Equilibrium between Metarhodopsin-I and Metarhodopsin-II Is Dependent on the Conformation of the Third Cytoplasmic Loop

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Rhodopsin is a G-protein-coupled receptor (GPCR) that is the light detector in the rod cells of the eye. Rhodopsin is the best understood member of the large GPCR superfamily and is the only GPCR for which atomic resolution structures have been determined. However, these structures are for the inactive, dark-adapted form. Characterization of the conformational changes in rhodopsin caused by light-induced activation is of wide importance, because the metarhodopsin-II photoprotein is analogous to the agonist-occupied GPCR conformations. In this work we characterize the interaction of antibody K42-41L with the metarhodopsin-II photoprotein. K42-41L is shown to inhibit formation of metarhodopsin-II while it stabilizes the metarhodopsin-I state. Thus, K42-41L recognizes an epitope accessible in dark-adapted rhodopsin and metarhodopsin-I that is lost upon formation of metarhodopsin-II. Previous work has shown that the peptide TGALQERSK is able to mimic the K42-41L epitope, and we have now determined the structure of the K42-41L-peptide complex. The structure demonstrates a central role for elements of the rhodopsin C3 loop, particularly Gln238 and Glu239, in the interaction with K42-41L. Geometric constraints taken from the antibody-bound peptide were used to model the epitope on the rhodopsin surface. The resulting model suggests that K42-41L locks the C3 loop into an extended conformation that is intermediate between two compact conformations seen in crystal structures of dark-adapted rhodopsin. Together, the structural and functional data strongly suggest that the equilibrium between metarhodopsin-I and metarhodopsin-II is dependent upon the conformation of the C3 loop. The biological implications of this model and its possible relations to dimeric and multimeric complexes of rhodopsin are discussed.

Rhodopsin is a G-protein-coupled receptor (GPCR) characterized by seven transmembrane helices (H1 through H7) (1). It is an efficient photoreceptor, with the chromophoric retinal cofactor undergoing photoisomerization from 11-cis to all-trans upon activation. The retinal isomerization propagates conformational changes through the light-excited rhodopsin protein to a meta-stable active state, metarhodopsin-II, which is in equilibrium with the inactive metarhodopsin-I precursor (see Fig. 1A). Metarhodopsin-II binds to the heterotrimeric G-protein transducin (Gt) and triggers GDP release followed by GTP uptake (2, 3).

A large fraction of existing drugs and drugs under development target GPCRs (4). The rhodopsin system is considered a prototypical model for the GPCR superfamily, and recent work has produced new insights on the biochemistry of rhodopsin (1, 2). However, many questions remain unanswered, particularly with respect to the mechanism of receptor and G-protein activation.

Metarhodopsin-II is characterized by its specific ability to bind and activate multiple copies of the heterotrimeric G-protein transducin (Gt,βγ) in rapid succession (5, 6). Transducin binds to the cytoplasmic face of metarhodopsin-II, involving interactions with the C2 and C3 loops (7), helix H8, and residues within the C-terminal tail (8, 9). There is, however, very little structural information at the atomic level regarding the interactions of these elements with transducin.

Five crystal structures of dark-adapted rhodopsin have been determined (10–14), greatly enhancing our understanding of the inactive, dark-adapted state. Collectively, these structures provide a framework within which detailed conformational changes associated with formation of metarhodopsin-I and metarhodopsin-II may be addressed. Further, the structures imply that several elements on the cytoplasmic face of dark-adapted rhodopsin are flexible, including the C3 loop and the C-terminal tail. Portions of the C3 loop are disordered in three of the five structures reported to date (10–12), whereas the structures of Li et al. (PDB ID 1GZM) (13) and Okada et al. (PDB ID 1U19) (14) show very different conformations for the C3 loop. The C3 loop is “ordered” in these structures (1GZM and 1U19) but the B-factors for the Cα atoms are high (rising above 100 Å²), suggesting that each of these models represents a family of related conformations, rather than a single, well-defined structure. These data, together with site-specific spin labeling (15), suggest that the C3 loop and C-terminal tail are dynamic structural elements in the dark-adapted state. Dunker and coworkers (16, 17) have...
recently pointed out that cell-signaling proteins often interact through highly flexible regions that form well-structured signaling complexes.

A 5.5-Å electron crystallographic study of metarhodopsin-I has recently been published (18). Comparison of this intermediate-resolution electron density map with the x-ray structures of dark-adapted rhodopsin shows that the structure of metarhodopsin-I is quite similar to that of the dark-adapted ground state. Thus, formation of metarhodopsin-I occurs without large rigid-body movements of the transmembrane helices, implying that the large conformational changes thought to accompany formation of metarhodopsin-II occur later in the photocleavage process. The electron crystallography map shows clear density for most of metarhodopsin-I, but as is the case for many crystal structures of dark-adapted rhodopsin, density for the C3 loop is absent, suggesting conformational flexibility for the C3 loop in the meta-I state. Structural information for metarhodopsin-II is more limited, although models have been proposed (19, 20). These models are based in large part on site-specific spin labeling studies that demonstrate a substantial conformational change upon formation of metarhodopsin-II (21–23) but do not provide atomic resolution.

The antibody imprint technique has been proposed to overcome difficulties inherent in the structural characterization of transient protein conformations or proteins that have eluded crystallization (24–27). This technique rests upon the primary immunological principle that the antigen recognition site of an antibody is complementary to the structure of the antigen and thus reflects the local topological features of its epitope (24, 26, 28). A combination of techniques is used to access this structural information. First, peptides that bind to an antibody are identified. These peptides might represent fragments of the protein of interest or, more commonly, are identified by phage display of random peptide libraries (29, 30). Peptides selected from phage display are particularly useful in dealing with discontinuous antibody epitopes, which are quite common (31, 32). Residues within the selected peptides are then mapped back to the primary structure of the target protein using techniques common to epitope mapping. We have been developing new computational techniques to determine the optimal epitope mapping (26, 27). In favorable cases more detailed information can be obtained by determining the structures of these peptides when they are bound to their complementary antibodies using NMR (24), or like the present work, by x-ray diffraction. The resulting structure of the peptide and the antibody surface provide numerous constraints that can be used to model the structure of the epitope on the surface of the native protein antigen.

In previous work, the antibody imprint technique has been used to identify a consensus peptide sequence that mimics a discontinuous epitope on the surface of flavocytochrome \( b \) (24, 29, 33). The peptide was mapped to two discontinuous regions of the protein that are folded closely together but are 150 residues and two transmembrane spans apart in the primary protein sequence. The structure of the antibody-bound peptide was determined by transferred nuclear Overhauser effect spectroscopy NMR studies and provided constraints that allowed modeling of the native epitope (24).

More recently, Bailey et al. (27) initiated antibody imprint studies of light-excited rhodopsin to probe its meta-stable conformations. This work utilized a library of monoclonal antibodies developed against opsin. Included in this library is K42-41L, a murine monoclonal antibody first described by Adamus et al. (34). Adamus et al. concluded that the primary epitope of K42-41L was contained within the C3 loop of rhodopsin (residues 230–252) by showing that a peptide fragment representing the C3 loop effectively competed against rhodopsin for binding to K42-41L. In contrast, peptides representing other surface loops did not show detectable competitive activity against this antibody.

Bailey et al. (27) employed a random sequence peptide library displayed on phage to select small peptides that bind to K42-41L. Ninety phage clones were selected from the J049 9-mer peptide library (29) and sequenced. Sequence alignment of the bound clones yielded the consensus sequence TGALQERSK. This peptide was found to compete effectively against rhodopsin for binding to K42-41L. The interaction of K42-41L with light-excited rhodopsin was also examined, providing preliminary evidence that this antibody inhibits formation of metarhodopsin-II (27).

In this work we report further characterization of the interaction of K42-41L with dark-adapted and light-excited rhodopsin. We show that K42-41L can discriminate between metarhodopsin-I and metarhodopsin-II, inhibiting formation of metarhodopsin-II under several conditions while stabilizing metarhodopsin-I. In addition, we have determined the crystal structure of Fab K42-41L bound to a peptide that mimics the native epitope (TGALQERSK). Geometric constraints taken from the structure of the peptide in the K42-41L-peptide complex were used to model the conformation of the native epitope. This model suggests that K42-41L recognizes a solvent-exposed conformation of the C3 loop that is intermediate between the compact conformations seen in two crystal structures of dark-adapted rhodopsin (1GZM and 1U19). The discriminating activity of K42-41L indicates that the conformation of the C3 loop that binds K42-41L can be attained in both dark-adapted rhodopsin and metarhodopsin-I, but this conformation must be significantly different from that present in metarhodopsin-II. Taken together, the structural and functional data suggest that the equilibrium between inactive metarhodopsin-I and active metarhodopsin-II is dependent upon the conformation of the C3 loop.

**EXPERIMENTAL PROCEDURES**

**Rod Outer Segment Preparation**—Bovine retinas were obtained from Schenk Packing Co. (Stanwood, WA). Manipulations of retinas, rod outer segments (ROS), and rhodopsin were carried out under dim red light unless otherwise indicated. Retinas were suspended in ice-cold Kuhn’s buffer (0.05 M monobasic potassium phosphate, 0.015 M dibasic potassium phosphate, 1 mM Mg(OAc)\(_2\), 1 mM dithiothreitol, 0.1 mM EDTA, pH 7.0, sparged with argon). ROS were sheared from the retinas by mechanical shaking (35), purified with discontinuous sucrose gradients (27.0%, 28.7%, 34.0%, and 38.0%), and stored in liquid nitrogen. The purity of the rod outer segments was determined by measuring the ratio of total protein absorbing at 280 nm to photoactivatable rhodopsin. Rhodopsin concentrations were calculated from the difference spectrum (unbleached-bleached) at 500 nm (\(\Delta A_{500}\)) in the presence of 3% lauryldimethylamine N-oxide using a molar extinction coefficient of 40,600 M\(^{-1}\) cm\(^{-1}\). A typical \(\Delta A_{500}/\Delta A_{500}\) ratio for the rod outer segments was 2.

**Light Titration Experiments**—Rhodopsin rod outer segment membranes used in light titration experiments (36) were suspended in buffer A (50 mM HEPES, 100 mM NaCl, 1 mM MgCl\(_2\)), and the final pH was adjusted for the given experiment. Prior to light titration experiments, working stock solutions of rod outer segments were briefly sonicated, and rhodopsin concentrations were determined by measuring the \(\Delta A_{500}\). The concentrations of rhodopsin were then adjusted to 7.5 \(\mu\)M by addition of buffer A at the appropriate pH. For each light titration experiment, an aliquot of rhodopsin was bleached with a series of millisecond flashes at 50-s intervals, and absorbance changes were measured during each interval. Each flash bleached \(\sim 8\%\) of the rhodopsin. The appearance of metarhodopsin-II was detected by monitoring the difference in
absorbance between 390 nm and 417 nm (36), using an Olis/Shimadzu UV-3000 dual wavelength spectrophotometer equipped with a temperature-controlled cuvette holder and a sampling compartment purged with dry nitrogen gas. All light titration experiments were performed at 4 °C.

For measurement of low pH-stabilized metarhodopsin-II, aliquots of rod outer segment were diluted in buffer A at pH 6.7. For assays that included K42-41L, the antibody was present at a concentration of 2.0 μM. The peptide Ac-VLEDLRS(Abu)GLF, a high affinity analog of the C-terminal tail of the transducin α subunit (37), was used to stabilize metarhodopsin-II at pH 8.0. Light titration assays involving peptide were done with buffer A at pH 8.0. Synthetic peptides were synthesized employing a Protein Technologies PS3 peptide synthesizer and purified by high-performance liquid chromatography. The purified peptide masses were determined by MALDI-TOF mass spectrometry. Stock solutions of the peptides were prepared by dissolving the peptide in buffer A at pH 8.0.

**Difference Absorbance Spectra**—Absorbance spectra of rhodopsin with and without antibody K42-41L were measured in a Cary 50 spectrophotometer with a temperature-controlled sample holder and sampling compartment purged with dry nitrogen. Absorbance spectra were acquired at 4 °C at pH 8.1. A rhodopsin stock solution (38 μM) was made by diluting rod outer segments in buffer A containing 5 mM dodecyl maltoside (Calbiochem). The final rhodopsin concentration was 3.2 μM, and the final dodecyl maltoside concentration was 2.0 mM. Rhodopsin samples were placed in the temperature-controlled sample holder, and the temperature was allowed to equilibrate. Spectra of unactivated rhodopsin were measured. The sample was then illuminated with >500 nm light from a Fiber Photics Illuminator 150 for 10 s while the absorbance spectra were acquired. The pH of the samples was measured following recording of spectra. Difference spectra were deconvoluted by fitting summed synthetic Gaussian curves for metarhodopsin-I, metarhodopsin-II and rhodopsin.

**ELISA**—Enzyme-linked immunosorbent assays (ELISAs) were performed using polystyrene chloride microtiter plates coated with bovine rhodopsin in purified rod outer segments (ROSs). The rhodopsin was prepared by adding 1 ml of 10 mM PBS, pH 7.4, to a 200-μl aliquot of 300 μM rhodopsin in dark- or light-exposed ROS, followed by brief sonication. 100-μl aliquots of rhodopsin solution were added to microtiter plates under dim red light and incubated at 4 °C for 8 h, washed three times with PBS, and then blocked with 5% (w/v) milk protein, 0.02% NaN₃ in PBS. A serial dilution of K42-41L was added to the coated and blocked microtiter plate to measure antibody binding to dark-adapted rhodopsin and light-exposed opsin. For competition ELISA, microtiter plates were prepared as described above with addition of antibody K42-41L to all wells of the microtiter plates at a concentration of 1 μg/ml. Stock solutions (5 mM) of synthetic peptides TQKAKEVTR-NH₂, TGALQERK, and TETSQVAPA were made in PBS, and the peptides were added in the amount indicated in each assay. The microtiter plates were incubated for 4 h on an orbital shaker at room temperature and then rinsed three times with PBS to remove unbound antibody. Binding of antibody K42-41L was detected by the addition of alkaline phosphatase conjugated goat anti-mouse antibody (GAM-AP, Zymed Laboratories Inc.) diluted 1:10,000 in PBS. Following the addition of GAM-AP, plates were incubated for 4 h at room temperature on an orbital shaker and washed three times with PBS. Following the addition of NBT/BCIP substrate, bound GAM-AP was detected by measuring absorbance at 405 nm.

**Sequencing of K42-41L**—The sequence of the antibody was obtained as follows: Total mRNA was extracted from a mouse hybridoma cell line producing antibody K42-41L (38) using the QuickPrep mRNA purification kit (Amersham Biosciences). Variable heavy and variable light chain cDNA were amplified from the total mRNA using a set of degenerate mouse variable heavy and variable light chain primers (39). Amplified variable heavy and variable light chain DNA was gel-purified and sequenced. The amino acid sequence of K42-41L was verified, in part, by mass spectrometry and Edman degradation.

**Expression and Purification**—Expression of the murine monoclonal antibody K42-41L was done as previously described (38). Fab fragments were generated by papain digestion (0.4 μg/ml, ICN) in the presence of 25 mM β-mercaptoethanol, with an enzyme:substrate ratio of 1:100 (w/w). The reaction was stopped by dialyzing against PBS to remove β-mercaptoethanol, and the completion of the digestion was verified by SDS-PAGE.

Fab fragments were purified from the Fab fragment by anion exchange chromatography using a Mono-Q column on an Amersham Biosciences fast-protein liquid chromatography system (Amersham Biosciences) with a linear elution gradient from 0 to 0.3 M NaCl in 10 mM Tris, pH 8.1. Fractions containing Fab K42-41L were concentrated and further purified by size-exclusion chromatography using a Superdex-75 column (Amersham Biosciences) in 10 mM MES, pH 6.5. Purified Fab fractions were pooled and concentrated to 4.0 mg/ml using Amicon Ultra 3500 MWCO centrifugal filters (Millipore, Inc.). Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (40). Purity was assessed by SDS-PAGE analysis with or without reducing agent (dithiothreitol). The overall yield was ~2.5 mg of purified Fab from 10 mg of pure monoclonal antibody.

**Crystallization and Data Collection**—Purified Fab K42-41L was crystallized at 18 °C by hanging drop vapor diffusion. Drops were assembled with 2 μl of Fab mixed with 1 μl of peptide TGLAQERK (16 mg/ml) and 2 μl of well solution (0.1 M MES pH 6.5, 0.1 M (NH₄)₂SO₄, 20% polyethylene glycol-monomethyl ether 5000). Crystals grew in ~4 weeks. The peptide stock solution was analyzed, post-crystallization, by MALDI-TOF mass spectrometry. It was shown that partial hydrolysis of the peptide had occurred, resulting in a mixed population in which some peptide had lost the serine and lysine residues. Therefore, a reduced occupancy of the C-terminal residues in the crystal is likely to have occurred, and may actually have promoted crystallization. Single crystals were soaked in 25% glycerol for 3 h and flash frozen in liquid nitrogen. Data were collected at 100 K using a Rigaku RU3HR rotating anode, CuKα x-ray source, and a MAR 345 image plate detector. Fab K42-41L crystals diffracted to a resolution of 2.7 Å and belonged to space group P2₁2₁2₁ with a = 109.72 Å, b = 196.65 Å, and c = 44.17 Å, with two Fab molecules per asymmetric unit. Data were integrated and reduced using the HKL software package (41).

**Structure Determination and Refinement**—The structure of Fab K42-41L was determined by molecular replacement. Rotation and translation functions were done using the program COMO (42). Due to the large variation in elbow angles of crystallized Fabs, the molecular replacement procedure typically breaks the chosen Fab search model into constant and variable domains, and each domain is positioned independently. However, in this case, we chose to use the family search option of COMO and a library of 195 Fab search models consisting of the entire Fab molecule. The large number of search models apparently allowed an effective search of the “elbow angle” space. The library was composed of 195 human, mouse and rat IgG Fab structures downloaded from the Protein Data Bank. Each structure was processed with a series of script files to generate a single Fab search model, free of small molecules, heterotomers, and complexed macromolecules. Upon completion...
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FIGURE 1. A, schematic diagram of the light-excitation pathway of rhodopsin. After exposure to visible light (hv) the pink-colored rhodopsin (Rho) undergoes a series of rapid (fast) conformational changes ending in an equilibrium mixture of the orange-colored metarhodopsin-I (MI) and yellow-colored metarhodopsin-II (MII). MI interacts with and is stabilized by the heterotrimeric G-protein (G) transducin or certain peptide segments of transducin (P) to form yellow-colored complexes (MI-G/P). The color changes of the different conformational forms of the protein allow spectroscopic monitoring of the photolysis of rhodopsin, the position of the equilibrium between MI and MII, and the formation of MI-G or MII-P complexes. The position of the MI/MII equilibrium is sensitive to pH and MII is favored under mild alkaline conditions (pH ~ 8) while MI is favored under mild acid conditions (pH ~ 6). MII slowly breaks down to orange-colored MIII, followed by formation of the colorless apoprotein opsin (chromophore-free). Opsin production is too slow to contribute under the conditions studied in the present experiments, unless explicitly indicated. B, ELISA demonstrating that the K42-41L antibody recognizes the original antigen, opsin (open diamonds), and dark-adapted rhodopsin in native retinal rod disk membranes (open squares) with essentially equal affinity. The dark-adapted or fully light-bleached membranes were bound to ELISA plates, washed, and presented with a dilution series of antibody K42-41L. Bound antibody was detected as described in the text.

of the library, the automated search process was rapid and successful. Mouse IgG FabR19.9 (PDB ID, 1FAI) was placed in the asymmetric unit, resulting in a correlation coefficient of 21.14% and an R-factor of 47.9%. The process was repeated, resulting in the placement of a second Fab (mouse IgG Fab 28, PDB ID, 1N6Q) and a final correlation coefficient of 33.94% yielding an initial R-factor of 45.8%.

All residues in the variable domains of this initial model were then converted to alanine, or glycine when appropriate, and the CDR loops were deleted. This initial model was then subjected to positional refinement using conjugant gradient energy minimization in CNS (43), followed by density modification (CCP4) (44). SigmaA-weighted 2mFo - dFc maps were calculated with independent NCS averaging across the variable and constant domains. All data between 20.0 and 2.7 Å were used in the refinement with 5% of the data randomly chosen for Rfree calculations. Rounds of iterative model building with O (45), and positional and independent B-factor refinement with CNS resulted in a final model with an R-factor of 23.3% (Rfree = 27.2%). The model generally has good stereochemistry, however, residue MetL51 in each Fab lies in a disallowed region of the Ramachandran plot (46). MetL51 is found at the apex of a strained gamma turn that is highly conserved among antibody structures and represents a canonical conformation for this residue (47). All structural figures were generated with PYMOL (www.pymol.org).

Coordinates—Atomic coordinates and structure factors for the Fab K42-41L-peptide TGALQERSK crystal structure have been deposited into the Protein Data Bank under accession code 1XGY.

Rhodopsin Modeling—The C3 loop is ordered in two different structures of dark-adapted rhodopsin, 1GZM and 1U19. The 1U19 structure (12) presents a more complete model for the C-terminal tail and is preferable in this regard. However, the conformation of the C3 loop is probably influenced by crystal contacts that are not present in the 1GZM structure. Therefore we created a starting model that is a hybrid of the two structures, replacing C3 loop residues 227–247 in the 1U19 structure with the same residues from the 1GZM structure. All modeling was done using CHARMM version 31b1 (48, 49) with the CHARMM22 force field (48). The rhodopsin modeling process was highly iterative. With the exception of atoms comprising the C-3 loop, residues 227–245, the rhodopsin molecule was held fixed throughout the modeling process. Our final model was generated as described...
The C3 loop controls metarhodopsin equilibrium.

Below, first, distance restraints were used to define the relative geometry of rhodopsin residues Gln238 and Glu239. These restraints were based upon interatomic distances in residues Gln238 and Glu239 of peptide TGALQERSK in the Fab K42-41L crystal structure. All possible distance restraints between the Cα of Gln238 and the Cα of Glu239 were included. Additional distance restraints to force the C3 loop away from the C2 loop were also imposed on the Cα atoms of the following residues: Pro142 to Ala233, 21.7 Å; Pro142 to Gln238, 26.1 Å; Pro142 to Ser240, 23.7 Å; Arg252 to Ala233, 7.6 Å; Arg252 to Gln238, 18.4 Å; Arg252 to Ser240, 15.2 Å; Lys311 to Ala233, 15.7 Å; Lys311 to Gln238, 21.0 Å; and Lys311 to Ser240, 15.2 Å. This latter set of restraints represents distances between residues in the C3 loop and the remainder of the rhodopsin molecule and was used to move the C3 loop into an extended, antibody-accessible conformation that is intermediate between those seen in the trigonal (1GZM) and tetragonal (1U19) crystal structures. This was necessary to allow flexible interactions with the C3 loop. These target distance restraints were satisfied using adopted-basis Newton-Raphson energy minimization to convergence. This was followed by a further adopted-basis Newton-Raphson energy minimization using a second set of distance restraints to further refine the relative positions and residues within the C3 loop. Cα–Cα distance restraints were as follows: Gln238 to Pro142, 26.1 Å; Gln238 to Arg252, 18.4 Å; Gln238 to Lys311, 21.0 Å; Glu239 to Pro142, 24.6 Å; Glu239 to Arg252, 18.0 Å; and Glu239 to Lys311, 19.1 Å. Gln238 to Cα distance restraints were: Cα of Gln238 to Cα of Pro142, 27.0 Å; Cα of Gln238 to Cα of Arg252, 22.1 Å; Cα of Gln238 to Cα of Lys311, 24.8 Å; Cα of Glu239 to Cα of Pro142, 21.7 Å; Cα of Glu239 to Cα of Arg252, 19.3 Å; and Cα of Glu239 to Cα of Lys311, 20.7 Å. These restraints made small adjustments to the relative positions of residues within the C3 loop to further facilitate interactions with the antibody. The Fab K42-41L crystal structure was then docked to the resulting rhodopsin model. This was accomplished with a least-squares superposition of peptide residues Gln238 and Glu239 of the K42-41L-peptide complex onto the Cα and side-chain atoms of Gln238 and Glu239. Bad contacts were alleviated by rigid-body energy minimization followed by 1.0 ns of restrained rigid-body Langevin dynamics, using the SHAPEs module of CHARMM to define the Fab molecule as a rigid unit, with the exception of the side-chain atoms in the CDR loop regions, which were allowed full flexibility within the context of the CHARMM22 force field. The temperature was held constant with the temperature bath set to 300 K and the radius of the Langevin buffered region set to zero. Forces were integrated across a 1.0-fs time step using the leapfrog Verlet integrator. Non-bonded electrostatics were modeled using the group electrostatic approximation model (49) with the constant dielectric term set to 1.0 and the Van der Waals energy term calculated with a long-range switching algorithm. The non-bonded interaction lists were updated heuristically. In the interest of keeping the Fab tightly bound to rhodopsin during the initial minimization and dynamics, distance restraints were placed between residues in the CDR of the Fab and Gln238 and Glu239 in rhodopsin. Specifically, the amide group in the side chain of Gln238 was constrained to be within H-bonding distance of Asn1435. Similarly, the carboxylate group of Glu239 was held close to the hydroxyl group of Tyr134 and the guanidinium group of Arg50. After 1.0 ns of Langevin dynamics, distance restraints between the Fab and rhodopsin were released, and the model was energy minimized and subjected to an additional 100 ps of Langevin dynamics.
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Finally, the model underwent unrestrained energy minimization in an implicitly solvated environment using the Generalized Born algorithm with a simple switching function as implemented by the GBSW module of CHARMM (50). In the implicitly solvated environment non-bonded interactions were calculated using an atomistic pairwise method, with a cutoff boundary of 20 Å.

Movement of the C3 loop between the trigonal 1GZM (13) and tetragonal 1U19 (14) conformations, passing through the putative antibody-bound conformation was modeled using a CNS-based morphing algorithm (51, 52). The script used stepwise linear interopolation of the beginning and end state coordinates to calculate the positions of the atoms followed by a short Powell energy minimization (60 cycles) to relax strained geometrical configurations. The resulting PDB files were then used to construct a movie (supplemental Fig. S1), illustrating a model for the motion of the C-3 loop between 1GZM (Fig. 6A), the K42-41L antibody bound model (Fig. 6B) and 1U19 (Fig. 6C). Movies were assembled from PNG format images generated by PYMOL (www.pymol.org) with subsequent conversion to mpeg format using CONVVERT (www.imagemagick.org).

RESULTS

Conformational States of Rhodopsin and the Activity of K42-41L—Photoisomerization of the retinal cofactor causes rhodopsin to rapidly progress through a series of intermediates that leads to an equilibrium between metarhodopsin-I and metarhodopsin-II, as diagrammed in Fig. 1A. The retinal is eventually lost, resulting in appearance of the apoprotein opsin (Fig. 1A). We found, using ELISAs, that the relative affinity of K42-41L for dark-adapted rhodopsin is quite similar to that for opsin, the original antigen used for production of K42-41L (Fig. 1B). This demonstrates that the K42-41L epitope is accessible in both opsin and dark-adapted rhodopsin.

Effect of K42-41L on the Metarhodopsin-I ↔ Metarhodopsin-II Equilibrium—Antibodies that preferentially recognize a specific metastable state of rhodopsin are expected to influence the equilibrium between metarhodopsin-I and metarhodopsin-II (27). Metarhodopsin-I is orange while metarhodopsin-II and its complex with transducin or certain transducin peptides are yellow (Fig. 1A). Thus the appearance of metarhodopsin-I and metarhodopsin-II, as well as the equilibrium between these two metastable species, can be followed spectrophotometrically. A convenient way to observe the metarhodopsin-I ↔ metarhodopsin-II equilibrium is to follow the change in absorbance between metarhodopsin-II (at 380 nm or with better signal to noise at 390 nm) and the nearby metarhodopsin-I/metarhodopsin-II isosbestic point at 417 nm (36). The native rhodopsin-containing ROS membrane suspensions are optically turbid, however, the light scattering of the suspensions is quite similar at 390 and 417 nm. Thus, differences in light scattering between the two wavelengths due to slight settling of membranes or changes in membrane scattering, are strongly attenuated in the ΔA(390 nm-417 nm) signals. The conformational equilibrium of light-excited rhodopsin is sensitive to several factors that preferentially stabilize particular metastable states (Fig. 1A), with pH ~8 stabilizing metarhodopsin-I and pH ~6 stabilizing metarhodopsin-II.

Binding of transducin peptides that mimic the C-terminal tail of the transducin α subunit (37, 53, 66), or the farnesylated N-terminal peptide of the transducin γ subunit (54) stabilize metarhodopsin-II. In the absence of transducin or transducin peptide mimetics, a series of light flashes results in light-activated rhodopsin in which the metarhodopsin-I ↔ metarhodopsin-II equilibrium greatly favors metarhodopsin-I, under typical assay conditions of pH 8 and 4°C. The addition of the high affinity transducin α subunit C-terminal peptide analog Ac-VLEDLRS-(Abu)GLF (53) shifts the metarhodopsin-I ↔ metarhodopsin-II equilibrium, greatly favoring the formation of metarhodopsin-II (Fig. 2A).

Optical monitoring of conditions that affect stability of metarhodopsin-II is termed the “extra meta-II assay.” In Fig. 2A each flash excited ~8% of the total rhodopsin present, and equilibrium is reached within ~40 s after each flash at 4°C.

To examine the interaction of K42-41L with light-excited rhodopsin, the antibody was added to the extra meta-II assay. Addition of K42-41L to the peptide-stabilized extra meta-II assay (Fig. 2A) resulted in a large decrease in formation of metarhodopsin-II. In a related version of the assay, shown in Fig. 2B, metarhodopsin-II is stabilized instead by low pH (6,7), and the kinetics for the formation of metarhodopsin-II are much faster. Introduction of K42-41L into this low pH extra meta-II assay results in a similar reduction in formation of metarhodopsin-II (Fig. 2B).

The decrease in metarhodopsin-II could be due to several factors. K42-41L might possibly inhibit formation of metarhodopsin-I, and thus the subsequent formation of metarhodopsin-II (Fig. 1A), or K42-41L might preferentially stabilize metarhodopsin-I, resulting in loss of the meta-II signal. Although the extra meta-II assay is very useful for measuring the kinetics of the light excitation process and for “light titration” of active rhodopsin, full absorption spectra are more definitive in confirming the identities of photolysis intermediates. However, the light scattering of the membrane suspensions tends to strongly distort the optical absorbance spectra. Thus dodecyl maltose, a mild detergent that dissolves the membrane, was added to reduce light scattering and greatly improve the clarity of the optical spectra. However, the presence of dodecyl maltose increases the rate of the MI → MII reaction and shifts the MI/MII equilibrium strongly toward metarhodopsin-II (55).

Under the conditions of this experiment (pH 7.2, 4°C, 2.0 mM dodecyl maltoside) the absorbance components show that the equilibrium ratio of MII/MI is 1.2 (accounting for the higher extinction coefficient of MI). Under the same conditions in the native membrane the MII/MI ratio would be ~3-fold lower (36). Deconvolution of the full light-dark difference spectra reveals components that are due to the loss of rhodopsin and the gain of metarhodopsin-I and metarhodopsin-II (Fig. 3). The loss of rhodopsin absorbance at 498 nm is evidenced by the negative lobe of the difference spectra in Fig. 3 (A and C), and the equilibrium ratio of

### TABLE 1

**Summary of crystallographic data**

| Parameter | Value |
|-----------|-------|
| Data collection | |
| Space group | P2₁ 2 2 |
| Unit cell dimensions (Å) | a = 109.721, b = 196.646, c = 44.173 |
| Solvent content (%) | 51.55 |
| Resolution | 98.3–2.71 |
| Total observations | 270,169 |
| Unique reflections | 21,162 (1,122)* |
| Rmerge (%) | 8.3 (23.4)* |
| Completeness (%) | 78.4 (43.2)* |
| Redundancy | 4.2 (1.2)* |
| l/σ | 12.7 (2.4)* |

* Values for the outer shell were from 2.80 to 2.71 Å. *Mel(31) exists in a well defined γ turn in almost all antibody structures but is designated by PROCHECK (46) as an outlier.

| Refinement | |
| Total residues | 875 |
| Water molecules | 75 |
| Refinement range | 20–2.71 |
| Rmerge (%) | 23.40 |
| Rfree (%) | 27.29 |
| Bond length deviation (Å) | 0.0083 |
| Bond angle deviation (°) | 1.49 |
| Residues in most favored regions of Ramachandran plot (%) | 85.1 |
| Residues in disallowed regions (%) | 0.3* |
| Average B value (Å²) | 40.5 |

(Abu)GLF (53) shifts the metarhodopsin-I ↔ metarhodopsin-II equilibrium, greatly favoring the formation of metarhodopsin-II (Fig. 2A).
metarhodopsin-I and metarhodopsin-II can be seen in the components of the absorbance spectra in Fig. 3 (B and D). Addition of the K42-41L antibody causes a decrease in metarhodopsin-II, with an absorbance maximum near 380 nm and an increase in the formation of metarhodopsin-I, with a maximum near 480 nm (Fig. 3, B and D). Thus the deconvoluted difference spectra show that K42-41L destabilizes metarhodopsin-II, pulling metarhodopsin-I into an antibody stabilized complex.

Adamus and coworkers (34) have demonstrated the reactivity of antibody K42-41L toward a peptide containing residues 230–252 of the C3 loop. The random peptide phage display epitope mapping data of Bailey et al. (27) indicated strongly that K42-41L epitope includes the rhodopsin residues Gln238 and Glu239. However FINDMAP, a computational epitope mapping algorithm designed to match target protein sequences for recognition by K42-41L, and this combination of loop lengths would be consistent with an interaction between K42-41L and an extended conformation of the C3 loop.

The antigen-binding Fab fragment of K42-41L was purified and co-crystallized with the TGALQERSK peptide. The structure was determined at 2.7 Å resolution by molecular replacement with two copies of the Fab-peptide complex in the asymmetric unit. There was clear electron density for K light-chain residues 1–211 (chains A and C) and IgG1 heavy-chain residues 1–129 and 133–214 (chains B and D). The antibody model exhibits good geometry with 85.1% of the residues in the most favored regions of the Ramachandran plot and 14.6% in generously allowed conformations. Data collection and refinement statistics are shown in Table 1.

One noteworthy structural feature of antibody K42-41L is its concave antigen recognition site (Fig. 4, A and B). This is a direct result of the long L1 and short H3 loops of the antibody. Strong density for the first 6 residues (TGALQE) of the peptide was found within the concave antigen recognition site of each Fab. These residues are well ordered with a

FIGURE 4. Stereo figures depicting the antigen recognition site of K42-41L. The relative orientation of K42-41L is identical in both panels. A, the structure of antibody K42-41L is depicted with a transparent blue surface and an underlying ribbon structure in green. The peptide TGALQE (carbon in yellow, oxygen in red, and nitrogen in blue) is bound in the concave antigen recognition site of the antibody. B, close up view of the structure depicted in panel A. The peptide adopts a helical conformation with one and a half turns of a-helix. The threonine, glycine, glutamine, and glutamate residues are found on the bottom half of the helix where they interact with the antibody. The alanine and leucine side chains emanate from the top surface of the helix and interact primarily with solvent. The orientation of the Gln238-Glu239 pair in the peptide is stabilized by H-bonds and a salt bridge (TGALQERSK), the peptide representing residues 243–252 of the rhodopsin sequence, namely the 243–252 region of the C3 loop.
mean $B$-value of 32 Å$^2$. The peptide adopts an $\alpha$-helical structure of approximately one and a half turns with the first, second, fifth, and sixth residues pointing down into the antigen binding site (Thr, Gly, Gln, and Glu), whereas the third and fourth residues of the peptide lie on the opposite side of the helix with their side chains solvent exposed (Ala and Leu). Not surprisingly, the four residues of the peptide that interact with the antibody are also the most strongly conserved among the peptides isolated from the diverse random peptide library displayed on phage (27). Threonine and glycine at positions P1 and P2 show 62 and 86% conserved identity, respectively, in peptides selected by the antibody from the random peptide library. The glutamine and glutamate at positions P5 and P6 show even greater conservation with nearly 100% identity in all peptide sequences from the phage display epitope mapping (27).

The threonine and glycine residues at the N terminus of the peptide interact with light chain residues 91–94. The threonine side chain forms a strong hydrogen bond to the main chain carbonyl of light-chain residue 92, whereas the main-chain NH group of the glycine forms a strong hydrogen bond to the carbonyl group of light-chain residue 91. The phi and psi values for the glycine are those of a typical $\alpha$-helix, thus the high sequence conservation at this position appears unrelated to the increased flexibility of this residue. A more likely explanation for the lack of a side chain is that a side chain at this position would result in steric clash with the antibody. In addition to these interactions, the amine group at the N terminus of the peptide appears to form a weak hydrogen bond with His$^{194}$. It is noteworthy that the threonine and glycine residues do not saturate the potential for formation of H-bonds to main chain atoms of the light chain. In particular, the main-chain NH of light-chain residue 94 is clearly accessible and might be expected to participate in recognition of the native epitope.

In keeping with their high degree of sequence identity in peptides selected by the antibody from phage display (27), the glutamine and glutamate residues of the peptide appear to form the most specific interactions between the peptide and the antibody in the crystal structure of the complex. The glutamine side chain extends into a pocket in which its amide group forms two complementary hydrogen bonds to the amide group of Asn$^{135}$, whereas the glutamate side chain extends into a complementary pocket, forming hydrogen bonds to the hydroxyl group of Tyr$^{135}$ and the guanidinium group of Arg$^{150}$. The apparent specificity with which these residues are bound strongly suggests that the Gln and Glu residues of the antibody-bound peptide are representative of the conformation of the C3 loop residues Gln$^{238}$ and Glu$^{239}$ in the K42-41L complex with rhodopsin. Gln$^{238}$ and Glu$^{239}$ are the only Gln-Glu pair in the rhodopsin sequence.

Modeling the C-3 Loop of Rhodopsin—An examination of the trigonal (1GZM) and tetragonal (1U19) crystal structures of dark-adapted rhodopsin in which the C3 loop is ordered shows vastly different conformations for the C3 loop in the two structures (Fig. 6, A and C). One indication of the magnitude of these differences is the 18 Å distance between the Ca atoms of Gln237 in these two crystal forms. This implies conformational flexibility that allows interconversion between these two forms. A first attempt to dock K42-41L to either of these structures with a simple superposition of the Gln-Glu pair of the peptide onto the Gln$^{238}$-Glu$^{239}$ pair of rhodopsin resulted in major steric clashes between the antibody and rhodopsin. Furthermore, the side chain of Glu$^{239}$ is not particularly accessible in either the 1GZM or the 1U19 structures (Fig. 6, A and C), because the side chain extends back toward the membrane, rather than out toward the solvent. Thus, the initial superposition of K42-41L with dark-adapted rhodopsin indicates that the antibody recognizes a conformation of the C3 loop that is quite different than those
seen in either of the crystal structures and is most likely intermediate between these two extremes. As the C3 loop moves back and forth between these two crystallographically observed conformations, it is likely that it passes through a conformation that is recognized by the antibody.

We have modeled the conformation of the C3 loop when it is bound to the K42-41L antibody using CHARMM. Constraints taken from the antibody-peptide complex defining the relative geometry of Gln238 and Glu239 were imposed along with distance constraints to push the C3 loop into a conformation that was antibody-accessible, allowing the antibody to be docked to the C3 loop of rhodopsin without a significant steric clash. The complex was then subjected to energy minimization and molecular dynamics.

The resulting model (Fig. 5, A and B) is free of bad contacts and presents the C3 loop in a conformation (Fig. 6A) that is intermediate between those seen in the two crystal structures of dark-adapted rhodopsin (Fig. 6, A and C). The model of the C3 loop bound to the antibody extends away from the membrane, into the cytoplasm, where it would be much more solvent-exposed in the absence of antibody than the C3 conformations observed in the rhodopsin crystal structures. The model of the complex suggests that the antibody interacts predominately with conformations observed in the rhodopsin crystal structures. The model of the complex suggests that the antibody interacts predominately with rhodopsin residues 232 through 244 (232EAAAQQQESATTQ244), with the apex of the C3 loop, including Gln238 and Glu239, projecting into the concave antigen recognition site of the antibody (Fig. 5). In a complementary fashion, the CDR L1 loop extends toward the membrane, terminating at the base of the C3 loop. The Gln238-Glu239 pair is nicely accommodated by the antibody, with interactions for the side chains that are essentially identical to those seen in the structure of the antibody-peptide complex. The model also makes new suggestions with regard to interactions between the rest of the C3 loop and the binding pocket of the antibody.

The side chain of Gln237 in the model of the complex is found in roughly the same position as the N-terminal threonine residue of the antibody-bound peptide. Gln237 has the potential to form three hydrogen bonds with the antibody; one with the carbonyl oxygen of Leu192, one with the main-chain amide group of His194, and one with the side chain of His194. It does not seem unreasonable that an N-terminal threonine might be able to substitute for a glutamine residue. Rhodopsin residues 232, 242, 243, and 244 also form side-chain-specific interactions with the CDR pocket in the model of the complex, but these lie away from the peptide binding site. Although minimal constraints for these regions of the C3 loop are dictated by the surface of the antibody, specific restraints on their positions relative to each other, or relative to the antibody, cannot be derived from the antibody-peptide complex. Thus the validity of the interactions predicted by the model for residues 232–235 and 242–244 are less certain, and we prefer to limit description of these putative interactions.

The lack of interaction between the antibody and the peptide sequence at the P3 and P4 positions in conjunction with the limited sequence identity at these same positions in phage display suggests that residues displaying <60% consensus sequence identity in epitope mapping may not interact directly with the antibody. Thus, residues that fall below this threshold should be mapped with caution. This also points to the need for further exploration of the amino acid substitution probabilities used in mapping consensus peptides from phage display, which is underway. Some preference for certain residues at low consensus positions may be due to subtle effects these residues exert on the structure of the peptide. It is also possible that crystal-packing forces could influence the interactions of some peptide residues with the antibody, especially those that are more weakly bound.

A superposition of the rhodopsin model generated in this work with the trigonal and tetragonal rhodopsin crystal structures discussed above suggests a potential pathway for conformational changes that take the C3 loop back and forth between the limiting states of the two crystal
Additional intermediates that might be associated with this conformational change were generated with CNS (51, 52). These potential intermediates have been depicted with PyMol and assembled into a movie that is included as on-line supplemental material (Movie S1). This sequence of conformations serves to illustrate the magnitude of conformational flexibility of the C3 loop in dark-adapted rhodopsin, and possibly in metarhodopsin-I as well. The depiction is not meant to imply detailed knowledge of the molecular events and represents only one of several possible pathways.

**DISCUSSION**

Spectroscopic assays show that the K42-41L antibody shifts the MI/MII conformational equilibrium away from agonist-activated metarhodopsin-II to the inactive metarhodopsin-I precursor. Such an analysis is inherently more difficult for other native GPCRs, because they lack the clear spectroscopic conformational signatures that are present in rhodopsin. K42-41L shifts the equilibrium toward metarhodopsin-I when metarhodopsin-II has been stabilized in either of two different ways. This occurs when metarhodopsin-II is stabilized "physiologically" by the binding of high affinity G\textsubscript{a} C-terminal peptides, or when metarhodopsin-II is stabilized "biochemically" at low pH (6.7) in the native membrane (Fig. 2). The antibody was also shown to bind the dark-adapted conformation and opsin (Fig. 1).

The ability of K42-41L to discriminate between metarhodopsin-II versus dark-adapted and metarhodopsin-I implies conformational similarity between the dark-adapted and meta-I states, followed by significant conformational change as metarhodopsin-I transitions to metarhodopsin-II. This is consistent with the electron crystallographic studies of Ruprecht et al. (18) on a photostationary state of rhodopsin that was highly enriched in metarhodopsin-I. The 5.5 Å structure indicated that the conformation of the meta-I state is similar to that of the ground state, and that conformational changes later in the photobleaching process (presumably at meta-II) are responsible for disruption of their two-dimensional protein crystals.

Biochemical and structural evidence strongly suggest that K42-41L recognizes an extended conformation of the C3 loop that is intermediate between the trigonal and tetragonal crystal structures of dark-adapted rhodopsin. Although energy minimization and molecular dynamics runs succeeded in pulling additional elements of the C3 loop into contact with the antigen recognition site of K42-41L in the docking model, these exercises failed to show significant antibody interactions with residues on the C2 loop or the C-terminal tail. This suggests that the activity of K42-41L is due primarily to its interaction with the C3 loop. Although the antibody is quite large and might physically block the MII-stabilizing G\textsubscript{a} C-terminal peptide from interacting with rhodopsin, the antibody also inhibits formation of low pH stabilized metarhodopsin-II that does not require an additional ligand. In the case of low pH-stabilized metarhodopsin-II, the activity of the antibody cannot be due to steric interference with a stabilizing ligand.

The structural model of the C3 loop presented in this work should be considered as a snapshot of an antibody accessible conformation. The dynamic simulation of the C3 loop conformation in dark-adapted rhodopsin (supplemental Movie S1) provides a plausible path for the conformational change associated with the transition between the trigonal and tetragonal crystal structures. The electron crystallographic structure of metarhodopsin-I, in which the C3 loop is disordered, and the ability of K42-41L to recognize metarhodopsin-I suggest that this simulation might also be relevant to the metarhodopsin-I conformation.
The C3 loop has been the subject of extensive mutational analysis. This includes the work of Yang et al. (23) who, using scanning cysteine mutagenesis, reported that the Q238C and E239C mutants exhibit normal photobleaching behavior and normal rates of Gt activation. They found that mutation of residues 226, 229, 230, 233, 234, 242, 243, or 244 all showed significant reduction in Gt activation but showed normal stabilization of metarhodopsin-II. More recently, Natochin et al. (9) demonstrated that conversion of the entire C3 loop to polyalanine inhibits activation of transducin. This does not, however, disrupt formation of metarhodopsin-II. The ability of these cysteine and polyalanine mutants to form metarhodopsin-II strongly suggests that the conformational role of the C3 loop in the meta-I/meta-II transition is independent of sequence-specific interactions.

Altenbach et al. (21) have used site-specific spin labeling to investigate the mobility of residues in the C3 loop. They found a general gradient of mobility such that mobility is lowest in the transmembrane helical segments and increases with distance from the aqueous/hydrophobic boundaries in the protein. The observed mobility is thus consistent with exposure of the apex of the C3 loop for interaction with the plasma membrane. The observed mobility is consistent with exposure of the apex of the C3 loop for interaction with the plasma membrane.

Thus, movement of these two helices relative to one another would separate the ends of the C3 loop, stretching it across the increased distance, pulling the loop toward the surface of the membrane. By binding to an extended conformation of the C3 loop that projects into the cytoplasm (Figs. 6 and 7), K42-41L might interfere with the movement of helix 6, essentially holding it on a short leash, and thus block formation of metarhodopsin-II. This model is consistent with the mutagenesis data in that it is sequence independent (Fig. 8A).

An alternative mechanism for the destabilization of metarhodopsin-II and the stabilization of metarhodopsin-I by the antibody (Fig. 8B) is suggested from evidence that rhodopsin may exist as a dimer, or even higher order oligomers or aggregates in the plane of the membrane (57). Filipek et al. (58) suggest that transducin may actually bind most favorably to a tetrmeric rhodopsin assembly, consisting of three dark-adapted and one light-excited species. Their favored model, referred to as "model IV–V", postulates a critical role for the C3 loop in formation of the active tetramer. Although the oligomeric IV–V model of Filipek et al. does not require cooperative protein binding, cooperative rhodopsin-G-protein interactions have been reported (59–61), further implying the possibility of multimeric interactions. There is also a variety of evidence relating dimerization or oligomerization of other GPCRs to the stability of the agonist-excited conformations (conformations thought to be homologous to metarhodopsin-II) (62). Thus, binding of K42-41L to the C3 loop of rhodopsin could inhibit the biological activity of the receptor by promoting dissociation of the tetramer, consequently destabilizing metarhodopsin-II as diagrammed schematically in Fig. 8B.

Once again, the mutagenesis data would suggest that such intermolecular contacts are largely sequence-independent with regard to the C3 loop. In addition, experiments using primarily monomeric and some dimeric rhodopsin in dodecyl maltose micelles indicate that K42-41L destabilizes metarhodopsin-II and stabilizes metarhodopsin-I (Fig. 3). Although the data data in Fig. 3 support the active-monomer model,
they do not necessarily exclude the active-tetramer model, because changes in aggregation could occur upon light excitation. Indeed, preliminary experiments at lower dodecyl maltoside concentrations, where formation of dimeric and tetrameric rhodopsin is favored (63, 64), result in somewhat greater stabilization of metarhodopsin-I by K42-41L.1 Thus, it is possible that the mechanism by which K42-41L inhibits formation of metarhodopsin-II includes elements of both models (Fig. 8, A and B). Arguments in support of the monomeric rhodopsin-G-protein activation mechanism have recently been summarized (65).

The flexibility of the C3 loop is likely to be related to its biological role. Highly flexible residues are frequently involved in protein-protein interactions during signaling events (16, 17). Entropic considerations suggest that increased flexibility allows the affinity of interactions to be modulated, while maintaining specificity (16, 17). In a similar fashion, the C-terminal tail of the transducin Gs subunit that stabilizes metarhodopsin-II (66) is also disordered in crystal structures (67–69) and becomes ordered when interacting with metarhodopsin-II (70, 71). Specific interaction of light-excited rhodopsin with transducin is required for visual excitation, but the complex cannot be too tight or the turnover of transducin would be too slow to provide the required amplification (5).

The observation that K42-41L can stabilize the dynamic C3 loop in a conformation that inhibits formation of metarhodopsin-II is significant. Dark-adapted rhodopsin is locked in an inactive conformation by the reverse agonist 11-cis-retinal. Photoconversion to the all-trans-retinal agonist releases the protein to allow an equilibrium between the inactive metarhodopsin-I and the active metarhodopsin-II, which excites the G-protein. Most other GPCRs have a much higher constitutive activity than rhodopsin (72) and presumably can fluctuate between M1-like and MII-like conformations in the absence of agonists. Further ongoing studies of antibodies or other compounds that stabilize metarhodopsin-I- or metarhodopsin-II-like states may elucidate novel methods for attenuating or activating GPCRs, leading to advances in experimental and biomedical applications.

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