The Location of the Chromophore in Rhodopsin: A Photoaffinity Study

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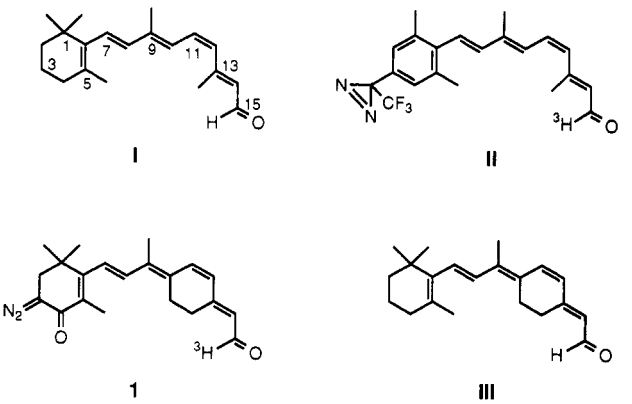
Abstract: A photoreactive analog of 11-*cis*-retinal was synthesized and used in photo-cross-linking studies to determine the orientation of the chromophore in bovine rhodopsin. The photoaffinity analog incorporated a tritium in the aldehyde group, a photoactivable diazo ketone moiety on the β-ionone ring, and a six-membered ring in the side chain; this six-membered ring fixes the 11-ene in its cisoid form, thus preventing photoisomerization and scrambling of cross-linked sites. The retinal analog was incorporated into bovine opsin to yield a rhodopsin analog absorbing maximmally at 483 nm. UV irradiation of the diazo ketone function resulted in almost exclusive cross-linking to Trp-265 and Leu-266 in α-helix F. These amino acids were identified by Edman degradation of a cyanogen bromide-cleaved peptide, which was separated by HPLC and gel electrophoresis. Since the labeled amino acids are located in the middle of the transmembrane helix F, while the Schiff-base linkage (Lys-296) at the other terminus of the chromophore also resides in the middle of helix G, the entire chromophore is positioned near the center of the lipid bilayer. A previous study (Nakayama, T. A.; Khorana, H. G. *J. Biol. Chem.* **1990**, *265*, 15762–15769) using a photoisomerizable and photoactive 11-*cis*-retinal analog demonstrated that both helices C and F were cross-linked. The present exclusive labeling of helix F with the nonisomerizable analog indicates that C-3 of the β-ionone ring is in contact with helix F in rhodopsin, and that light-induced isomerization moves the C-3 region to come in contact with helix C. This motion could be responsible for triggering the conformational changes leading to metarhodopsin-II, the species directly involved in initiating the enzymatic cascade leading to visual transduction.

Introduction

Rhodopsin, which is found in the outer segment of vertebrate rods, is the photoreceptor that mediates dim-light vision. This membrane protein comprises a 348 amino acid polypeptide[1-3] and a chromophore, 11-*cis*-retinal (**I**), which is linked to the ε-amino group of Lys-296 through a protonated Schiff base.[4-7] The protein constitutes a cluster of seven α-helices, which traverse the membrane and are connected by six water-soluble loops.[2,8] The bound retinal resides within the interior of the transmembrane region.[9] Absorption of visible light leads to an 11-cis to all-trans isomerization of the chromophore within the binding pocket. Subsequent conformational changes in the polypeptide portion lead to activation of a G-protein, transducin.[10] Transducin in turn activates a cyclic GMP phosphodiesterase, which acts to deplete this second messenger from the cytoplasma, thereby causing closure of cGMP-gated sodium channels at the plasma membrane, leading to membrane hyperpolarization.[11,12] During the rhodopsin activation process, a number of spectroscopic intermediates can be observed,[13] the crucial intermediate being meta-II, which absorbs maximally at 380 nm and directly activates transducin.[14] Techniques such as FTIR difference spectroscopy[15] show that the meta-II intermediate has undergone a profound conformational change, both in the transmembrane and in the water-soluble regions. The cytosolic loops of this altered conformer bind and hence activate transducin.[16] In this visual transduction process triggered by the 11-cis to all-trans photo-



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isomerization, charge translocation in the excited state[17] does occur; however, a *complete cis–trans isomerization around the 11-ene involving the entire polyene moiety* is required for efficient transduction.[18]

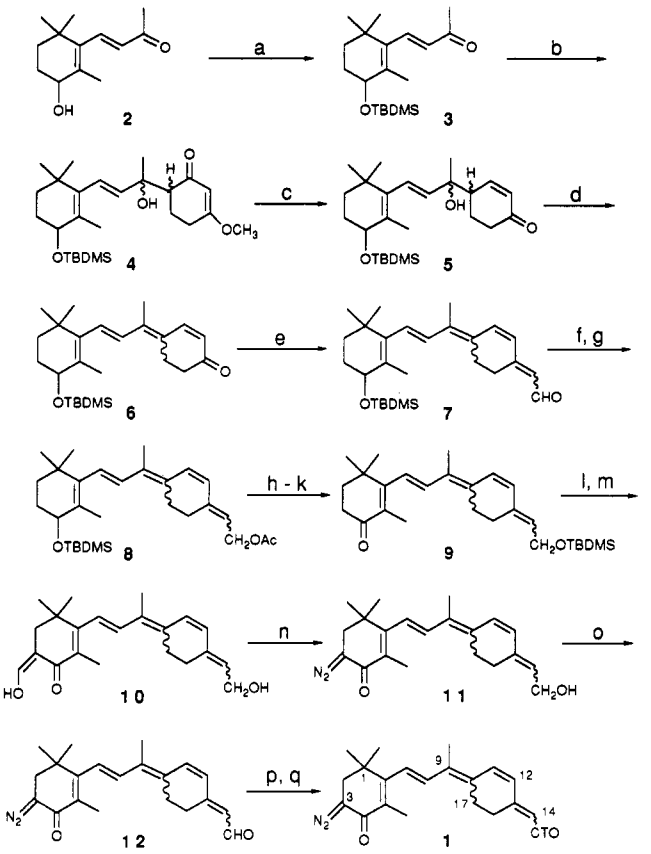
An understanding of rhodopsin tertiary structure, including the orientation of the chromophore, is essential to clarify details of the visual transduction mechanism. However, high-resolution structural data are lacking due to the difficultires in obtaining crystals or applying solution NMR techniques to rhodopsin. Thus alternate techniques, such as fluorescence energy transfer[19] and X-ray scattering,[20] have been applied to elucidate various aspects of the tertiary structure. A projection density map has recently been obtained by electron cryomicroscopy of two-dimensional crystals.[21] Photoaffinity labeling has also yielded important information on the orientation of retinal in the protein core.[22,23] In a recent study, Nakayama and Khorana photolyzed a functional rhodopsin containing *o*-dimethyl *p*-(trifluoromethyl)diazirine phenyl analog **II** as its chromophore, to cross-link to six amino acids located in helices C and F; this indicated that the β-ionone ring was in close proximity to these two helices, perhaps residing between them.[22] The authors speculated that the large number of cross-linked amino acids and the large distance between some labeled residues (13.5 Å) might result from isomerization during photolysis, so that both the original 11-cis and the photoisomerized all-trans chromophores cross-linked to amino acids in the protein core. It is expected that isomerization in the binding procket should lead to movement of the retinal β-ionone ring and/or the helices.

We thus decided to examine the orientation of 11-*cis*-retinal in rhodopsin by using the photoaffinity analog **1**, [15-^{3}H]-3-diazo-4-oxo-10,13-ethano-11-*cis*-retinal (N_2-Rh6), which was obtained by modification of the known 10,13-ethano-11-*cis*-retinal,[24] Rh6, **III**; this analog has been reported to adopt a conformation similar to that of native 11-*cis*-retinal within the protein binding pocket.[25] The tritiated analog **1** has a photoreactive diazo ketone moiety on the β-ionone ring in addition to being blocked from 11-cis → all trans isomerization by virtue of the six-membered ring. This locked analog almost exclusively cross-linked to Trp-265 and Leu-266 located around the middle of helix F, thus yielding one of the most clear-cut results in retinal protein photoaffinity labeling.[26] This result also indicates that isomerization moves C-3, which is close to helix F, toward helix C. This apparent movement of the β-ionone ring toward helix C might be responsible for triggering the protein conformational changes that lead to transducin coupling.

Results

Chromophore Synthesis. The synthetic outline of photoaffinity analog **1** is shown in Scheme 1. Protection of 4-hydroxy-β-ionone (**2**)[27] with TBDMSCl afforded a 99% yield of silyl ether **3**. Aldol condensation between **3** and 3-methoxy-2-cyclohexen-1-one[28] yielded ketone **4** as a mixture of isomers (85%).[24] LAH reduction of **4** and subsequent sulfuric acid acidification generated enone alcohol **5**, 56%. Acetylation of **5** with acetic anhydride followed by acetoxy elimination with DBN gave enone **6** as a mixture of cis/trans isomers in 91%.[24] Horner–Emmons coupling of **6** with diethyl (cyanomethyl)phosphonate and subsequent DIBAL-H reduction afforded, in 85%, retinal analog **7** as a mixture of four cis/trans isomers.[25] $NaBH_4$ reduction of **7** provided a retinol analog (83%), which upon acetylation with acetic anhydride generated retinyl **8** in 81%. Deprotection of **8** with *n*-$Bu_4N^+F^-$ and subsequent MnO_2 allylic oxidation yielded the retinyl ester in 70% yield. This ester was deacetylated upon basic hydrolysis and then protected with TBDMSCl to give silyl ether **9** (89%). Formylation of **9** with ethyl formate yielded a formylated **10** in 98% yield.[29] Diazotization of **10** with mesyl axide[30,31] produced retinol analog **11** (74%), MnO_2 oxidation of which gave retinal analog **12** as a mixture of four cis/trans isomers (75%). The 11-cis-locked retinal analog **12** was isolated and identified by NOE studies after HPLC separation. Selective reduction of

Scheme 1. Synthesis of Retinal Analog **1**[a]



[a] Reagents: (a) TBDMSCl, imidazole, DMAP, CH_2Cl_2; (b) (i) 3-methoxy-2-cyclohexen-1-one, LDA, THF, (ii) HOAc; (c) (i) LAH, ether, (ii) H^+; (d) (i) Ac_2O, DMAP, toluene, (ii) DBN; (e) (i) $(EtO)_2P(O)CH_2CN$, NaH, THF, (ii) DIBAL, hexanes; (f) $NaBH_4$, MeOH–THF; (g) DMAP, Ac_2O, Et_3N, CH_2Cl_2; (h) *n*-$Bu_4N^+F^-$, THF; (i) MnO_2, CH_2Cl_2; (j) K_2CO_3, MeOH–H_2O; (k) TBDMSCl, CH_2Cl_2; (l) NaH–absolute EtOH, HC(O)OEt, THF; (m) *n*-$Bu_4N^+F^-$, THF; (n) NaH–absolute EtOH, MsN_3, ThF; (o) MnO_2, CH_2Cl_2; (p) $NaBT_4$–0.1 N NaOH, MeOH–THF; (q) MnO_2, CH_2Cl_2.

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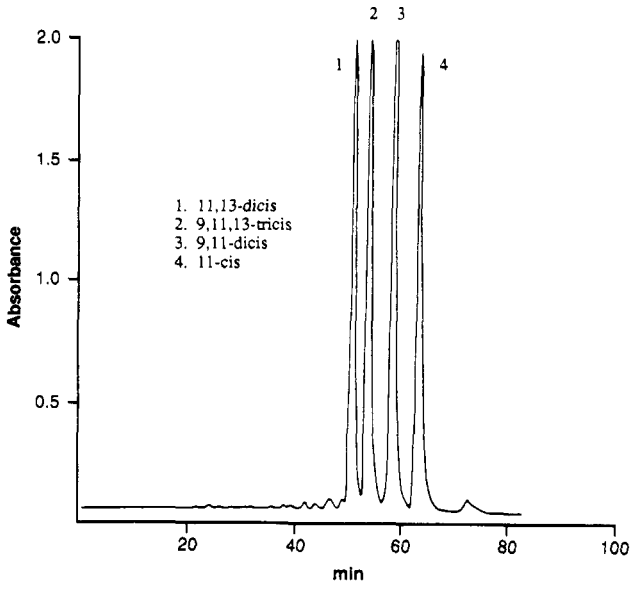

Figure 1. Normal-phase HPLC of retinal analog **1** isomers. The 11-cis isomer (peak 4) was resolved from its contaminating geometric isomers (column, YMC S-5 silica, 30 × 250 mm; eluent, 2-propanol–EtOAc–hexane = 1.3:7.5:91.2; flow rate, 6 mL/min; detection, 367 nm).

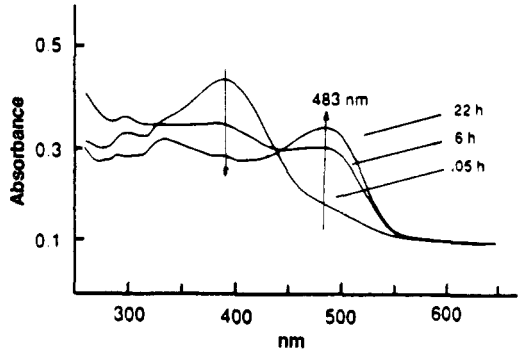

Figure 2. UV–vis spectrum of pigment reconstituted from retinal analog **1**. Incubation of retinal analog **1** with bovine opsin yielded a pigment absorbing maximally at 483 nm after 1 day; native rhodopsin has a λ_{max} of 500 nm. The bleached opsin was incubated with a substoichiometric amount of retinal analog **1** (1:0.65), and the spectrum was measured using bleached opsin as the blank.

retinal analog **12** with $NaBT_4$, followed by MnO_2 oxidation, gave tritiated anlog **1** as an isomeric mixture, overall yield ca. 60% based on TLC. The desired 11-cis-locked [^{3}H]retinal analog **1** was isolated by following the HPLC retention time of the non-radioactive analog **12**. HPLC purification of the desired 11-cis isomer from the mixture of four isomers is shown in Figure 1.

Incorporation of Analog 1 into Apoprotein. HPLC-purified analog **1** was incorporated into bovine opsin to form a rhodopsin analog that absorbed maximally at 483 nm (Figure 2). The reconstitution was essentially complete after 1 day, with a reconstitution yield of ~50% based on apoprotein as determined spectrophotometrically. In the reconstitution mixture the apoprotein was used in excess (1:0.65) in order to avoid random cross-linking by unincorporated chromophore during photolysis. The CD spectrum of the reconstituted pigment showed a strong positive Cotton effect at 308 nm, which differed from that of native rhodopsin exhibiting two positive Cotton effects at 340 nm (β-band) and 490 nm (α-band) (Figure 3).

The reconstituted pigment was irradiated at 254 nm (10 min, −5 °C) to generate the carbene, which would cross-link to amino acid residues close to the β-ionone ring. Analog **1** cross-linked to the protein in 23% yield, as estimated from the portion of radioactivity associated with denatured polypeptide after separation from free retinal chromophore by size-exclusion HPLC. The

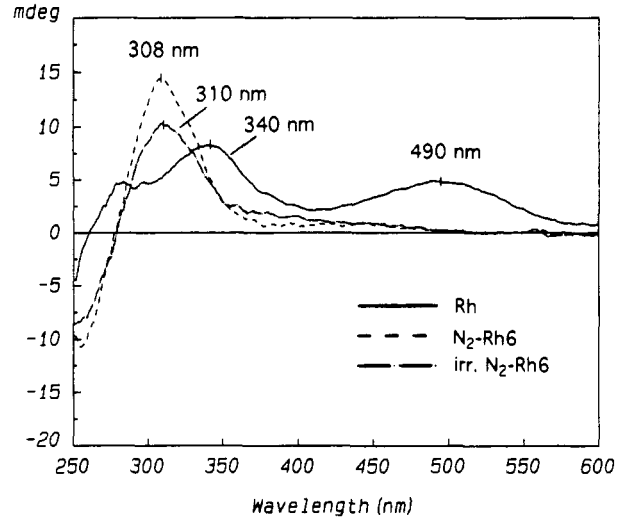

Figure 3. CD spectrum of reconstituted rhodopsin analog. A detergent solution (0.5 mg protein/mL) of pigment reconstituted to 50% apoprotein yield from retinal analog **1** was measured from 600 to 250 nm in a 1 cm cell, both before (dashed line) and after (dashed, dotted line) irradiation. The solid line represents native rhodopsin with the same protein concentration. The analog spectra are similar before and after irradiation; however, the positive Cotton effect at longer wavelength is lacking in the pigment reconstituted from retinal analog **1**.[42]

CD of the photolyzed rhodopsin analog had a decreased positive Cotton effect at 310 nm, which was very similar to that of the original pigment (Figure 3). The Schiff-base linkage was then cleaved,[32] and the protein suspension was washed with bovine serum albumin (BSA) to remove unbound photolytic products derived from analog **1**.[33]

Limited Proteolysis. The cross-linked apoprotein in membrane suspension was cleaved by V8 protease into large (V8-L) and small (V8-S) fragments (Figure 4).[7] The cleavage yield was about 50%, as judged by gel electrophoresis (Figure 5, lane A); for un-cross-linked opsin the cleavage yield is ~85%. After detergent solubilization and carboxymethylation of cysteine residues,[34] the cleaved fragments were precipitated with 80% ethanol. The V8-L and V8-S fragments were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE),[35] and the cross-linked fragment was identified by fluorographic enhancement of the tritium signal (Figure 5, lane B). *No radioactivity was associated with the V8-L portion of rhodopsin*; however, a radiolabeled band corresponding to the V8-S fragment was detected, indicating that the major cross-linking site resided in α-helix F or G (Figure 4). To identify the cross-linked amino acid, the V8-S polypeptide was isolated from the V8 digestion by gel filtration HPLC[36] (Figure 6). Some weak radioactivity was associated with the chromatographed V8-L peak, but this was due to coelution of V8-S dimer, since electrophoresis and fluorography of the V8-L peak revealed that the V8-L band was not radioactive but some contaminating V8-S dimer, which migrated below V8-L and above V8-S on the gel, was labeled. It was very fortunate that radioactivity was associated with the V8-S HPLC peak since this was the only peak base-line separated from the rest (Figure 6).

Exhaustive Cleavage and Peptide Sequencing. The isolated V8-S polypeptide was cleaved into smaller peptides by cyanogen

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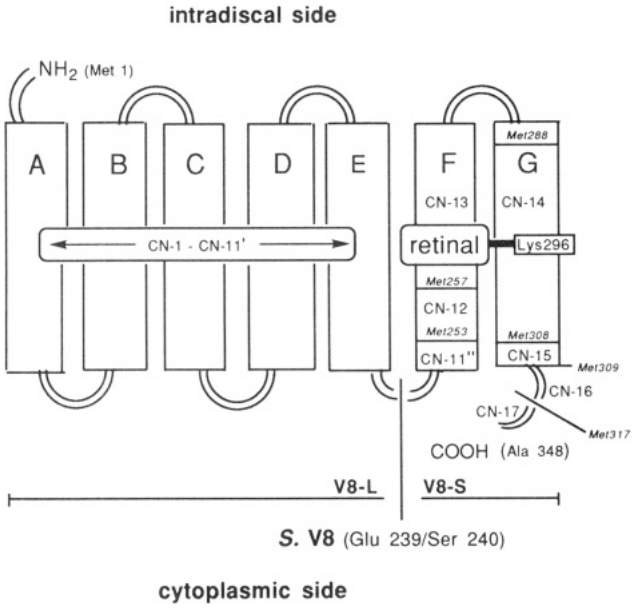

Figure 4. Rhodopsin secondary structure showing cleavage sites. The seven transmembrane α-helices are depicted by rectangles A–G. The V8 cleavage site (Glu-239/Ser-240) is indicated by a vertical line, while the cyanogen bromide cleavage sites at methionine residues are represented by horizontal lines. The 17 CNBr-cleaved fragments are numbered consecutively (1–17) from the amino terminus. Predigestion with V8 protease cleaves between CN-11′ (Phe-208 to Glu-239) and CN-11″ (Ser-240 to Met-253).

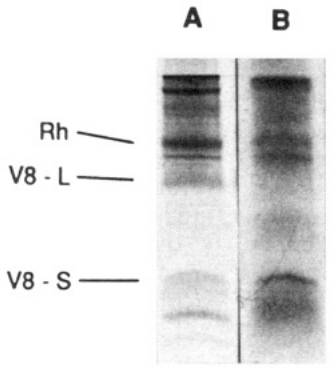

Figure 5. Electrophoretic identification of cross-linked peptide generated by V8 limited proteolysis. Lane A is the coomassie-stained gel showing uncleaved rhodopsin (Rh) and the large (V8-L) and the small (V8-S) fragments resulting from limited proteolysis with *S. aureus* V8 protease. Lane B is the fluorograph revealing that fragment V8-S was cross-linked by tritiated retinal analog **1**, while fragment V8-L was not. The minor radioactivity between V8-S and V8-L on the fluorograph is due to V8-S dimer. (The cross-linked rhodopsin in membrane suspension, after hydrolysis of the Schiff base, was digested with V8 protease, and the resulting peptides were separated on a 16.5% SDS–polyacrylamide gel, which was stained with coomassie blue and then impregnated with fluorographic enhancer to detect the tritium-labeled peptides).

bromide[37] (Figure 4). The resulting mixture was separated by SDS–PAGE, visualized by staining with coomassie blue (Figure 7, lane A), and the labeled peptide was identified by fluorography (Figure 7, lane B). The cyanogen bromide cleavage was essentially complete, the V8-S fragment being converted to low-molecular-weight peptides, with very little aggregated material seen in the high-molecular-weight region of the gel (the diffuse radioactive band above the CN–Br band, Figure 7, lane B). The major radioactive band corresponded to a weakly coomassie blue stained band, which was identified as cyanogen bromide peptide-13 (Figure 4) by Edman degradation after the band was blotted

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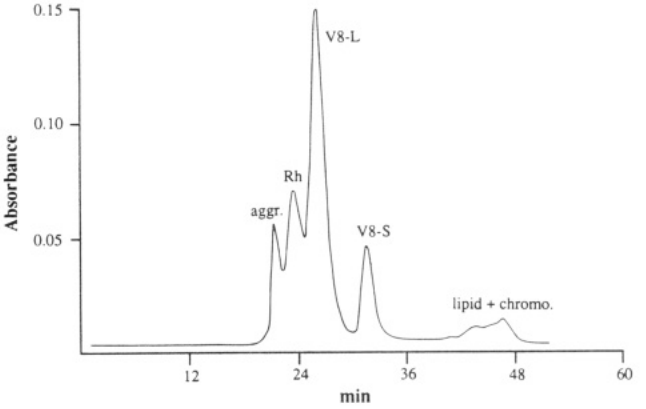

Figure 6. HPLC isolation of cross-linked peptide generated by V8 limited proteolysis. Aggregated (aggr) and uncleaved rhodopsin (Rh) eluted early in the chromatography, followed by the large (V8-L) and the small (V8-S) V8 protease fragments. Small molecular contaminants came out much later. The V8-S fragment was collected, dried, and prepared for further cleavage by cyanogen bromide. (The V8 digest was dissolved in 96% formic acid, injected onto a TSK G-3000 SW column, and eluted as described in the Experimental Section. Fractions were monitored at 280 nm, collected, and dried under vacuum.)

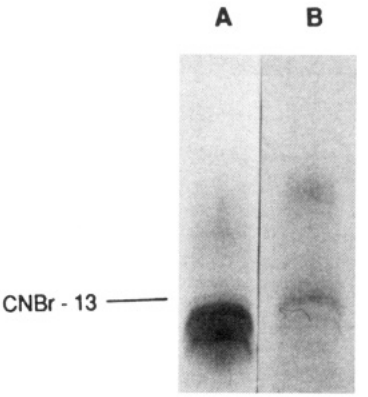

Figure 7. Electrophoretic identification of cross-linked peptide CN-13. Lane A shows the coomassie-stained peptide bands, and lane B is the corresponding fluorograph showing the radioactive band. This radiolabeled band corresponds to the weakly-stained CN-13 peptide (Figure 4), which was identified by sequencing the band from a PVDF-blotted gel. The cross-linked V8-S fragment, isolated by gel-filtration HPLC (Figure 6), was digested with cyanogen bromide, and the resulting peptides were separated on a 16.5% SDS–polyacrylamide gel, stained with coomassie blue, impregnated with fluorographic enhancer, and exposed to X-ray film to detect the radiolabeled peptide.

onto a PVDF membrane.[38] This band was slightly contaminated with peptide-17 and peptide-16+17 (from incomplete cleavage), which migrated directly below peptide 13 and stained much more darkly; however, the radioactivity was due to peptide 13 because the signal on the fluorogram corresponded to this higher band and not to the more darkly stained lower band, which mainly consisted of peptides-17 and -16+17 with little peptide-13.[39]

Edman degradation of peptide-13 after the band was blotted onto a PVDF membrane revealed that cycle 9 had the maximum radioactivity, and that cycles 8 and 9, which represented Trp-265 and Leu-266, respectively, accounted for about 20% of the total

(38) Speicher, D. W. *Techniques in Protein Chemistry*; Hugli, T., Ed.; Academic Press: New York, 1989; pp 24–35.

(39) Both the weakly-stained upper band and the darkly-stained lower band were sequenced from PVDF-blotted gels to determine the major and minor peptide constituents.

(40) Cyanogen bromide-cleaved peptide-13 was subjected to 31 cycles of Edman degradation because this peptide comprises 31 amino acids. A radioactivity recovery yield of 20% is reasonable in photoaffinity studies (e.g., see ref 22). The other 80% of the radioactivity mainly represents loosely bound peptide that washed off the filter nonspecifically during the cycling.

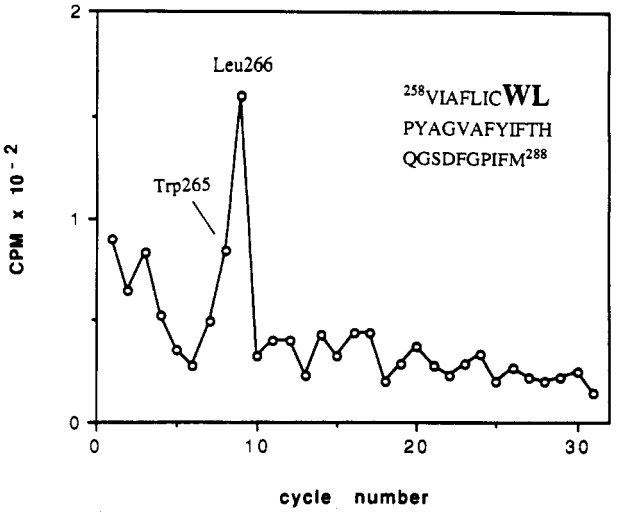

Figure 8. Identification of cross-linked amino acids by Edman degradation. The major radioactive peak at cycles 8 and 9 represents Trp-265 and Leu-266 of α-helix F, respectively. The minor radioactivity in the first four cycles represents weakly bound peptide and is due to incomplete precycling wash of the protein-bound filter. (The fragment CN-13 band, after electrophoresis and blotting to a PVDF membrane, was excised and subjected to 31 cycles[40] of Edman degradation, and the elutes were scintillation counted.)

tritium recovered from the sample filter (Figure 8).[40] The signal to noise ratio (>2) demonstrates the reliability of these labeling assignments.

Discussion

The OS (opsin shift) denotes the energy difference, expressed in wavenumbers (cm^{-1}), between the retinal protein, e.g., bovine rhodopsin λ_{max} = 500 nm (20 000 cm^{-1}), and the *n*-BuNH_2-protonated Schiff base of the corresponding retinal, e.g., 11-*cis*-retinal λ_{max} = 440 nm (22 727 cm^{-1}, measured in MeOH); in this case the OS is ca. 2700 cm^{-1}.[41] The OS gives a measure of the influence of the binding site on the maxima of pigments. The diazo ketone retinal analog **1** (N_2-Rh6), the λ_{max} of its protonated Schiff base with *n*-BuNH_2 being 435 nm (23 000 cm^{-1}), smoothly formed a rhodopsin analog with λ_{max} = 483 nm (20 700 cm^{-1}); the OS of 2300 cm^{-1} is 85% that of native rhodopsin. The environment of the six membered ring locked 11-*cis*-retinal analog **III** (Rh6) in the apoprotein has been reported to be similar to that of the natural chromophore in native rhodopsin.[25] Therefore, the OS of the present pigment derived from diazo ketone analog **1**, coupled with the similarity in CD between the present pigment (Figure 3) and that reconstituted from analog **III**, which has an exclusive positive Cotton effect at 340 nm,[18] show that the environment of analog **1** within the binding pocket is similar to that of the native chromophore.

The lack of the red-shifted α-band in the CD spectrum of analog **1** is in line with the CD spectra of rhodopsins Rh5 and Rh6 reconstituted from analogs containing *planar* five-membered[42] and six-membered rings,[18] respectively, at the 11-ene position in the polyene chain (as in **III**). Rh5 and Rh6 both lack the α-band, showing only the β-bands at 336 nm[42] and 340 nm (a stronger positive Cotton effect at 293 nm is seen in Rh6, but this is presumably mostly due to protein),[18] respectively; Ito and co-workers[42] have attributed the α-band arising from distortion around the 12–13 bond and the β-band to distortion around the 6–7 bond. Although the CD spectrum of the photolyzed Rh analog had a somewhat weaker Cotton effect at 310 nm, the overall shape was similar to its unirradiated precursor, thus showing that the environment of the chromophore had not undergone drastic changes upon cross-linking (Figure 3).

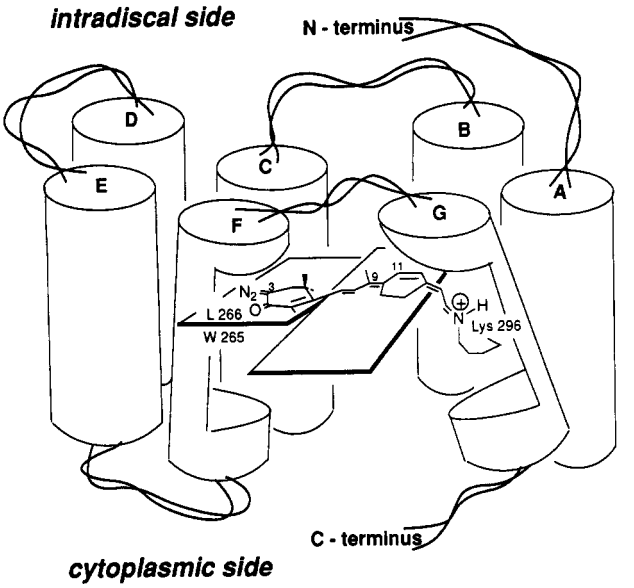

Figure 9. Model of the chromophore binding site in rhodopsin. The cylinders labeled A–G represent the seven transmembrane α-helices, while the interconnecting loops are located in the aqueous environment. The rectangles denote two slightly offset planes of the chromophore.[67] The main features of this model are that (i) C-3 of the β-ionone ring is in close contact with helix F and (ii) the entire chromophore resides near the center of the lipid bilayer.

Photolysis of the diazo ketone *in situ* led to cross-linking of amino acid residues in close proximity to the C-3 of the β-ionone ring. These residues were identified by initially subjecting the cross-linked protein to enzymatic and chemical cleavages followed by HPLC and electrophoretic purification of the resulting peptides. These experiments revealed that cyanogen bromide peptide-13 contained the major cross-linking site. Sequencing of this peptide revealed the virtually exclusive labeling of Trp-265 and Leu-266, which reside in α-helix F (Figure 9). The helices in Figure 9 are arranged on the basis of recent electron cryomicroscopic data[21] and a published theoretical assignment of the seven transmembrane helices.[43] A current transmembrane model of the rhodopsin helices[44] places both the labeled Trp-265/Leu-266 residues and the Schiff-base linkage to Lys-296 in the middle of the lipid bilayer. Since the plane of the polyene is essentially parallel to the membrane plane,[45] *the entire chromophore must reside near the center of the bilayer, with the polyene long axis tilted only slightly relative to the membrane plane*; this is in good agreement with previous fluorescence energy transfer and optical dichroic studies.[19,46]

Theoretical studies[43] suggested that Trp-265 is rotated more toward helix E than we have shown in Figure 10, and Leu-266 is directly facing the lipid bilayer, away from the cavity center. This model suggests that Leu-266 is an unlikely target for cross-linking, a discrepancy we cannot explain at present. However, both the theoretical study and the present study are consistent with Trp-265 being in close proximity to the β-ionone ring. In agreement with this, point substitution of Trp-265 with Tyr, Phe, or Ala resulted in rhodopsin mutants with low regeneration yields, blue-shifted λ_{max}, and reduced transducin stimulation.[47] Furthermore, previous photoaffinity labeling with retinal analog **II**

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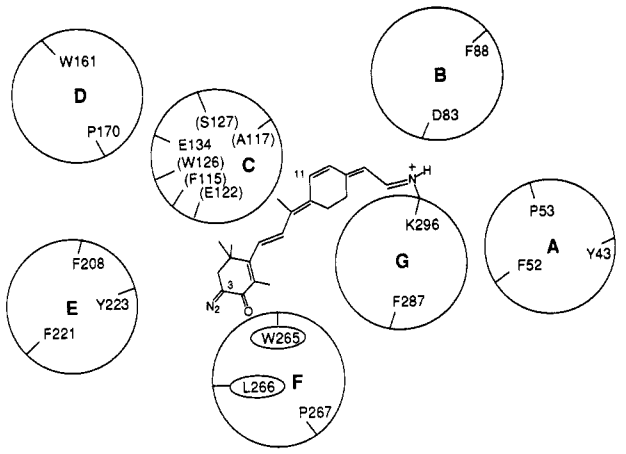

Figure 10. Helical wheel projection model of rhodopsin. This view is looking down from the intradiscal side of the membrane. The residues cross-linked in the present study are shown in ovals. The coordinates of the helices are based upon electron cryomicroscopic data[21] and a theoretical assignment of the seven helices.[43] The amino acid residues on the helices are oriented according to ref 22. The anti conformation of the Schiff base was determined by resonance Raman spectroscopy.[68] Cross-linked residues from a previous photoaffinity labeling[22] are shown in parentheses.

also resulted in cross-linking of Trp-265, along with several other amino acids on helices F and C.[22] Since this earlier analog **II** isomerized upon photolysis, our exclusive labeling of helix F with the 11-ene nonisomerizable analog suggests that *C-3 of the β-ionone ring is in van der Waals contact with helix F*, the opposing *gem*-dimethyl group pointing away from helix F (Figure 10).[48] Light-induced isomerization could then move C-3 to come in contact with the adjacent helix C by rotation of the moiety comprising C-11 to the ionone ring by 90–180° relative to helices C and F. This would account for the residues in helix C, including Glu-122, that were labeled in the previous photoaffinity study. This conclusion is supported by FTIR studies which identified a membrane-embedded Glu, most likely Glu-122 of helix C, that was perturbed upon formation of bathorhodopsin (an early intermediate with a distorted all-trans chromophore).[15,49]

Our model of rhodopsin structure depicted in Figures 9 and 10, which is based on work including electron cryomicroscopy,[21] site-specific mutagenesis,[47,50,51] and photoaffinity labeling[22] including the present study, places the β-ionone ring between helices C and F, with Glu-122 of helix C less than 3 Å from the ionone ring. Since photoexcitation of the chromophore leads to considerable redistribution of positive charge from the protonated Schiff-base nitrogen to the C-11 → C-5 segment,[52] this cationic region could form a transient salt bridge with the carboxylate of Glu-122 while the chromophore is in the excited state. This excited-state salt bridge could assist isomerization by stabilizing the excited state and/or inducing movement of the C-11 → C-5 segment toward helix C. This model of protein-assisted isomerization is supported by site-specific mutagenesis of Glu-122 to Gln, since with the neutralized mutant the λ_{max} was blue-shifted to 480 nm, and it displayed reduced transducin-stimulating activity.[47,50,51] Assistance of isomerization by Glu-122 might explain why this residue, along with the Schiff-base counterion Glu-113, is the most highly conserved membrane-embedded carboxylate found in rhodopsins,[47,53,54] and why the quantum yield of retinal isomerization in rhodopsin (0.67) is much higher than that in free solution (0.04–0.2).[55]

A popular view of rhodopsin activation is that the initial isomerization to the highly distorted all-trans retinylidene of the photo and batho intermediates, which retain the original polypeptide conformation, is followed by sequential relaxation of the chromophore to an undistorted all-trans species, namely, metarhodopsin-II, which has an altered protein conformation and activates transducin.[14,56] Since release of *all-trans*-retinal causes the polypeptide to revert to the original conformation,[15,56,57] the all-trans isomer appears to maintain (as well as generate) the active protein conformer. Examination of the electron density map[21] of rhodopsin in the inactive ground-state conformation reveals a contorted binding pocket that lacks a straight portion to accommodate the linear all-trans chromophore (Figure 10); thus only cis isomers (except 13-cis, the majority of which is still linear) can be incorporated into the protein core, which is formed predominantly by helices B, C, F, and G.[58] Therefore, isomerization to the all-trans geometry of the chromophore in the photo and batho intermediates is expected to exert a force upon the protein in the region of these four helices. The rigid, all-trans polyene could interact with nearby amino acids, possibly Glu-122 or neighboring amino acids on helix C, and/or amino acids near Lys-296 on helix G, in an allosteric fashion to bring about the altered conformation in the cytoplasmic-loop region that is critical for transducin binding; this might occur via a subtle realignment of one or more helices (specific helices unknown) that occurs in the process of generating a cavity more accommodating to *all-trans*-retinal.

Photoaffinity labeling studies are sometimes complicated by undesired photochemistry of the photolabile analog. One example is the Wolff rearrangement of α-carbonyl diazo compounds, such as diazo ketones and diazoacetates, to ketenes via the formation of the carbene intermediate, a process which can reduce the efficiency of cross-linking.[59] Another problem can occur if the original ligand itself is inherently photoreactive such as retinals. In such cases one can use a photolabile group not conjugated to the natural chromophore, so that photolysis does not excite the ligand appreciably.[60] In this study we chose to fix the isomerizable 11-cis bond with a bridged structure. Such photostable analogs have been used extensively to study the excited state[61] and very early intermediates of rhodopsin.[18,62–64] The present studies with the nonisomerizable analog **1** have yielded a clear-cut and specific labeling pattern in the difficult area involving retinal proteins.

Conclusion

Photoaffinity labeling of bovine rhodopsin with the 11-cis nonisomerizable retinal analog **1** has revealed that C-3 of the

(48) In this projection the plane of the polyene is placed parallel to the membrane plane as determined previously[45] and the amino acid residues on the helices are oriented according to ref 22.

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(52) Theoretical calculations[17a] and biophysical studies (Mathies, R.; Stryer, L. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 2169–2173) indicate that the dipole moments of protonated polyene Schiff-base moieties increase in the excited state, respectively, by 33 and 12 D.

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β-ionone ring is in close contact with Trp-265 and Leu-266 located around the middle of helix F. Since the Schiff-base linkage to Lys-296 at the other terminus of the chromophore is also close to the middle of helix G, this shows that the entire retinal chromophore resides near the center of the lipid bilayer.

Experimental Section

General Information. Chemicals were purchased from Aldrich, Bio-Rad, and Sigma. *Staphylococcus aureus* V8 protease was obtained from ICN Biochemicals. Bovine retinae were obtained from J. A. Lawson Co., Lincoln, NE. Anhydrous solvents were prepared by distillation with appropriate drying methods. Glassware used for anhydrous conditions was flame-dried and cooled under vacuum immediately prior to use. Reactions were checked by thin-layer chromatography, which was visualized by phosphomolybdic acid reagent or UV light. Flash column chromatography was carried out using 32–63 mesh silica gel from ICN. HPLC of nonradioactive retinals was performed on a Waters 6000A solvent delivery system using a YMC-Pack S-5 silica, 30 × 250 mm preparative column. The radioactive retinals were separated on a Rainin Rabbit HP solvent delivery system using a YMC-Pack S-5 silica, 10 × 250 mm column. ^{1}H NMR spectroscopy was measured on a Varian VCR 200 or VCR 400 spectrometer. UV/vis spectra were recorded on a Perkin-Elmer 320 spectrophotometer, CD spectra on a JASCO Model 720 spectrometer, MS spectra on a Nermag R10-10 mass spectrometer, and FTIR spectra on a Perkin-Elmer 1600 series spectrometer. Radioactivity was determined with a Beckman LS 3901 scintillation counter using Scintiverse II as the scintillation cocktail. Fluorography was with Kodak X-Omat AR5 film. Autoradiographic enhancer was from NEN Research products.

Synthetic Procedures. 4-[(*tert*-Butyldimethylsilyl)oxy]-β-ionone (3). To a solution of 4-hydroxy-β-ionone (**2**) (4.5 g, 21.3 mmol), imidazole (2.9 g, 42.6 mmol), and DMAP (0.8 g, 6.4 mmol) in dry CH_2Cl_2 (70 mL) was added TBDMSCl (5.1 g, 34.0 mmol) at 0 °C. The mixture was stirred overnight and washed with water (2 × 20 mL) and then with brine (20 mL). The organic phase was separated, dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 10% EtOAc–Hexanes) to provide β-ionone derivative **3** (6.84 g, 99% yield): CI-MS m/z 323 (M + 1); ^{1}H NMR ($CDCl_3$) δ 0.05 (s, 6H), 0.88 (s, 9H), 0.98 (s, 3H), 1.03 (s, 3H), 1.30–1.67 (m, 4H), 1.72 (s, 3H), 2.25 (s, 3H), 3.99 (t, 1H, J = 5.4 Hz), 6.11 (d, 1H, J = 16.4 Hz), 7.19 (d, 1H, J = 16.4 Hz).

6-{(2*E*)-1-Hydroxy-1-methyl-3-[3-[(*tert*-butyldimethylsilyl)oxy-2,6,6-trimethylcyclohexenyl]-2-propenyl}-3-methoxy-2-cyclohexenone (4). To a stirred solution of diisopropylamine (2 mL, 14.4 mmol) in dry THF (10 mL) was added *n*-BuLi solution (2.5 M in hexanes, 5.3 mL, 13.2 mmol) at −70 °C. After being stirred at −70 °C for 20 min, the solution was warmed to 0 °C and stirred for an additional 15 min. A solution of 3-methoxy-2-cyclohexen-1-one (1.52 g, 12 mmol) in dry THF (15 mL) was added dropwise to the prepared LDA solution at −70 °C, and the mixture was stirred at −70 °C for 30 min then at 0 °C for an additional 10 min. A solution of β-ionone derivative **3** (3.87 g, 12 mmol) in dry THF (15 mL) was then added slowly. The mixture was quenched by HOAc (0.76 mL) after 1.5 h of stirring. The mixture was warmed to room temperature, diluted with ether, and washed with water (2 × 20 mL) and then with brine (20 mL). The organic phase was separated, dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 15–40% EtOAc–hexanes) to yield ketone **4** as an isomeric mixture (4.54 g, 85% yield): CI-MS m/z 449 (M + 1); ^{1}H NMR ($CDCl_3$) δ 0.063 + 0.073 (2 s, 6H), 0.89 (s, 9H), 0.92 + 0.96 (2 s, 3H), 0.97 + 1.01 (2 s, 3H), 1.32 + 1.33 (2 s, 3H), 1.30–1.70 (m, 4H), 1.61 + 1.71 (2 s, 3H), 1.80–2.20 (m, 2H), 2.30–2.50 (m, 3H), 3.68 + 3.70 (2 s, 3H), 3.96 + 3.99 (2 t, 1H, J = 5.8 Hz), 5.32 + 5.38 (2 s, 1H), 5.51 (d, 1H, J = 16.0 Hz), 5.70 + 5.84 (2 s, 1H), 6.12 (d, 1H, J = 16.0 Hz).

4-{(2*E*)-1-Hydroxy-1-methyl-3-[3-[(*tert*-butyldimethylsilyl)oxy]-2,6,6-trimethylcyclohexenyl]-2-propenyl}-2-cyclohexenone (5). To a solution of LAH (0.80 g, 21 mmol) in anhydrous ether (35 mL) was added ketone **4** (4.5 g, 10 mmol) in ether (10 mL) at 0 °C. The mixture was stirred at 0 °C for 1 h and then at room temperature for 15 min. A solution of H_2SO_4/ice-cold water (5 mL, v/v = 1/9) was added to the reaction mixture at 0 °C over 30 min until the layers separated. The organic layer was separated, washed with water (2 × 20 mL) and then with brine (20 mL), dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 8–35% EtOAc–hexanes) to produce enone **5** as an isomeric mixture (2.38 g, 56%): CI-MS m/z 419 (M^+); ^{1}H NMR ($CDCl_3$) δ 0.06 (s, 6H), 0.88 (s, 9H), 0.93 (s, 3H), 0.99 (s, 3H), 1.35 + 1.40 (2 s, 3H), 1.50–1.90 (m, 5H), 1.63 + 1.67 (2 s, 3H), 2.10–2.60 (m, 5H), 3.98 (t, 1H, J = 5.8 Hz), 5.49 + 5.54 (2 d, 1H, J = 16.0 Hz), 6.06 (m, 1H), 6.11 + 6.13 (2 d, 1H, J = 16.0 Hz), 7.09 + 7.13 (2 d, 1H, J = 9.5 Hz).

(4*E*/*Z*)-4-{1-Methyl-3-[3-[(*tert*-butyldimethylsilyl)oxy]-2,6,6-trimethylcyclohexenyl]-2-propenylidene}-2-cyclohexenone (6). To a solution of enone **5** (2.38 g, 5.7 mmol) in toluene (100 mL) was added DMAP (1.6 g, 13.1 mmol) at 0 °C. Ac_2O (1.17 g, 11.4 mmol) was then added dropwise to the solution after 5 min of stirring. The mixture was gradually warmed to room temperature and stirred for 70 h. DBN (1.43 g, 11.4 mmol) was then added to the reaction mixture, and the solution was refluxed at 122 °C for 2.5 h. After cooling to room temperature, water (40 mL) was added and layers were separated. The aqueous layer was extracted with ether (2 × 20 mL). The combined organic layers were washed with water (2 × 20 mL) and then with brine (20 mL), dried over $MgSO_4$, filtered, concentrated, and purified by chromatography (SiO_2, 8–20% EtOAc–hexane) to give enone **6** as cis/trans isomers (2.08 g, 91%): CI-MS m/z 401 (M^+); UV (MeOH) 351 nm; IR (neat) 1672 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$) δ 0.07 + 0.08 (2 s, 6H), 0.89 (s, 9H), 0.98 + 0.99 (2 s, 3H), 1.03 + 1.04 (2 s, 3H), 1.30–1.80 (m, 4H), 1.73 + 1.74 (2 s, 3H), 2.02 + 2.05 (2 s, 3H), 2.50 + 2.52 (m, 2H), 2.82 + 2.86 (m, 2H), 4.02 (t, 1H, J = 5.5 Hz), 5.88 + 5.89 (2 d, 1H, J = 10.1 Hz), 6.29 + 6.42 (2 d, 1H, J = 16.0 Hz), 6.61 + 6.67 (2 d, 1H, J = 16.0 Hz), 7.58 + 7.66 (2 d, 1H, J = 10.1 Hz).

4-[(*tert*-Butyldimethylsilyl)oxy]-10,13-ethano-(7*E*,9*E*/*Z*,11*Z*,13*E*/*Z*)-retinal (7). To a suspension of NaH (60% in mineral oil, 0.25 g, 6.38 mmol) in dry THF (15 mL) at 0 °C was slowly added diethyl (cyanomethyl)phosphonate (1.172 g, 6.38 mmol). After the mixture was stirred at 0 °C for 15 min and then at room temperature for an additional 30 min, enone **6** (2.05 g, 5.1 mmol) in THF (20 mL) was added dropwise at 0 °C. After the reaction mixture was stirred at room temperature for 2 h, ice-cold water (20 mL) was added and layers were separated. The aqueous layer was extracted with ether (2 × 10 mL). The combined organic extracts were washed with water (2 × 20 mL) and then with brine (20 mL), dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 5% EtOAc–hexane) to provide the nitrile (2.2 g, ~100%). To a solution of the nitrile (~2.2 g, 5.1 mmol) in dry hexanes (40 mL) at −78 °C was added DIBAL-H (1.0 M in hexanes, 6.6 mL, 6.6 mmol) dropwise. The mixture was stirred for 2 h at −78 °C and then warmed gradually to −10 °C over 30 min to complete the reaction. The solution was then poured into ice-cold wet silica gel in ether (H_2O, 0.5 mL; SiO_2, 2.5 g; ether, 40 mL) and stirred for an additional 1 h. The mixture was dried over $MgSO_4$–K_2CO_3 (0.1 g of each) and filtered. The filter cake was washed with ether–hexanes (ether–hexanes, 1:1, 30 mL). The solution was concentrated and chromatographed (SiO_2, 6% EtOAc–hexanes) to yield retinal analog **7** as a mixture of four isomers (1.84 g, 85%): CI-MS m/z 427 (M + 1); UV (hexane) 362 nm; IR (neat) 1661 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$) δ 0.08 + 0.09 (2 s, 3H), 0.90 (s, 9H), 0.99 (s, 3H), 1.03 (s, 3H), 1.30–1.90 (m, 4H), 1.74 (s, 3H), 1.96 + 1.98 + 2.02 + 2.04 (4 s, 3H), 2.40–3.10 (m, 4H), 4.03 (t, 1H, J = 5.2 Hz), 5.73 + 5.85 (2 d, 1H, J = 8.2 Hz), 6.17–6.36 (m, 1.5H), 5.56–6.80 (m, 1.5H), 6.99–7.15 (m, 1H), 10.04 (d, 0.6H, trans, J = 8.3 Hz), 10.14 + 10.15 (2 d, 0.4H, cis, J = 8.1 Hz).

Ethyl 4-[(*tert*-Butyldimethylsilyl)oxy]-10,13-ethano-(7*E*,9*E*/*Z*,11*Z*,13*E*/*Z*)-retinyl Ester (8). To a solution of aldehyde **7** (1.75 g, 4.1 mmol) in MeOH–THF [MeOH–THF, 1:2 (v/v), 30 mL] at 0 °C was added dropwise a solution of $NaBH_4$ (0.32 g, 8.6 mmol) in MeOH (10 mL). The mixture was gradually warmed to room temperature and stirred for 1.5 h. A saturated aqueous NH_4Cl solution (10 mL) was then added to quench the reaction, and the mixture was diluted with ether. The organic phase was separated, washed with water (2 × 10 mL) and then with brine (10 mL), dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 10–25% EtOAc–hexanes) to give the retinol (1.46 g, 83%): CI-MS m/z 428 (M^+); UV (hexane) 322 nm; IR (neat) 3409 cm^{-1} (OH); ^{1}H NMR ($CDCl_3$) δ 0.078 + 0.083 (2 s, 6H), 0.90 (s, 9H), 0.97 (s, 3H), 1.03 (s, 3H), 1.30–1.85 (m, 4H), 1.74 (s, 3H), 1.91 + 1.94 (2 s, 3H), 2.30–2.70 (m, 4H), 4.02 (t, 1H, J = 5.3 Hz), 4.26 + 4.28 (2 d, 2H, J = 7.0 Hz), 5.43 + 5.56 (2 t, 1H, J = 8.6 Hz), 6.04–6.81 (m, 4H). To a solution of the retinol (1.45 g, 3.4 mmol) and DMAP (0.1 g, 1.0 mmol) in dry CH_2Cl_2 (40 mL) at 0 °C were added Et_3N (0.7 g, 6.8 mmol) in CH_2Cl_2 (5 mL) and Ac_2O (0.69 g, 6.8 mmol) in CH_2Cl_2 (5 mL). The mixture was gradually warmed to room temperature and stirred for 14 h. The reaction mixture was washed with water (2 × 10 mL) and then with brine (10 mL), dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 5% EtOAc–hexanes) to give ester **8** (1.29 g, 81%): CI-MS m/z 470 (M^+); UV (hexane) 325 nm; IR (neat) 1743 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$) δ 0.08 (s, 6H), 0.90 (s, 9H), 0.97 (s, 3H), 1.02 (s, 3H), 1.30–1.85 (m, 4H), 1.74 (s, 3H), 1.91 + 1.93 (2 s,

3H), 2.04 (s, 3H), 2.30–2.70 (m, 4H), 4.02 (t, 1H, J = 5.6 Hz), 4.68 + 4.70 (2 d, 2H, J = 7.2 Hz), 5.35 + 5.48 (2 t, 1H, J = 8.6 Hz), 6.03–6.86 (m, 4H).

4-Oxo-10,13-ethano-(7*E*,9*E*/*Z*,11*Z*,13*E*/*Z*)-retinyl *tert*-Butyldimethylsilyl Ether (9). To a solution of ester **8** (1.22 g, 2.6 mmol) in dry THF (20 mL) at 0 °C was added n-$Bu_4N^+F^-$ (1.0 M in THF, 7.8 mL, 7.8 mmol). The solution was gradually warmed to room temperature and stirred for 3 h. The mixture was diluted with ether, washed with water (10 mL) and then with brine (10 mL), dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 15% EtOAc–hexanes) to afford the ester (0.746 g, 80%): CI-MS m/z 356 (M^+); UV (hexane) 323 nm; IR (neat) 3415 cm^{-1} (OH), 1741 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$) δ 1.00 (s, 3H), 1.02 (s, 3H), 1.30–1.85 (m, 4H), 1.83 (s, 3H), 1.91 + 1.93 (2 s, 3H), 2.04 (s, 3H), 2.30–2.70 (m, 4H), 4.00 (m, 1H), 4.68 + 4.70 (2 d, 2H, J = 7.2 Hz), 5.35 + 5.49 (2 t, 1H, J = 7.6 Hz), 6.03–6.84 (m, 4H). To a solution of the ester (0.72 g, 2 mmol) in dry CH_2Cl_2 (12 mL) at 0 °C was added MnO_2 (8.1 g, 92 mmol). The slurry was stirred at 0 °C for 7 h. The mixture was then diluted with ether and filtered through Celite, and the filter cake was washed with EtOAc (20 mL). The solution wws concentrated and chromatographed (SiO_2, 10% EtOAc–hexanes) to yield the ketone ester (0.632 g, 88%): FAB-MS m/z 354 (M^+); UV (hexane) 337 nm; IR (neat) 1738 cm^{-1} (COOR), 1660 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$) δ 1.17 (s, 1.17 (s, 6H), 1.80–1.88 (m, 2H), 1.86 (s, 3H), 1.94 + 1.97 (2 s, 3H), 2.04 (s, 3H), 2.30–2.70 (m, 6H), 4.68 + 4.71 (2 d, 2H, J = 7.2 Hz), 5.39 + 5.52 (2 t, 1H, J = 7.4 Hz), 6.09–6.96 (m, 4H). To a solution of the ketone ester (0.61 g, 1.72 mmol) in methanol (40 mL) at 0 °C was added a solution of K_2CO_3 [4.1 g, 10% (w/v)] in ice-cold water (10 mL). The mixture was gradually warmed to room temperature and stirred for 15 h. The solution was then evaporated to remove most of MeOH, diluted with ether, washed with water (2 × 10 mL) and then with brine (10 mL), dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 20% EtOAc–hexanes) to give the ketone alcohol (0.505 g, 94%): CI-MS m/z 313 (M + 1); UV (hexane) 337 nm; IR (neat) 3407 cm^{-1} (OH), 1660 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$) δ 1.17 (s, 6H), 1.82–1.86 (m, 2H), 1.86 (s, 3H), 1.94–1.98 (m, 3H), 2.30–2.65 (m, 6H), 4.28 (m, 2H), 5.48 + 5.60 (m, 1H), 6.11–6.94 (m, 4H). To a solution of the ketone alcohol (0.49 g, 1.57 mmol), imidazole (240 mg, 3.53 mmol), and DMAP (57.6 mg, 0.47 mmol) in dry CH_2Cl_2 (40 mL) at 0 °C was added TBDMSCl (425 mg, 2.83 mmol). The mixture was gradually warmed to room temperature and stirred for 12 h. The organic solution was washed with water (20 mL) and then with brine (10 mL), dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 5% EtOAc–hexanes) to produce silyl ether **9** (0.639 g, 95%): CI-MS m/z 427 (M + 1); UV (hexane) 336 nm; IR (neat) 1665 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$) δ 0.056 (s, 6H), 0.88 (s, 9H), 1.17 (s, 6H), 1.80–1.86 (m, 2H), 1.86 (s, 3H), 1.93 + 1.96 (2 s, 3H), 2.30–2.65 (m, 6H), 4.30 + 4.32 (2 d, 2H, J = 6.6 Hz), 5.38 + 5.52 (2 t, 1H, J = 7.2 Hz), 6.08–6.97 (m, 4H).

3-Formyl-4-oxo-10,13-ethano-(7*E*,9*E*/*Z*,11*Z*,13*E*/*Z*)-retinol (10). To a suspension of NaH (60% in mineral oil, 144 mg, 3.5 mmol) in dry THF (2 mL) was added absolute EtOH (200 proof, 4 drops), and the reaction mixture was stirred at room temperature for 1 h. Silyl ether **9** (0.332 g, 0.78 mmol) and ethyl formate (275 mg, 3.5 mmol) in THF (4 mL) were then added at –10 °C. The mixture was stirred at –10 °C for 1 h and then at room temperature for an additional 3.5 h. The reaction was quenched with water (1 mL) and diluted with ether. The organic phase, after being washed consecutively with saturated aqueous NH_4Cl (10 mL), water (10 mL), and brine (10 mL), was dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 5% EtOAc–hexanes) to provide the formyl derivative (0.321 g, 90%): CI-MS m/z 455 (M + 1); UV (hexane) 371 nm; IR (neat) 1638 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$) δ 0.059 (s, 6H), 0.89 (s, 9H), 1.10 (s, 6H), 1.94 (s, 6H), 2.26 (s, 2H), 2.30–2.65 (m, 4H), 4.30 + 4.33 (2 d, 2H, J = 6.4 Hz), 5.39 + 5.53 (2 t, 1H, J = 7.0 Hz), 6.09–7.01 (m, 4H), 7.47 (d, 1H, J = 8.6 Hz). To a solution of the formyl derivative (0.32 g, 0.7 mmol) in dry THF (15 mL) at 0 °C was added dropwise n-$Bu_4N^+F^-$ (1.0 M in THF, 2 mL, 2.0 mmol). The mixture was gradually warmed to room temperature and stirred for 2 h. The reaction solution was diluted with ether, washed with water (10 mL) and then with brine (10 mL), dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 15% EtOAc–hexanes) to give formylretinol **10** (0.235 g, 98%): CI-MS m/z 341 (M + 1); UV (hexane) 369 nm; IR (neat) 3404 cm^{-1} (OH), 1635 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$) δ 1.09 (s, 6H), 1.93 (s, 6H), 2.25 (s, 2H), 2.30–2.65 (m, 4H), 4.26 + 4.28 (2 d, 2H, J = 6.8 Hz), 5.48 + 5.59 (2 t, 1H, J = 6.4 Hz), 6.09–7.00 (m, 4H), 7.47 (s, 1H).

3-Diazo-4-oxo-10,13-ethano-(7*E*,9*E*/*Z*,11*Z*,13*E*/*Z*)-retinol (11). To a suspension of NaH (60% in mineral oil, 79.5 mg, 2.0 mmol) in dry THF (2 mL) was added absolute EtOH (200 proof, 46 mg, 1 mmol), and the reaction mixture was stirred at room temperature for 1 h. Formylretinol **10** (0.225 g, 0.66 mmol) in THF (4 mL) was then added, and the mixture was stirred at –10 °C for 25 min and then at room temperature for an additional 15 min. MsN_3 (240 mg, 2.0 mmol) in THF (1 mL) was then added, and the mixture was stirred at room temperature for 3 h. The mixture was quenched with ice-cold water (1 mL) and diluted with ether. The organic phase was washed with water (10 mL) and then with brine (10 mL), dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 15–50% EtOAc–hexanes) to afford diazoretinol **11** (165 mg, 74%): CI-MS m/z 338 (M^+); UV (hexane) 347 nm; IR (neat) 3443 cm^{-1} (OH), 2074 cm^{-1} (diazo), 1610 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$) δ 1.18 (s, 6H), 1.92 (s, 6H), 2.30–2.60 (m, 4H), 2.63 (s, 2H), 4.25 + 4.27 (2 d, 2H, J = 6.6 Hz), 5.46 + 5.58 (2 t, 1H, J = 6.8 Hz), 6.05–6.92 (m, 4H).

3-Diazo-4-oxo-10,13-ethano-(7*E*,9*E*/*Z*,11*Z*,13*E*/*Z*)-retinal (12). To a solution of diazo compound **11** (0.165 g, 0.49 mmol) in dry CH_2Cl_2 (2 mL) at 0 °C was added MnO_2 (1.2 g, 14.7 mmol). The slurry was stirred at 0 °C for 3 h. The mixture was then diluted with ether and filtered through Celite, and the filter cake was washed with EtOAc (20 mL). The solution was concentrated and chromatographed (SiO_2, 15% EtOAc–hexanes) to give 11-cis-locked diazoretinal analog **12** as a mixture of four geometric isomers (120 mg, 75%). The flash chromatographed isomers were further separated by preparative HPLC (column, YMC S-5 silica, 30 × 250 mm; solvent, 2-propanol–EtOAc–hexane, 1.3:7.5:91.2; flow rate, 6 mL/min; detection, 367 nm) (Figure 1). Each isomer was then identified by NOE studies. The spectroscopic data of the desired 11-cis-locked isomer (peak 4 in the HPLC profile shown in Figure 1) are as follows: EI-HRMS m/z 336.1856, calcd for $C_{21}H_{24}N_2O_2$ 336.1838; UV (MeOH) 386 nm; IR (neat) 2076 cm^{-1} (diazo), 1661 cm^{-1} (CHO), 1601 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$) δ 1.21 (s, 3H), 1.23 (s, 3H), 1.94 (s, 2H), 1.94 (m, 1H), 2.03 (s, 3H), 2.67 (s, 3H), 2.67 (m, 1H), 2.98 (m, 2H), 5.89 (d, 1H, J = 7.7 Hz), 6.31 (d, 1H, J = 9.8 Hz), 6.32 (d, 1H, J = 16.2 Hz), 6.77 (d, 1H, J = 16.0 Hz), 7.00 (d, 1H, J = 9.9 Hz), 10.05 (d, 1H, J = 8.2 Hz).

[15-^{3}H]-3-Diazo-4-oxo-10,13-ethano-(7*E*,9*E*/*Z*,11*Z*,13*E*/*Z*)-retinal (1). To a solution of retinal analog **12** (34.0 mg, 0.1 mmol) in MeOH–THF (1:1, 4 mL) at 0 °C was added slowly a solution of $NaBT_4$ (11.12 Ci/mmol, 0.025 mmol) in 0.1 N NaOH (0.224 mL). The mixture was stirred at 0 °C for 1 h and then quenched with ice-cold water (5 mL) (TLC indicated that the reaction was over 80% complete). The solution was evaporated to remove most of the organic solvents and diluted with ether. Brine (10 mL) was added to the reaction mixture, which was then stirred for 2 min. The organic layer was separated, and the aqueous phase was extracted with ether (3 × 10 mL). The combined organic extracts were dried over $MgSO_4$, concentrated, and then dried under vacuum. To a solution of the dried mixture in dry CH_2Cl_2 (1 mL) at 0 °C was added MnO_2 (260 mg, 3 mmol). The slurry mixture was then stirred at 0 °C for 3 h, diluted with ether (10 mL), and filtered through Celite. The filter cake was wash with EtOAc (10 mL). The filtrate was concentrated and chromatographed (SiO_2, 15% EtOAc–hexanes) to give tritiated retinal analog **1** as a mixture of four geometric isomers (TLC revealed over 80% yield). The flash chromatographed isomers were further separated by analytical HPLC (column, YMC S-5 silica, 10 × 250 mm; solvent, 16% EtOAc–hexane; flow rate, 2.5 mL/min; detection, 367 nm). The 11-cis-locked tritiated retinal analog was isolated on the basis of the HPLC retention time of the non-radioactive analog.

Biochemical Procedures. Isolation and Reconstitution of Rhodopsin. Rod outer segment membranes, which are enriched to >80% rhodopsin, were isolated from bovine retinae by sucrose density gradient centrifugation.[65] After the pellet was washed three times with 67 mM phosphate buffer (pH = 7.0) to remove sucrose, the endogenous retinal was removed by bleaching with a lamp equipped with a 470 nm cutoff filter at 4 °C for 10 min in the presence of 0.05 M hydroxylamine. The bleached pellet was washed three times with 5% (w/v) BSA in 0.02 M Tris buffer, pH = 7.0, followed by three washes with the phosphate buffer. The protein suspension (~2 mg/mL) was stored at –70 °C. For reconstitution the 11-*cis*-retinal analog **1** was added from an ethanol stock solution (final ethanol concentration: <1%) to the opsin suspension (2 mg/mL, in 10 mM HEPES buffer, pH = 7.0) at a molar ratio of 0.65:1. The suspension was stirred in the dark for 1 day at room temperature, and incorporation into apoprotein was monitored spectrophotometrically. The reconstitution yield was estimated from the A_{483} by applying the ϵ_{500} of native Rh.

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CD Spectrum of Rh Analog 1. The CD spectra of a 67 mM phosphate buffer solution of rhodopsin analog containing 2% digitonin (0.5 mg of protein/mL, 50% reconstitution yield) were measured in a 1 cm quartz cell before and after irradiation, respectively, scanning from 600 to 250 nm and subtracting the spectrum of the same protein concentration Rhopsin blank. Native Rh, with the same protein concentration, was measured under identical conditions.

Photolysis and Limited Proteolysis. Photolysis of the reconstituted rhodopsin analog (suspended in HEPES buffer) was accomplished by irradiating for 10 min at −5 °C in the dark, with stirring, under a low-pressure mercury lamp (New England Ultraviolet) having a 254 nm narrow-band emission. The Schiff base was hydrolyzed by irradiating at 4 °C with a projector equipped with a 430 nm cutoff filter in the presence of hydroxylamine (0.05 M NH_2OH, 5% BSA, 0.02 M Tris, pH = 7.0) until the red color disappeared (~10 min), and the sample was centrifuged. The pellet was washed three times with 5% BSA and then three times with phosphate buffer (67 mM, pH = 7.0) to remove retinaloxime and residual hydroxylamine. The cross-linking yield of radiolabel to protein was obtained by separating the cross-linked protein from free chromophore using size-exclusion HPLC and counting the radioactivity associated with the protein. To the cross-linked protein suspension (2 mg/mL) in phosphate buffer was added V8 protease to 2% (w/w) from a buffered stock solution, and the digestion was allowed to proceed for 3 h at 30 °C with vigorous shaking in the dark.[7] After centrifugation the pellet was washed two times with phosphate buffer to remove residual V8 protease.

Cysteine Alkylation and Size-Exclusion HPLC. The V8-cleaved polypeptide was solubilized in a solution of 0.3 M Tris-acetate buffer, pH = 8.0, containing 10 M urea, 2% sodium dodecyl sulfate, 2 mM EDTA, and 0.02 M DTT to give a protein concentration of 3 mg/mL. The solution was then shaken at 20 °C for 1 h in the dark. Iodoacetic acid (in Tris-acetate buffer, pH = 8.5) was then added to 10% v/v from a 1 M stock, and alkylation of thiols was allowed to proceed for 40 min with shaking at 20 °C in the dark.[34] Four volumes of ice-cold ethanol was then added, and after centrifugation the protein pellet was washed two times with phosphate buffer to remove contaminating small molecules. The protein was then dissolved in 96% formic acid (~0.5 mg/0.2 mL), and the V8-L and V8-S fragments were separated by size-exclusion HPLC on a TSK G 3000 SW column (pore size, 250 Å; 21.5 mm × 600 mm) using a solvent system of H_2O–MeOH–$CHCl_3$ (2:5:2, 0.1% TFA).[36] The flow rate was 4 mL/min, and the separation was monitored at 280 nm while fractions were collected and then lyophilized.

Cyanogen Bromide Cleavage. The isolaterd V8-S polypeptide was further cleaved into smaller peptides by cyanogen bromide.[37] The lyophilized peptide material was dissolved in 70% formic acid to 2 mg/mL, and 100-fold molar excess (over methionine) cyanogen bromide was added from a 70% formic acid stock solution. The cleavage was allowed to proceed for 24 h at room temperature in the dark, and samples were diluted 5-fold with water and lyophilized.

Electrophoresis and Fluorography. Polypeptides were dissolved in sample buffer and separated on sodium dodecyl sulfate–polyacrylamide gels using tricine as the trailing ion.[35] Peptide bands were visualized by staining with coomassie blue G in 10% aqueous acetic acid and destaining with 10% aqueous acetic acid. For fluorographic detection of tritium-labeled bands, the gels were impregnated with autoradiographic enhancer (NEN research products), dried, and exposed to Kodak X-Omat AR 5 film for 1–7 days. For sequence analysis of electrophoretically separated bands, gels were blotted to PVDF membranes (Millipore corporation) in 10 mM CAPS, pH 11, with 10% methanol (80 V, 1 h), and the blots were stained with 0.1% coomassie blue in 40% methanol and destained with 45% methanol, 5% acetic acid.[66] The desired bands were excised with a razor, air dried, and stored at −20 °C for sequence analysis.

Edman Degradation. The PVDF-blotted peptide bands were sequenced on an Applied Biosystems Model 477A sequencer. For identification of peptide bands, the PTH program was used with HPLC identification of PTH amino acids. For radioactive sequencing, the ATZ-1 program was used, and the collected fractions were scintillation counted to identify tritium-containing fractions. For the radioactive sequencing run the protein-bound filter was washed three times using a cycling program in which the phenyl isothiocyanate was omitted, in order to wash out loosely bound peptide.

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