This hydrogen bonding network is further influenced by Cys¹⁰⁵, Cys¹⁸⁰, Ser¹⁸¹, and Cys¹⁸². The assumed cysteine bond between Cys¹⁰⁵ and Cys¹⁸² is shown. This model was used as a guide to target residues for mutagenesis. Oxygen atoms appear in red, nitrogen in blue, carbon in gray, and hydrogen in green. A hydrogen bond interaction between atoms is specified with a dashed blue line. Where necessary, the blue line is bent for visual aesthetics only. B, Predicted hydrogen bonding network derived from homology modeling in the extracellular domain of the single amino acid substitution mutants VCOP L280G (*left*) and L280P (*right*). Both mutant protein models indicate an interaction with Thr284 not present in the wild type VCOP protein. In the L280G model, the hydrogen bonding interaction between the EC3 and EC2 loops is disturbed, whereas in L280P the hydrogen bonds are shifted. Oxygen atoms appear in red, nitrogen in blue, carbon in gray, and

hydrogen in green. A hydrogen bond interaction between atoms is specified with a dashed blue line. Where necessary, the blue line is bent for visual aesthetics only.

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A

B

Figure 5.

A, Changes in conformation of the extracellular loop region accompany substitution of L280. A ribbon representation of EC2 and EC3 with selected amino acid residues (portions of TM6 and TM7 are also shown in the background). The orientation of the EC3 residue side chains with respect to the EC2 loop is altered in all of the mutants. The WT VCOP protein displays β -strand orientation similar to bovine rhodopsin whereas the double mutant L280G/T284A shows significant disruption of the β -strand formation. These models suggest that partial misfolding of the EC region alters the mutants ability to stably bind 11-cis retinal. B, Charge density model of WT VCOP and EC3 VCOP mutants. The charge density of each residue is displayed in either red (*negative*) or blue (*positive*). The absence of charge is indicated by white. D²⁷⁹ and R²⁸¹ are shown for each model. The charge density model indicates the L280(G/P) mutation induces an interaction between D²⁷⁹ and R²⁸¹ in which the charge on each moiety is partially or completely neutralized.

Figure 6.

Ribbon diagram of EC2 loop chimera models. The WT VCOP model EC2 loop is shown in 9A, along with portions of TM2 and TM3. A model of the EC2 loop chimera VCOP protein is shown in 9B from the same orientation. The β strands of the EC2 loop are shown for WT VCOP (9C), the EC2 loop chimera (16D) and bovine rhodopsin (16E). As shown, fewer H-bonding interactions are observed in the chimera model compared to either WT protein.

Table 1

Mutants	$\Delta\lambda_{\max}/\text{nm}$ ¹	$A(\lambda_{\max})/A(\lambda_{280})$ ²	$\Delta(\Delta\text{HOF})/(\text{kcal/mol})$ ³	Shift of key residues/ $\text{\AA}$ ⁴	$\Delta\beta_3 \text{ Length}/\text{\AA}$ ⁵	$\Delta\beta_4 \text{ Length}/\text{\AA}$ ⁵	$\Delta\text{Hairpin Turn / deg.}$ ⁶
Wild Type	$\lambda_{\max}=427$	1 Normalized	$\Delta\text{H}=-23,264$	0	0	0	0
C105A	N/A	0	-77	0.781	0.325	-0.067	-0.394
C180A	-9.5	0.490	-4	0.247	0.000	-0.002	0.002
C182A	N/A	0	180	0.637	0.392	-0.064	-0.495
C180/C182	-18	0.141	238	0.507	0.001	-0.009	-0.007
L178A	-7	0.397	322	0.956	0.348	-0.058	-0.705
L178D	N/A	0	152	0.523	-0.755	0.295	0.512
L178H	-16.5	0.178	619	0.620	0.433	-0.043	0.812
L178M	-0.5	0.686	468	0.349	0.938	-0.058	0.295
P184I	-2	0.485	262	0.846	0.140	-0.172	12.847
W186Y	N/A	0	402	0.411	0.571	-0.079	-0.821
E176D	-13.5	0.104	395	0.686	0.370	-0.295	-0.935
E176H	N/A	0	685	0.868	0.521	-0.044	-1.602
E176G	N/A	0	333	1.020	0.463	-0.022	-1.046
E176Q	N/A	0	332	0.942	0.325	0.017	-1.270
E176S	N/A	0	322	0.956	0.348	-0.058	-0.706
E176D/S181T	N/A	0	-17	0.466	0.005	0.004	0.078
E176S/S181E	N/A	0	158	1.140	-0.668	-0.219	2.136
E176G/L178E	-18	0.114	198	0.521	-0.001	-0.003	2.080
S181A	N/A	0	443	0.604	0.525	0.117	-0.961
S181C	N/A	0	50	0.078	0.001	0.000	0.003
S181E	N/A	0	-101	0.040	-0.002	0.002	0.017
S181T	N/A	0	280	0.546	0.476	-0.063	-0.958
D279Q	N/A	0	757	0.576	0.448	-0.228	-0.955
R281Q	N/A	0	176	0.775	0.455	-0.222	-1.042
D279Q/R281Q	N/A	0	-82	0.376	0.000	0.000	0.000
D279R/R281D	N/A	0	173	0.518	0.000	0.000	0.000

Mutants	$\Delta\lambda_{\max}/\text{nm}$ ¹	$A(\lambda_{\max})/A(\lambda_{280})$ ²	$\Delta(\Delta\text{HOF})/(\text{kcal/mol})$ ³	Shift of key residues/Å ⁴	$\Delta\beta 3$ Length/Å ⁵	$\Delta\beta 4$ Length/Å ⁵	$\Delta\text{Hairpin Turn}$ / deg. ⁶
L280A	1	0.250	438	0.561	0.490	-0.162	-0.889
L280G	-1	0.150	414	0.773	0.442	-0.206	-0.890
L280P	-17	0.980	419	0.727	0.467	-0.200	-0.846
BOP / VCOP Chimera	N/A	0	-1446	3.843	-0.478	-2.431	4.199
Chimera W186Y	N/A	0	-1420	4.101	-0.525	-2.513	3.885

¹ A negative $\Delta\lambda_{\max}$ indicates a blue-shift.

² For each mutant the absorbance at the mutant's $\lambda_{\max}$ was divided by the absorbance of the amino acids at λ_{280} . The ratio [$A(\lambda_{\max})/A(\lambda_{280})$] for each mutant was then divided by the same ratio for the wild type protein to normalize wild type to unity.

³ The enthalpy of formation of the wild type protein was subtracted from the enthalpy of formation of the mutant protein. Therefore a negative $\Delta(\Delta\text{HOF})$ indicates the mutant protein is more enthalpically favored than the wild type protein.

⁴ The shift in the center of mass of charged residues and residues within 5 angstroms of the chromophore was determined. The chromophore was used to align the WT and mutant proteins.

⁵ The length of each beta strand was determined by calculating the distance between the center of mass of the residues at either end of each strand.

⁶ The angle between beta strands was calculated by creating vectors for each beta strand, determined by the center of mass of the residues belonging to the strand. The two vectors were then used to trigonometrically determine the angle between them.