Orientation of the Retinal 9-Methyl Group in Bacteriorhodopsin As Studied by Photoaffinity Labeling

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Abstract: High-resolution electron cryomicroscopy has clarified the tertiary structure of the proton-pumping bacteriorhodopsin (BR) at a resolution of 3.5 Å in the direction parallel to the membrane. However, due to the lower resolution in the perpendicular direction, it could not be determined whether the retinal side-chain methyls at C-9 and C-13 were directed toward the extracellular surface or the cytoplasmic side of the membrane. The direction of the Me groups is of fundamental importance because it is directly related to the mechanisms of proton pumping. Our past bioorganic studies with C-9 substituents containing ω-sulfate groups suggested that the methyls are directed to the exterior. In contrast, measurements of linear dichroism, neutron diffraction and solid-state deuterium NMR have concluded the opposite. In order to secure further evidence, two tritiated analogs of retinal (**5** and **6**), each with a photolabile phenyl azide moiety appended through a 13-Å spacer arm, one pointing in the same direction and the other pointing opposite to the polyene methyl groups, were synthesized and incorporated into the apoprotein to reconstitute functional bacteriorhodopsin analogs. The cross-linked residues labeled by analog **5**, in which the photoactive group is directed opposite to the methyl groups, were identified as Arg-175 and Asn-176 by Edman degradation of cyanogen bromide-cleaved peptides. These two amino acids are located on the cytoplasmic side of α-helix F, indicating that the *9-Me in this photolabile analog points toward the extracellular space.* These studies also revealed that the chromophore plane is closely perpendicular to the plane of the membrane and that the β-ionone ring of retinal is tilted toward the extracellular space at a 27° angle relative to the plane of the membrane. Photoaffinity studies with analog **6** did not give conclusive results.

Introduction

Bacteriorhodopsin (BR)[1] is a 26-kDa hydrophobic protein found in the purple outer membrane of *Halobacterium halobium*, functioning as a light-driven proton pump.[2] It comprises a 248 amino acid polypeptide chain[3,4] and a chromophore, *all-trans*-retinal, which is attached to the ε-amino group of Lys-216 through a protonated Schiff base.[5–7] The chromophore is located within the seven discrete hydrophobic segments of the polypeptide that form the seven transmembrane α-helices.[8,9] Many studies, including computations,[9,10] neutron diffraction,[11–13] X-ray diffraction,[14] site-specific mutagenesis,[15] and photoaffinity labeling,[16–18] have elucidated various aspects of the BR tertiary structure. Recent electron cryomicroscopic measurements performed at a resolution of 3.5 Å in the direction parallel to the membrane plane have greatly clarified the tertiary structure of BR.[19]

The chromophore of BR absorbs light to initiate a photocycle in the protein that results in translocation of protons across the membrane, leading to an electrochemical gradient that is coupled to ATP synthesis. During the photocycle the chromophore undergoes cis/trans isomerizations about the C-13, C-14 double bond and deprotonation–reprotonation of the Schiff base. Recent studies have shown that only one proton is translocated in each photocycle,[20–23] suggesting that the Schiff-base proton is directly involved in proton pumping. Since the chromophore plays a critical role in proton translocation, knowledge of its detailed orientation within the binding site is essential to account for the interactions with neighboring amino acids leading to transmembrane proton pumping.

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(1) Abbreviations: BR, bacteriorhodopsin; CNBr, cyanogen bromide; PTH, phenylthiohydantoin; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

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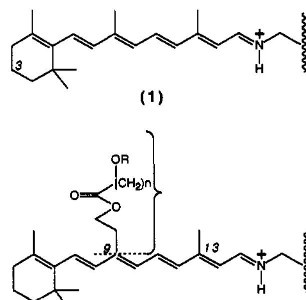
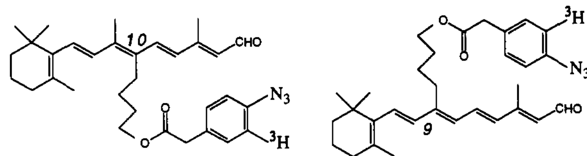
Although the electron cryomicroscopic studies of Henderson and Colleagues[19] elucidated the tertiary structure of BR, the orientation of the chromophore could not be determined because of the low 10-Å resolution perpendicular to the membrane. Earlier studies showed that the plane of the retinal polyene is nearly perpendicular to the membrane plane[24-26] and that the β-ionone ring is tilted toward the extracellular side.[16,27,28] The direction of the C-9 and C-13 methyls is critical for an understanding of the proton translocation mechanism, since it dictates the direction of the protonated-Schiff-base proton, which is opposite to that of the methyls (see **1**).



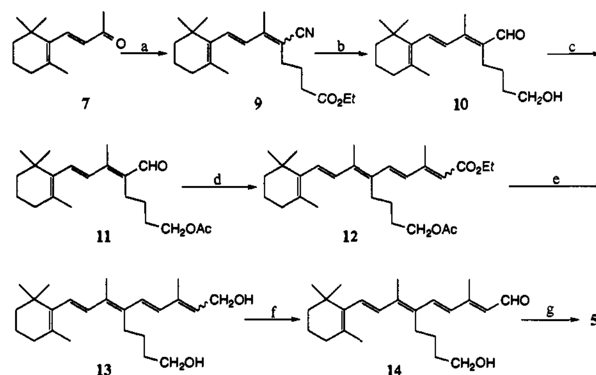
(**2**) R : H n = 3, 5, 7, 9; λmax 452 nm, functional
(**3**) R : OSO_3K n = 7, 9; λmax 475 nm, functional
(**4**) R : OSO_3K n = 3,5; no BR analog formation



(**5**) C-10 azidophenyl analog (**6**) C-9 azidophenyl analog

We had prepared BR analogs generated from retinals having C-9 branches with ω-hydroxyl (**2**) or sulfate groups (**3** and **4**).[29] The chain lengths from C-9 of alcohols **2** for $n = 3, 5, 7$, and 9 were 10, 13, 15, and 18 Å, respectively. The fact that the 452-nm maxima of these BR analogs differ from the native 560-nm value means that the long branches affect the chromophoric environment; the retinal analog with a 22 Å long 9-*n*-heptadecanyl branch also absorbs at 450 nm. However, series **2** analogs do pump protons with an efficiency of 7–40% relative to native BR. Sulfates **3** with branch lengths of 15 and 18 Å, respectively, for $n = 7$ and 9, also yield BR analogs, λ_{max} 475 nm, that pump protons at 12% efficiency. However, sulfated retinals with branch lengths less than 13 Å (10 and 13 Å for $n = 3$ and 5, respectively, in **4**) do not regenerate BR, presumably because the electrostatic interaction between the sulfate anions and charges on the membrane surface prevent the retinal from reaching the binding site. Since BR analogs were reconstituted from intact lipid vesicles and since *the direction of proton pumping was the same for native and artificial BRs*, we concluded that the *C-9 branches and therefore the side-chain methyls both face the extracellular side* of the membrane and that the depth of C-9 is ca. 15 Å from the surface. In contrast, studies with linear dichroism,[30] neutron diffraction,[31] and solid-state deuterium NMR[32] supported a model in which the methyls are directed toward the cytoplasm. It should also be noted that the distance of 15 Å from C-9 to the external lipid surface leads to a tilt angle of ca. 25° for the side chain,[17] which is in agreement with other experiments which give tilt angles ranging from 15° to 25°.[24,30]

Scheme 1[a]



[a] Reagents: (a) $(EtO)_2P(O)CH(CN)(CH_2)_3CO_2Et$ (**8**)/NaH, THF; (b) (i) DIBAL-H, ether, (ii) wet SiO_2; (c) Ac_2O, DMAP, pyridine; (d) $(EtO)_2P(O)CH_2C(CH_3){=}CHCO_2Et$/NaH, THF; (e) (i) DIBAL-H ether, (ii) wet SiO_2; (f) (i) MnO_2, CH_2Cl_2, (ii) HPLC; (g) (i) p-$N_3C_6H_3(^3H)CH_2CO_2H$, DCC, DMAP, CH_2Cl_2, (ii) HPLC.

We have sought further chemical evidence to solve this discrepancy by utilizing tritiated azide retinal analogs **5** and **6** with photoaffinity labels at C-9 or C-10 extending in opposite directions. Unlike the three previous photoaffinity-labeling studies with BR which led to multiple labeling sites,[16-18] the present experiments with analog **5** cross-linked to only two amino acids Arg-175 and Asn-176 of α-helix F and hence greatly simplified interpretation of results. Photo-crosslinking to specific amino acid residues revealed that (1) the 9-Me is oriented toward the extracellular space in BR; (2) the chromophore plane is closely perpendicular to the plane of the membrane; and (3) the β-ionone ring is tilted toward the exterior at an angle of approximately 27°. These results are in agreement with previous reconstitution studies using C-9 sulfated retinal analogs.[29]

Results

Synthesis of Retinal Analogs 5 and 6. β-Ionone (**7**) was the starting material for azide retinal analog **5** (Scheme 1). Olefination of **7** with triethyl 5-cyano-5-phosphonopentanoate (**8**)[33] gave ester **9**, which consisted of 1:1 cis/trans isomers, 77% yield. Treatment of the trans isomer of **9** with excess DIBAL-H reduced both nitrile and ester groups to yield aldehyde **10** (83%), which was acetylated to ester **11**, 90% yield. Horner–Emmons coupling[34] of **11** with triethyl 3-methyl-4-phosphonocrotonate provided a 3:2 mixture of all-trans and 13-cis ester **12**, 60% yield. DIBAL-H reduction of **12** gave diol **13**, a mixture of cis/trans isomers (75%), which was oxidized with MnO_2 to afford retinal analog **14**, an 80:20 mixture of all-trans and 13-cis, 75% yield.[35] Esterification of HPLC-purified all-trans retinal analog **14** with p-azidophenylacetic acid gave crude azide retinal analog **5** (92%), which upon HPLC purification afforded all-trans azide analog **5**. Similarly, esterification of **14** with [^{3}H]-p-azidophenylacetic acid[36] followed by HPLC provided tritiated all-trans azide retinal analog **5** in 53% radiochemical yield.

The synthesis of analog **6** used ethyl 3-methyl-4-oxocrotonate (**15**) as starting material (Scheme 2). Horner-Emmons reaction

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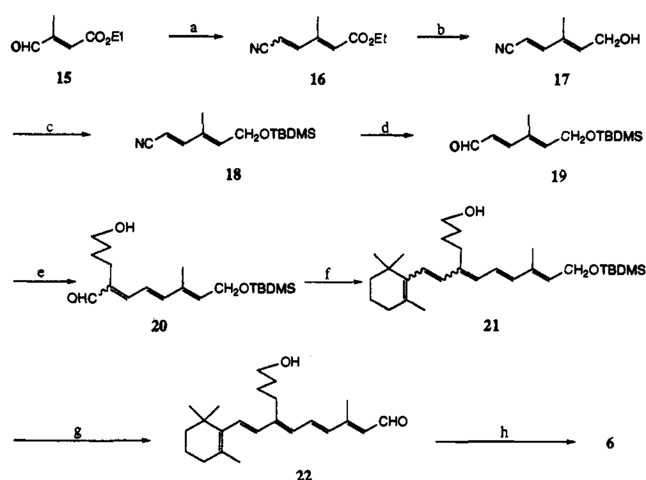
Scheme 2[a]



[a] Reagents: (a) $(EtO)_2P(O)CH_2CN$/NaH, THF; (b) (i) DIBAL-H, THF, (ii) wet SiO_2; (c) TBDMSCl/Et_3N, CH_2Cl_2; (d) (i) DIBAL-H, ether, (ii) wet SiO_2; (e) (i) $(EtO)_2P(O)CH(CN)(CH_2)_3CO_2Et$ (**8**)/NaH, THF; (ii) DIBAL-H, ether; (iii) wet SiO_2; (f) β-cyclogeranyltriphenylphosphonium bromide/*n*-BuLi, THF; (g) (i) n-$Bu_4N^+F^-$, THF, (ii) MnO_2, CH_2Cl_2, (iii) HPLC; (h) (i) p-$N_3C_6H_3(^3H)CH_2CO_2H$, DCC, DMAP, CH_2Cl_2, (ii) HPLC.

of **15** with diethyl(cyanomethyl)phosphonate produced ester **16**, 90% yield (trans/cis = 4:1), of which the trans isomer was reduced with DIBAL-H to yield alcohol **17** (75%). Protection of **17** with TBDMSCl gave silyl ether **18** (86%), which upon DIBAL-H reduction yielded aldehyde **19**, 87% yield. Horner–Emmons reaction of **19** with triethyl 5-cyano-5-phosphonopentanoate (**8**) followed by DIBAL-H reduction gave, in 60% yield, aldehyde **20** as a 3:1 mixture of trans and cis isomers, which upon coupling with β-cyclogeranyltriphenylphosphonium bromide[37] yielded silyl ether **21** as a mixture of four isomers, 52% yield. Deprotection of **21** with n-$Bu_4N^+F^-$ and subsequent MnO_2 oxidation generated an isomeric mixture of retinal analogs, which upon HPLC purification gave all-trans retinal analog **22** (27%). Esterification of **22** with [^{3}H]-*p*-azidophenylacetic acid followed by HPLC afforded tritiated all-trans azide retinal analog **6** in 26% radiochemical yield under conditions used for the non-radioactive analog **6**.

Incorporation of Retinal Analogs into Apoprotein. Incubation of bacterioopsin with azido retinal analog **5** yielded a pigment with a λ_{max} of 503 nm (Figure 1a). The pigment formed slowly, reaching the maximum absorption in 5–8 days, which corresponded to a 50% reconstitution yield. The incorporation of analog **6** took only about 2 h to yield the final pigment with a λ_{max} of 475 nm corresponding to a 35% reconstitution yield (Figure 1b). The 503- and 475-nm peaks were blue-shifted relative to the 568-nm maximum of natural BR, the corresponding 2340- and 1430-cm^{-1} opsin shifts being less than half that of BR, 5100 cm^{-1}. The CD spectrum of the reconstituted BR with analogs **5** (Figure 2, solid line) showed strong positive and negative Cotton effects at 496 and 290 nm, respectively, which were similar in sign and intensity to the 544- and 320-nm peaks of the natural BR reconstituted from *all-trans* retinal[38] (Figure 2, dashed line). The proton-pumping activities of BR analogs **5** and **6** (ΔpH/μmol of BR), i.e., the measured ΔpH values resulting from irradiation of the reconstituted pigments,[39] were roughly equal to that of natural BR.[40] The *p*-azidophenylacetoxy group of retinal analogs has a strong absorption at 253 nm in aqueous suspensions of the reconstituted pigments. Photolysis with a 254-nm low-pressure Hg lamp, 4 °C, 8 min, resulted in a decrease of the

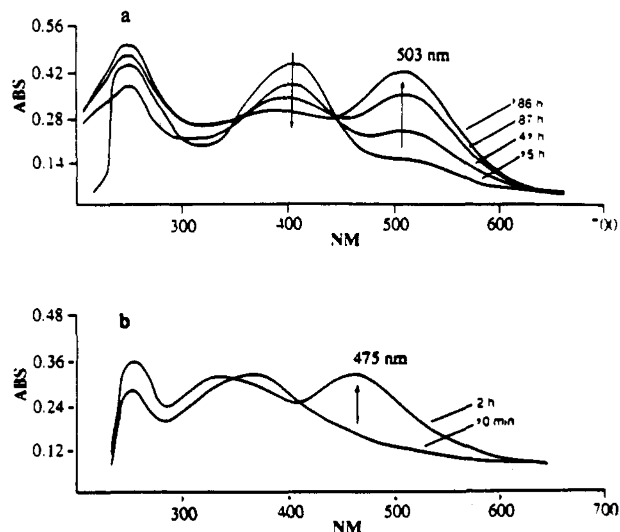


Figure 1. UV/vis spectrum of pigments reconstituted from retinal analogs **5** and **6**. The spectrum was taken by using BO as the reference. (a) Reconstitution with analog **5**. The formed pigment had a λ_{max} at 503 nm and required 5–8 days to reach the maximum absorption. (b) Reconstitution with analog **6**. The pigment, which formed in 2 h, had a λ_{max} at 475 nm. Natural BR, with an all-trans retinal chromophore, has λ_{max} at 568 nm.

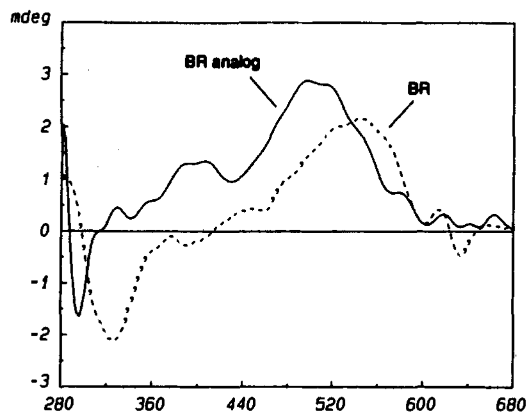


Figure 2. CD spectrum of pigment reconstituted from retinal analog **5**. A membrane suspension (0.5 mg of protein/mL) of monomeric pigment reconstituted from retinal analog **5** was measured from 680 to 280 nm in a 1-cm cell (solid line). The dashed line represents BR reconstituted to 35% from the natural *all-trans*-retinal. The spectrum of the BR analog as a shape similar to that of the authentic BR, in particular the strong positive peak in the visible region and the strong negative peak in the near-UV region, as well as the fine structure.

253-nm band and a blue shift to 246 nm, indicating generation of the nitrene. Beyond 8 min of irradiation, no further change in the UV spectrum was observed. Analog **5** was cross-linked to the polypeptide to the extent of ca. 7%, as estimated from the amount of radioactivity associated with denatured polypeptide after separation from free ligand by size-exclusion HPLC. Irradiation of the phenylazido group with 254-nm light did not cause concomitant isomerization of the retinal polyene, which could have complicated these experiments. This was evident from the absorption spectrum, since the 503-nm peak did not decrease upon UV irradiation; however, it decreased noticeably upon irradiation longer than 1 min with a 470-nm-wavelength-cutoff filter, which activates proton pumping. This decrease in visible absorbance results from bleaching of the pigment, i.e., hydrolysis of the Schiff base.

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(40) Equal concentrations of apoprotein were reconstituted with ligands; the reconstitution yield of BR from *all-trans*-retinal was 85%, while those of BR analogs **5** and **6** were 50% and 35%, respectively. Under these conditions, the ΔpH values for the BR analogs were over 60% and 40%, respectively, of the value of natural BR; therefore, consideration of the different reconstitution yields shows that the two artificial pigments had activities approximately equal to that of natural BR (for examples see ref 17).

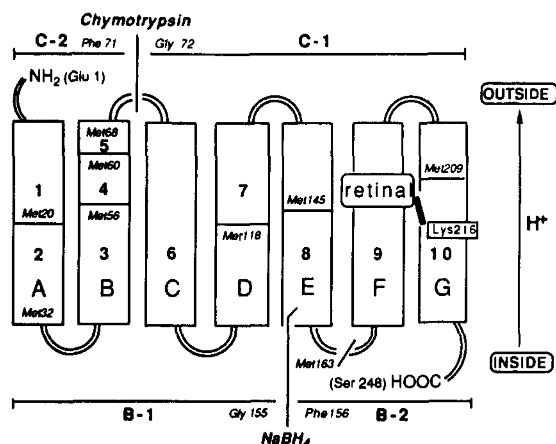

Figure 3. BR secondary structure showing cleavage sites. The seven transmembrane α-helices are depicted by the rectangles A–G. The chymotryptic cleavage site (Phe-71/Gly-72) and sodium borohydride site (Gly-155/Phe-156) are indicated with vertical lines. The chymotryptic fragments are named C1 and C2, whereas the sodium borohydride fragments are called B1 and B2. The cyanogen bromide cleavage sites, at methionine residues, are denoted by short horizontal lines. The ten cyanogen bromide fragments are numbered consecutively (1–10) from the amino terminus. The arrow labeled by H^+ shows the direction of proton pumping from the intracellular to the extracellular side of the membrane.

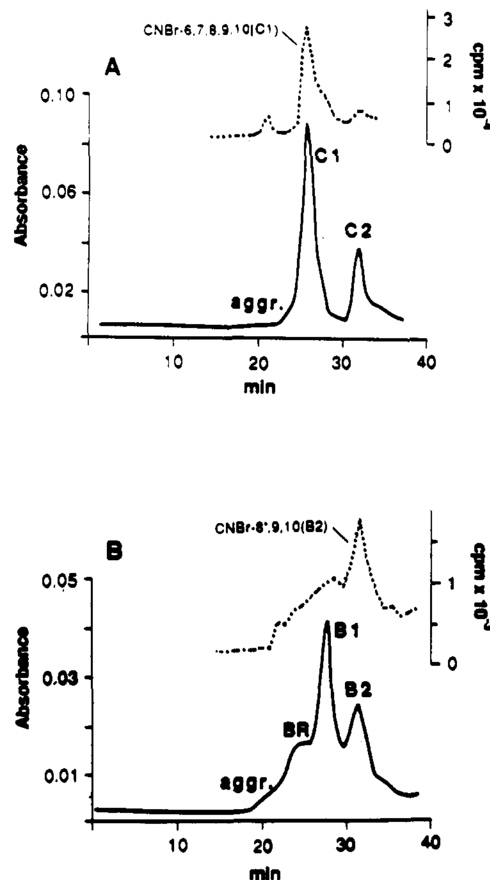

Figure 4. HPLC determination of region cross-linked by retinal analog 5. Panel A. Size-exclusion HPLC profile of the chymotryptic fragments C1 and C2, showing the absorbance at 280 nm (solid line) and the corresponding radioactivity (dotted line). Aggregated polypeptide is indicated by aggr. Panel B. Size-exclusion HPLC profile of the sodium borohydride fragments B1 and B2, again showing the absorbance at 280 nm (solid line) and the corresponding radioactivity (dotted line). Uncleaved BR analog is represented by BR, and aggregated polypeptide is denoted by aggr. The major cross-linking site (~85% of incorporated label) was on the chymotryptic C1 fragment and on the sodium borohydride B2 fragment, indicating that cyanogen bromide peptides 8',[39] 9, or 10 (Figure 3) contained the major cross-linking site. The radioactivity on the shoulder of the B2 peak was due to the residual uncleaved pigment (BR) and aggregate (aggr.), as well as the minor labeling of the B1 fragment (i.e., 14% of total B1 + B2).

Limited Cleavage Experiments. When BR is cleaved either by chymotrypsin or sodium borohydride under mild conditions, only one peptide bond is cleaved to yield two fragments.[4,41] Figure 3 depicts the BR secondary structure and shows that chymotrypsin cleaves at the Phe-71/Gly-72 bond to give amino-terminal (C2) and carboxy-terminal (C1) fragments. Figure 4A shows the size-exclusion HPLC separation of the two chymotryptic fragments C1 and C2, which were derived from cross-linked proteins with analog **5**. The absence of an uncleaved BR peak in the A_{280} profile, which normally appears after the aggregate on the shoulder of the C1 peak, revealed that the digestion was nearly complete; the corresponding radioactivity profile showed that about 85% of the label was associated with the C1 fragment and that about 15% was associated with C2. This meant that the major cross-linking sites were distributed on the C1 fragment among the cyanogen bromide peptides 6–10 (Figure 3). In the case of analog **6**, the HPLC profile of the chymotrypsin-cleaved fragments as monitored by absorbance and radioactivity was similar to that of analog **5**, i.e., Figure 4A.

In the pigment incorporating analog **5**, the possibility that cyanogen bromide fragments 6 or 7 were labeled was excluded by a sodium borohydride cleavage experiment. Sodium borohydride cleaves at the Gly-155/Phe-156 bond to yield amino-terminal (B1) and carboxy-terminal (B2) fragments (Figure 3). In contrast to the essentially quantitative cleavage by chymotrypsin, sodium borohydride cleaved the protein at only about 60% yield,[42] as judged from the HPLC profile (data not shown). Since size-exclusion HPLC could not baseline separate the uncleaved BR analog from the B1 fragment, the pooled B1 and B2 peaks from the first HPLC run were reinjected in order to reduce the amount of contaminating uncleaved BR analog. The resulting profile is shown in Figure 4B. After subtracting the radioactivity belonging to the residual uncleaved and aggregated BR analog (~30% of total), the radioactivity profile revealed that the B2 fragment contained about 86% of the remaining radioactivity, whereas the B1 fragment contained the other 14%. This 14% of label incorporated into B1 corresponds to the 15% of label found in the chymotryptic C2 fragment, revealing that *essentially no radioactivity was present in cyanogen bromide fragments 6 or 7*, which are simply appended onto C2 to give B1 (Figure 3), in the labeling with analog **5**.

Identification of Cross-Linking Site. The limited cleavage experiments described above revealed that the major cross-linking site (~85% of incorporated label) in the analog **5**-labeled protein was in sodium borohydride fragment B2, i.e., among cyanogen bromide fragments 8',[43] 9, and 10 (Figure 3). Since the B2 fragment could not be baseline-resolved from B1, further studies utilized the HPLC-purified chymotryptic C1 fragment (i.e., CNBr fragments 6–10). This was adequate, since the potentially troublesome minor labeling site was on C2. To determine which of the three possible peptides (8, 9, and 10) was labeled, the chymotryptic C1 fragment was subjected to cyanogen bromide cleavage and the resulting fragments 6–10 were separated by SDS[1]-polyacrylamide gel electrophoresis, followed by fluorographic enhancement of the gel to detect the tritium-labeled bands. Figure 5a (lane A) shows that cyanogen bromide had cleaved C1 to completion, with the five derived fragments appearing as three bands on a coomassie blue-stained gel. Edman degradation of a PVDF[1]-membrane blot demonstrated that the upper band was cyanogen bromide fragment 10, the middle band was fragments 6 and 9, and the lower band was fragments 7 and 8 (data not shown). The fluorogram (Figure 5a, lane B) showed that only

(41) Huang, K. S.; Liao, M. J.; Gupta, C. M.; Royal, N.; Biemann, K.; Khorana, H. G. *J. Biol. Chem.* **1982**, *257*, 8596–8599.
(42) This is similar to the original report of 70% yield (see ref 41).

(43) Peptide 8' is the sodium borohydride-truncated form of cyanogen bromide fragment 8, i.e., Phe-156/Met-163.

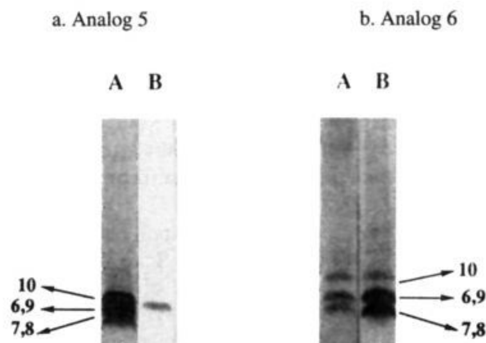

Figure 5. Electrophoretic identification of the cross-linked peptide. The cross-linked chymotryptic C1 fragment was digested with cyanogen bromide, and the resulting peptides were separated on a 16.5% SDS–polyacrylamide gel, which was stained with coomassie blue and then fluorographed to detect the tritium-labeled peptide. (a) The analog **5**-labeled CNBr fragments. Lane A shows the coomassie blue-stained peptide bands. The upper band was fragment 10, the middle band was fragments 6 and 9, and the lower band was fragments 7 and 8, as determined by Edman degradation of the PVDF-blotted bands. Lane B is the fluorograph showing the radiolabeled band. Since only the middle band was labeled, it demonstrate that cyanogen bromide fragment 9 was the cross-linked peptide because the possibility that fragment 6 could be labeled was excluded by the sodium borohydride cleavage experiment (Figure 4B). (b) The analog **6**-labeled CNBr fragments. Lane A shows the fluorographic results, and lane B represents the coomassie blue-stained peptide bands. In contrast to the limited labeling by analog **5**, analog **6** radiolabeled all three bands, indicating that it cross-linked a number of amino acids in multiple helices and/or loops.

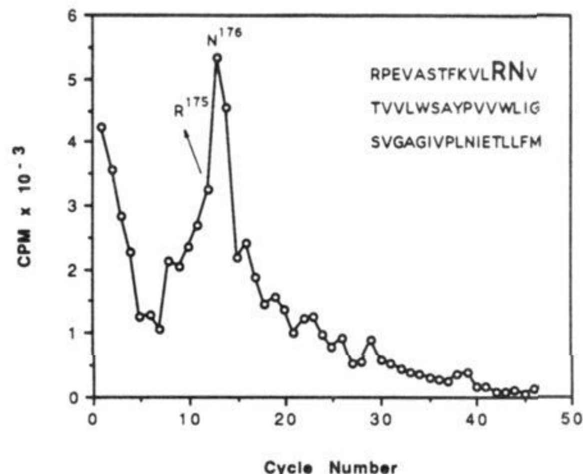

Figure 6. Identification of cross-linked amino acids by Edman degradation. The mixture of cyanogen bromide fragments 6–10, in which only BR fragment 9 was labeled, was obtained by CNBr cleavage of the analog **5**-labeled chymotryptic C1 fragment. This mixture was spotted onto a PVDF membrane and subjected to 46 cycles of Edman degradation, and the elutes were scintillation counted. The tritium in the first four cycles represented peptide that had bound weakly to the membrane and was washed off in the early cycles. The main radioactivity peak corresponded to cycles 12, 13, and 14, which represented Arg-175, Asn-176, and Val-177, respectively.

the middle band was tritium-labeled, proving that *fragment 9 was the major cross-linked peptide in the analog 5 case*, since the above sodium borohydride cleavage experiment excluded fragment 6 as a labeling site.

In contrast to the clear-cut labeling by analog **5**, analog **6** cross-linked a number of amino acids in several helices and/or loops because all three electrophoretic bands were radiolabeled (Figure 5b); it was therefore not possible to characterize the cross-linked amino acids.

The cross-linked amino acids of cyanogen bromide fragment 9 labeled by analog **5** were identified by subjecting the mixture of fragments 6–10, which was obtained by cyanogen bromide cleavage of the chymotryptic C1 fragment, to Edman degradation. This mixture gave an unambiguous result, since fragment 9 was the only labeled peptide in the mixture, the minor labeling site being on C2 (Figures 4 and 5). Scintillatin counting of the eluent from each of the 46 cycles[44] revealed that cycle 13 had the maximum radioactivity and that cycles 12, 13, and 14, which represented Arg-175, Asn-176, and Val-177, respectively, accounted for about 30% of the total tritium recovered from the filter (Figure 6).[45] The high ratio of signal to noise (ca. 15:1) attested to the reliability of these labeling assignments, as random labeling by free ligand would not be so selective. The high radioactivity in the first four cycles represented weakly bound peptide that was stripped off the filter in the early washes, which is a commonly-observed phenomenon.[16] In addition, the radioactivity corresponding to Val-177 was probably due to the incomplete cleavage in the previous cycle, as this residue is known to be on the lipid-exposed outer surface of helix F.[19]

Discussion

Pigment reconstitution for CD measurements was performed using an apoprotein/ligand ratio of 1/0.35 for the native pigment and 1/0.65 for analog **5**. This was to maintain a reconstitution yield of around 35% so that BR monomers, and not dimers or trimers, would be formed. Thus, since most of the BR molecules are monomers at the 35% reconstitution yield, the positive and negative CD peaks in Figure 2 arise from protein-induced asymmetry of the polyene chain and not from exciton coupling. At higher reconstitution yields which give rise to trimers, the longer-wavelength CD peak is split into positive and negative components; this splitting may[46] or may not[47] be due to exciton splitting. The similarity in sign and intensity of the CD spectra of the analog (although peaks are blue-shifted) and natural pigment, taken in conjunction with the equal proton-pumping activity, provides strong evidence, although not proof, that analog **5** is binding naturally. However, in view of the fact that proof of natural binding is not possible with current techniques, an alternative mode of binding is also proposed (see below, end of Discussion section). A totally different and unambiguous approach to differentiate the two modes of binding has been initiated.

The almost exclusive labeling of Arg-175 and Asn-176 on the cytoplasmic side of helix F by analog **5** strongly favors that *the 9-Me (and 13-Me) of retinal be directed toward the extracellular environment* in bacteriorhodopsin (Figure 7). The nitrene precursor of the azidophenyl group is probably located midway between the two cross-linked residues. The possibility that the 9-Me is disposed in the opposite orientation can be excluded, since a fully-extended C-10 branch is needed to reach Arg-175 and Asn-176 and any bending would cause the phenyl azide to fall short of its cross-linked targets.[48] This point can be clarified by comparing the length of the fully-extended branch, i.e., 13 Å from C-10 to the nitrene-precursor nitrogen, with the 13-Å distance between C-10 and the cross-linked Arg-175 and Asn-176 observed in the electron-microscopic structure (Figure 8).[19,49] The requirement for full extension of the C-10 branch to reach the cross-linked amino acids also indicates that the polyene plane is closely perpendicular to the plane of the membrane, in agreement with previous studies.[24–26]

(44) Cyanogen bromide peptide 9 comprises 46 amino acids.

(45) This is a fairly high recovery (see ref 16). The other 70% of the radioactivity mainly represents loosely bound peptide that washed off the filter nonspecifically during the cycling. Errors in radioactivity measurements were negligible at these high radioactivity levels (i.e., >1000 cpm; water blank 0~20 cpm).

(46) Becher, B.; Ebrey, T. G. *Biochem. Biphys. Res. Commun.* **1976**, *69*, 1–6.

(47) Wu, S.; Awad, E. S.; El-Sayed, M. A. *Biophys. J.* **1991**, *59*, 70–75.

(48) This assumes that the polyene is tilted toward the exterior, for which these is ample evidence (see ref 16, 27, 28).

(49) The reported distance was 14 Å vertically from the β-ionone ring, which can be extrapolated to 13 Å from the C-10, assuming a tilting angle of ~25°.

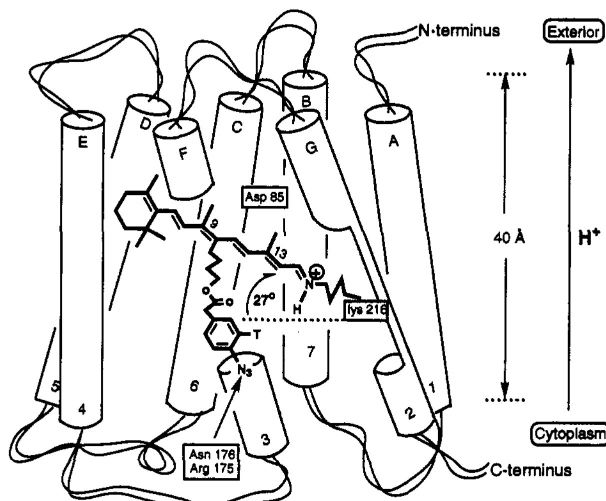

Figure 7. Model of the retinal binding site in BR. The cylinders labeled A–G represent the seven transmembrane α-helices, and the loops connecting them are in the aqueous environment. The arrow labeled by H^+ shows the direction of proton pumping toward the extracellular side. The main features of this model are that (i) the retinal 9- and 13-methyl groups point toward the exterior of the cells, (ii) the polyene plane is perpendicular to the plane of the membrane, and (iii) the retinal is tilted to the extracellular side by 27° with respect to the membrane plane.

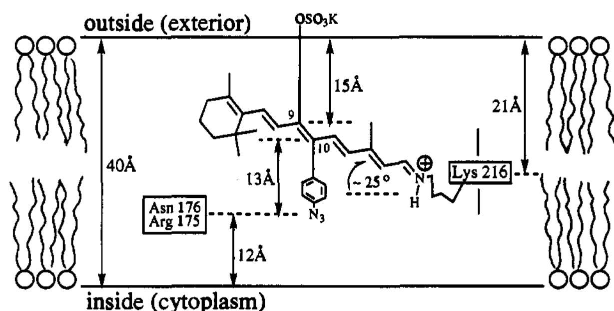

Figure 8. Spatial relationships in BR binding site determined from two bioorganic studies. This is a schematic representation of the retinal binding site based on results from this study and that of Park et al.[29] The earlier study had determined that the C-9 was 15 Å from the extracellular surface, as this was the shortest length of the sulfate side chain that allowed the molecule to reach the binding site. The present study reached a similar conclusion, because the C-10 phenylazido side chain reached 13 Å to the cross-linked Arg-175 and Asn-176, which are 12 Å from the cytoplasmic side;[19] since the membrane is 40-Å thick, the C-10 is thus 15 Å from the exterior. These distances of 15 Å from C-9[29] or 15 Å from C-10 (present study) to the extracellular space lead to retinal tilt angles of 25° and 27°, respectively, when combined with the 21-Å distance of Lys-216 to the extracellular surface.

Assuming a zig-zag conformation for the methylene chain, the distance between C-10 to the azido nitrogen that binds to Arg-175 and Asn-176 is 13 Å, while these two amino acids are about 12 Å from the cytoplasmic surface (see Figure 8);[19] the present study thus leads to a distance of 25 Å from C-10 to the intracellular surface. Since the membrane is ca. 40-Å thick, then C-10 is about 15 Å from the extracellular surface. This distance of 15 Å is in good agreement with the previous study that used retinal analogs with branches extending from C-9 which terminated with a hydroxyl or a sulfate group.[29] This study showed that retinal analogs with uncharged (i.e., hydroxyl-terminated) C-9 side chains could reconstitute with bacterioopsin to form pigments independent of the side-chain length; however, if these chains were terminated with a sulfate group, only analogs with side chains longer than 15 Å could form pigments in the vesicles, presumably because the retinal aldehye group could thus reach Lys-216 (Figure 8). This earlier study also indicated that the 9-Me of retinal pointed toward the exterior, because if it were directed toward the interior and the analog with the 15-Å branch was bending its sulfate back toward the exterior to reach the aqueous extracellular medium, a tilting angle of much greater than 25° would be required for the polyene. This is not in accord with most data which suggest a tilting angle of 10–25° toward the extracellular side.[24,30,50] Thus, this earlier bioorganic study is in excellent agreement with the present results concerning the orientation of the polyene 9- and 13-methyl groups, the distance of the polyene from the extracellular surface, and the polyene tilting angle.

Helix G of BR has been shown to contain residues 203–227,[19] meaning that Lys-216, which forms the Schiff base with retinal, is located approximately in the middle of the helix at ~21 Å from the extracellular surface (Figure 8). Our studies indicate that C-10 is ca. 15 Å from the extracellular surface; from these distances, a tilt angle of ca. 27° (Figures 7 and 8) can be estimated for the polyene chain, assuming the distance from Lys-216 to C-10, including the four carbon side chain of the lysine to be ca. 13 Å. This angle is in good agreement with tilt angles of 10–25° that have been derived from several physical measurements,[24,30,50] and the tilt angle of 25° that can be estimated using data from the earlier bioorganic study[29] (Figure 8).

In contrast to the reconstitution of apoprotein with analog **6**, the incorporation of analog **5** is about 90-fold slower (2 h vs 186 h). This might be due to the long azide side chain of analog **5** requiring a longer time to reach the cytoplasmic half. This finding together with results of a previous photoaffinity-labeling study, in which the 3*R* enantiomer (3β in structure **1**) of 3-(diazoacetoxy)-*all-trans*-retinal only labeled helices E and F, is also in favor of the extracellular direction of the methyls.[17,51]

It is generally agreed that in the BR photocycle a proton is transferred from the Schiff base to Asp-85, which is the primary counterion, during the L_{550} to M_{412} transition.[52] Recent FTIR studies have suggested that a favorable spatial orientation of the protonated Schiff base, water molecule, and Asp-85 could account for the anomalously high pK_a (~13.3 ± 0.3) of BR.[53] The model of the retinal orientation depicted in Figure 7 predicts a favorable configuration for the proton transfer. It shown the N–H vector of the Schiff base pointing toward the interior in ground-state BR; therefore, after isomerization the N–H vector should point toward the exterior,[54] thereby presenting the proton in close proximity and in a favorable orientation for transfer to Asp-85 (Figure 7). Such a scenario has been proposed earlier on the basis of data obtained by Fourier-transform infrared spectroscopy.[55]

Although analog **6** gave a pigment absorbing at 475 nm that displayed similar proton-pumping ability compared with that of natural BR, it cross-linked a number of amino acids in multiple helices and/or loops.[56] Due to difficulties encountered in the separation of sticky peptides, the labeled amino acids could not be identified.[57]

(50) Heyn, M. P.; Cherry, R. J.; Muller, U. *J. Mol. Biol.* **1977**, *117*, 607–620.

(51) Because the optically active 3*R* analog only labeled helices E and F, the methyl groups must be toward the extracellular surface if the chromophore plane is assumed to be perpendicular to the membrane plane, for which there is a lot of evidence (see refs 24–26); if the side-chain methyls were directed toward the cytoplasmic side, helices D and/or C should have been labeled (see Figure 7).

(52) Fodor, S. P. A.; Ames, J. B.; Gebhard, R.; van den Berg, E. M. M.; Stoeckenius, W.; Lugtenburg, J.; Mathies, R. A. *Biochemistry* **1988**, *27*, 7097–7101.

(53) Gat, Y.; Sheves, M. *J. Am. Chem. Soc.* **1993**, *115*, 3772–3773.

(54) This is because the imine bond remains in the anti conformation. See: Smith, S. O.; Myers, A. B.; Pardoen, J. A.; Winkel, C.; Mulder, P. P. J.; Lugtenberg, J.; Mathies, R. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 2055–2059.

(55) Braiman, M. S.; Mogi, T.; Marti, T.; Stern, L. J.; Khorana, H. G.; Rothschild, K. J. *Biochemistry* **1988**, *27*, 8516–8520.

(56) Since the photoactive azide group of analog **6** resides close to the extracellular surface, a relatively unhindered environment (see ref 19), the multiple labeling sites probably result from flexibility of the side chain in this region.

(57) The cross-linked polypeptide C1 was cleaved by CNBr into five smaller fragments. Unfortunately, these sticky labeled fragments could not be completely separated by conventional methods and therefore could not be submitted to Edman degradation analysis.

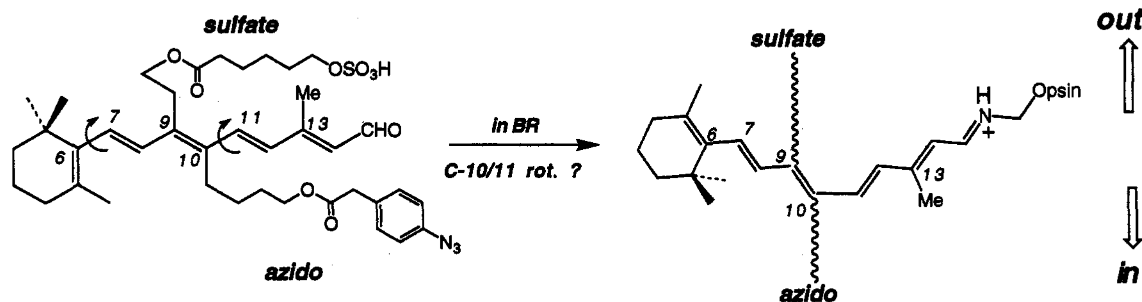

Figure 9. Rotation around C-10/C-11 (as well as C-6/C-7) upon incorporation into bacterioopsin.

The assignment of the methyl groups pointing toward the extracellular surface is in conflict with several physical studies, i.e., linear dichroism,[30] neutron diffraction,[31] and solid-state deuterium NMR,[32] all of which led to the conclusion that the retinal methyl groups are facing the cytoplasmic side of the membrane. The reason for this discrepancy is not clear. The polyene conformation of the chromophore in the protein binding pocket might be distorted so that the conformation is s-cisoid around some single bond(s). A possibility suggested from preliminary computational and molecular dynamics studies of the C-10 photolabile analog is that a single bond near C-9 adopts an s-cisoid conformation so that 9- and 13-methyls are not pointing in the same direction as in native BR.[58] As depicted in Figure 9, the 9-sulfate and 10-azido retinal analogs adopting the 6-s-cis conformation with an all-trans polyene chain in solution may rotate around the 10/11 single bond, in addition to the known rotation of the 6/7 single bond,[59] upon incorporation into bacterioopsin, so that the chromophore becomes 10-s-cis. In fact the possibility of a single-bond twist in the chromophore of native BR, especially in the photocycle intermediates, had been raised to account for the absence of a C14–C15/N–H coupling in the FTIR.[60] Experiments that will unambiguously prove the occurrence or nonoccurrence of such side-chain single-bond rotations and hence solve the discrepancy between the present and previous bioorganic experiments[29] and biophysical[30–32] experiments are underway.

Conclusion

The C-9 methyl group of the chromophore has been determined to point toward the extracellular surface of BR on the basis of direct chemical evidence from photoaffinity labeling. This conclusion is in agreement with an independent study employing retinal analogs with C-9 branches terminating with a sulfate group.[29] Results of these two bioorganic studies are self-consistent, both revealing that the polyene tilts toward the extracellular surface from the linkage point at Lys-216 at an angle of 25–27° and that the 9-Me points toward the extracellular side of the membrane in the two BRs incorporating the two retinal analogs.

Experimental Section

General Information. Chemicals were purchased from Aldrich, Bio-Rad, Fisher, Fluka, and Sigma. 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 by using 32–63 mesh silica gel from ICN. HPLC-grade chromatographic solvents were degassed by sonication. HPLC of non-radioactive retinals was performed on a Waters 6000A solvent delivery system using a YMC-Pack S-5 silica, 10 mm × 250 mm semipreparative column. The radioactive retinals were separated on a Beckman 331 HPLC system using a Lichrosorb silica 5 μ 10 mm × 250 mm column. ^{1}H NMR spectroscopy was measured on a Bruker WM-250 spectrometer operating at 250.13 MHz for observation of hydrogen. UV/vis absorption spectra were measured on a Perkin-Elmer 320 spectrophotometer, and CD spectra were measured on a JASCO model 720 spectrometer. Radioactivity was determined on a Beckman LS 3901 scintillation counter using Scintiverse II as the scintillation fluid.

Synthesis of Retinal Analogs. Triethyl 5-Cyano-5-phosphonopentanoate (8). To a suspension of NaH (60% in mineral oil, 1.2 g, 30 mmol) in dry THF (20 mL) at 0 °C was slowly added diethyl (cyanomethyl)-phosphonate (5.31 g, 30 mmol). After the mixture was stirred at room temperature for 1 h, ethyl 4-bromobutyrate (5.85 g, 30 mmol) was added dropwise at 0 °C. After being stirred at room temperature for an additional 24 h, the mixture was neutralized with saturated aqueous NH_4Cl solution (20 mL) and partitioned between H_2O and EtOAc (20 mL). The organic layer was separated, dried over $MgSO_4$, filtered, and concentrated. Phosphonate **8** was obtained as a residue in vacuum distillation (5.51 g, 60%, 160 °C/4 mm): CI-MS m/z 292 (M + 1); ^{1}H NMR ($CDCl_3$) δ 1.23 (t, 3H, J = 7.1 Hz), 1.35 (t, 3H, J = 6.5 Hz), 1.77–1.99 (m, 4H), 2.33 (t, 2H, J = 4.8 Hz), 2.90 (ddd, 1H, J = 4.4, 10.0, 24.0 Hz), 4.10 (q, 2H, J = 7.2 Hz), 4.16–4.26 (m, 4H).

Ethyl 5-Cyano-8-(2',6',6'-trimethyl-1'-cyclohexenyl)-6-methyl-5,7-octadienoate (9). To a suspension of NaH (60% in mineral oil, 0.48 g, 12 mmol) in dry THF (20 mL) at 0 °C was slowly added triethyl 5-cyano-5-phosphonopentanoate (**8**; 3.49 g, 12 mmol). After the mixture stirred at room temperature for an additional 1 h, the mixture was poured into ice-cold water (20 mL). The organic layer was separated, dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 5% EtOAc–hexanes) to give ester **9** (trans, 1.59 g, 40.6%; cis, 1.43 g, 36.4%): CI-MS m/z 392 (M^+); ^{1}H NMR ($CDCl_3$) of the trans isomer δ 1.00 (s, 6H), 1.23 (t, 3H, J = 7.2 Hz), 1.45 (m, 2H), 1.60 (m, 2H), 1.68 (s, 3H), 1.85 (m, 2H), 2.02 (t, 2H, J = 6.0 Hz), 2.20 (s, 3H), 2.33 (t, 2H, J = 7.3 Hz), 2.38 (t, 2H, J = 7.4 Hz), 4.10 (q, 2H, J = 7.2 Hz), 6.38 (d, 1H, J = 16.0 Hz), 6.52 (d, 1H, J = 16.0 Hz).

2-(4'-Hydroxybutyl)-5-(2',6',6'-trimethyl-1'-cyclohexenyl)-3-methyl-2,4-pentadienal (10). To a solution of ester **9** (1.49 g, 3.8 mmol) in anhydrous diethyl ether (10 mL) at −78 °C was slowly added DIBAL-H (1.0 M in hexanes, 16 mL, 16 mmol). The mixture was stirred at 0 °C for 1 h, poured onto ice-cold wet silica gel in ether (30 mL), and stirred for an additional 1 h. The mixture was dried over $MgSO_4$ and filtered. The filter cake was washed with EtOAc (10 mL). The combined solution was concentrated and purifed by flash chromatography (SiO_2, 15% EtOAc–hexanes) to give aldehyde **10** (0.91 g, 83%): CI-MS m/z 291 (M + 1); ^{1}H NMR ($CDCl_3$) δ 1.04 (s, 6H), 1.36 (m, 2H), 1.47 (m, 2H), 1.62 (m, 4H), 1.72 (s, 3H), 2.04 (t, 2H, J = 6.0 Hz), 2.30 (s, 3H), 2.40 (t, 2H, J = 7.8 Hz), 3.63 (t, 2H, J = 6.4 Hz), 6.58 (d, 1H, J = 16.1 Hz), 6.75 (d, 1H, J = 16.1 Hz), 10.24 (s, 1H).

2-(4'-Acetoxybutyl)-5-(2',6',6'-trimethyl-1'-cyclohexenyl)-3-methyl-2,4-pentadienal (11). To a solution of aldehyde **10** (0.87 g, 3 mmol) and DMAP (0.11 g, 0.9 mmol) in dry pyridine (10 mL) at 0 °C was added Ac_2O (1.0 mL, 11 mmol). The mixture was stirred at room temperature for 12 h before water (1 mL) was added to quench the reaction. The mixture was concentrated under reduced pressure, dissolved in ether (20 mL), and washed with water (2 × 20 mL) and then brine (10 mL). The organic phase was separated, dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 5% EtOAc–hexanes) to yield ester **11** (0.90 g, 90%): CI-MS m/z 333 (M + 1); ^{1}H NMR ($CDCl_3$) δ 1.04 (s, 6H), 1.35 (m, 2H), 1.47 (m, 2H), 1.62 (m, 4H), 1.72 (s, 3H), 1.99 (s, 3H), 2.04 (t, 2H, J = 5.8 Hz), 2.30 (s, 3H), 2.40 (t, 2H, J = 7.8 Hz), 4.02 (t, 2H, J = 6.6 Hz), 6.58 (d, 1H, J = 16.1 Hz), 6.75 (d, 1H, J = 16.1 Hz), 10.24 (s, 1H).

Ethyl 6-(4'-Acetoxybutyl)-9-(2',6',6'-trimethyl-1'-cyclohexenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate (12). To a suspension of NaH (60%

(58) Professors Mudi Sheves and Klaus Schulten. Private communication.
(59) Harbison, G. S.; Smith, S. O.; Pardeon, J. A.; Courtin, J. M. L.; Lugtenburg, J.; Herzfeld, J.; Mathies, R. A.; Griffin, R. G. *Biochemistry* **1985**, *24*, 6955–6962.
(60) Livnah, N.; Sheves, M. *Biochemistry* **1993**, *32*, 7223–7228.

in mineral oil, 0.12 g, 3 mmol) in dry THF (10 mL) at 0 °C was slowly added triethyl 3-methyl-4-phosphonocrotonate (0.80 g, 3 mmol). After the mixture was stirred at room temperature for 1 h, ester **11** (0.80 g, 2.4 mmol) in THF (2 mL) was added dropwise at 0 °C in the dark. After being stirred at room temperature for an additional 1 h, the reaction solution was poured into ice-cold water (10 mL). The aqueous phase was extracted with ether (2 × 10 mL). The combined organic solution was dried over $MgSO_4$, filtered, concentrated, and purified by flash chromatography (SiO_2, 5% EtOAc–hexanes) to provide ester **12** as a mixture of 60% all-trans and 40% 13-cis (0.64 g, 60%): CI-MS m/z 443 (M + 1); ^{1}H NMR ($CDCl_3$) of the trans isomer δ 1.02 (s, 6H), 1.26 (t, 3H, J = 7.1 Hz), 1.46 (m, 4H), 1.61 (m, 4H), 1.71 (s, 3H), 2.01 (s, 3H), 2.02 (s, 3H), 2.03 (m, 2H), 2.33 (s, 3H), 2.44 (m, 2H), 4.02 (t, 2H, J = 6.6 Hz), 4.16 (q, 2H, J = 7.1 Hz), 5.81 (s, 1H), 6.32 (d, 1H, J = 15.7 Hz), 6.33 (d, 1H, J = 15.9 Hz), 6.53 (d, 1H, J = 15.9 Hz), 7.15 (d, 1H, J = 15.7 Hz).

6-(4'-Hydroxybutyl)-9-(2',6',6'-trimethyl-1'-cyclohexenyl)-3,7-dimethyl-2,4,6,8-nonatetraenol (13). To a solution of esters **12** (0.62 g, 1.4 mmol) in anhydrous diethyl ether (10 mL) at -78 °C was slowly added DIBAL-H (1.0 M in hexanes, 5.6 mL, 5.6 mmol) in the dark. The mixture was stirred at 0 °C for 1 h, poured onto ice-cold wet silica gel in ether (30 mL), and stirred for an additional 1 h. The crude product was dried over $MgSO_4$ and filtered. The filter cake was washed with EtOAc (10 mL). The combined organic solution was concentrated and chromatographed (SiO_2, 30% EtOAc–hexanes) to give diol **13** as a mixture of 60% all-trans and 40% 13-cis (0.38 g, 75%): CI-MS m/z 357 (M - 2H + 1); ^{1}H NMR ($CDCl_3$) of the trans isomer δ 1.02 (s, 6H), 1.45 (m, 4H), 1.60 (m, 4H), 1.71 (s, 3H), 1.87 (s, 3H), 1.98 (s, 3H), 2.01 (m, 2H), 2.44 (m, 2H), 3.63 (t, 2H, J = 6.5 hz), 4.31 (d, 2H, J = 7.0 Hz), 5.71 (t, 1H, J = 7.0 Hz), 6.24 (d, 1H, J = 16.0 Hz), 6.34 (d, 1H, J = 15.9 Hz), 6.54 (d, 1H, J = 16.0 Hz), 6.75 (d, 1H, J = 15.9 Hz).

6-(4'-Hydroxybutyl)-9-(2',6',6'-trimethyl-1'-cyclohexenyl)-3,7-dimethyl-2,4,6,8-nonatetraenal (14). To a solution of diols **13** (0.36 g, 1.0 mmol) in anhydrous hexane (4 mL) at 0 °C was added MnO_2 (1.74 g, 20.0 mmol) in the dark. After being stirred at 0 °C for 1 h, the reaction mixture was filtered through Celite, and the filter cake was washed with EtOAc (20 mL). The organic solution was concentrated and purified by flash chromatography (SiO_2, 20% EtOAc–hexanes) to afford retinal analog **14** as a mixture of 80% all-trans and 20% 13-cis (0.27 g, 75%). The retinal analog isomers were further separated by semipreparative HPLC (SiO_2, 10 mm × 250 mm, 45% EtOAc–hexane, flow rate 2 mL/min, detection 360 nm) to give all-trans retinal analog **14**: CI-MS m/z 357 (M + 1); UV (hexane) 373 nm; ^{1}H NMR ($CDCl_3$) δ 1.03 (s, 6H), 1.46 (m, 4H), 1.59 (m, 4H0, 1.71 (s, 3H), 2.03 (t, 2H, J = 6.0 Hz), 2.05 (s, 3H), 2.34 (s, 3H), 2.47 (m, 2H), 3.64 (t, 2H, J = 6.3 Hz), 6.00 (d, 1H, J = 8.2 Hz), 6.40 (d, 1H, J = 15.9 Hz), 6.42 (d, 1H, J = 15.7 Hz), 6.58 (d, 1H, J = 15.9 Hz), 7.30 (d, 1H, J = 15.7 Hz), 10.10 (d, 1H, J = 8.2 Hz).

6-{4'-[(4''-Azido-3''-[^{3}H]phenyl)acetoxy]butyl}-9-(2',6',6'-trimethyl-1'-cyclohexenyl)-3,7-dimethyl-2,4,6,8-nonatetraenal (5). To a solution of all-trans retinal analog **14** (10 mg, 0.028 mmol) in dry CH_2Cl_2 (3 mL) at 0 °C were added *p*-azidophenylacetic acid (6 mg, 0.034 mmol) and DCC (7 mg, 0.034 mmol) in the dark. After the mixture was stirred at room temperature for 3 h, DMAP (7 mg, 0.057 mmol) was added, and the reaction solution was stirred for an additional 1 h. One drop of HOAc was then added to quench the reaction. The mixture was dried through a Pasteur pipet filled with $MgSO_4$, concentrated, and purified by a Pasteur pipet column (SiO_2, 10% EtOAc–hexanes) to give azide retinal analog **5** as a mixture of isomers (13.2 mg, 92%). The flash-chromatographed product was further purified by semipreparative HPLC (SiO_2, 10 mm × 250 mm, 20% EtOAc–hexane, flow rate 2 mL/min, detection 370 nm) to provide all-trans azide retinal analog **5**: FAB-HRMS m/z 515.3157 (M^+), calcd for $C_{32}H_{41}N_3O_3$ 515.3148; UV (MeOH) 385 nm; ^{1}H NMR ($CDCl_3$) δ 1.03 (s, 6H), 1.46 (m, 4H0, 1.59 (m, 4H), 1.71 (s, 3H0, 2.03 (t, 2H, J = 6.0 Hz), 2.06 (s, 3H), 2.34 (s, 3H), 2.44 (m, 2H), 3.55 (s, 2H), 4.08 (t, 2H, J = 6.6 Hz), 5.99 (d, 1H, J = 8.2 Hz), 6.37 (d, 1H, J = 15.8 Hz), 6.41 (d, 1H, J = 15.9 Hz), 6.55 (d, 1H, J = 15.9 Hz), 6.94 (d, 2H, J = 8.6 Hz), 7.22 (d, 2H, J = 8.6 Hz), 7.29 (d, 1H, J = 15.8 Hz), 10.11 (d, 1H, J = 8.2 Hz). [^{3}H]-Azide retinal analog **5** was obtained by the reaction of retinal analog **14** with [^{3}H]-*p*-azidophenylacetic acid (2 Ci/mmol) under same conditions. The reaction mixture was chromatographed with a pipet column (SiO_2, 5% MeOH–CH_2Cl_2) prior to HPLC purification. The all-trans tritiated azide retinal analog was isolated on the basis of the HPLC retention time of the non-radioactive analog (53% radiochemical yield).

Ethyl 5-Cyano-3-methyl-2,4-pentadienoate (16). To a suspension of NaH (80% in mineral oil, 4.9 g, 0.165 mol) in dry THF (350 mL) at 0 °C was slowly added diethyl (cyanomethyl)phosphonate (26.8 g, 0.151 mol). After the mixture was stirred at room temperature for 1 h, ethyl 3-methyl-4-oxocrotonate (**15**; 19.5 g, 0.137 mol) was added dropwise at 0 °C. After being stirred at room temperature for an additional 1 h, the reaction solution was poured onto a mixture of ice, concentrated HCl (13 mL), and saturated aqueous NH_4Cl solution (160 mL). The organic layer was separated, dried over $MgSO_4$, filtered, concentrated, and purified by flash chromatography (SiO_2, 25% EtOAc–hexanes) to give ester **16** (20.4 g, 90%). The trans/cis isomer ratio was 4/1. CI-MS m/z 165 (M^+); ^{1}H NMR ($CDCl_3$) of the trans isomer δ 1.27 (t, 3H, J = 7.1 Hz), 2.22 (s, 3H), 4.18 (q, 2H, J = 7.1 Hz), 5.67 (d, 1H, J = 16.4 Hz), 5.97 (s, 1H), 7.00 (d, 1H, J = 16.4 Hz).

5-Cyano-3-methyl-2,4-pentadienol (17). To a solution of all-trans ester **16** (10.0 g, 60.5 mmol) in dry THF (150 mL) at -78 °C was added dropwise DIBAL-H (1.0 M in hexanes, 150 mL, 150 mmol). The reaction mixture was stirred at -78 °C for 1 h, poured onto ice-cold wet silica gel in ether (500 mL), and stirred at 0 °C for an additional 1 h. $MgSO_4$ was added to the mixture. The whole mixture was filtered, and the filter cake was washed with EtOAc (30 mL). The organic solution was concentrated and flash chromatographed (SiO_2, 60–67% EtOAc–hexanes) to yield allylic alcohol **17** (5.6 g, 75%). CI-MS m/z 124 (M + 1); ^{1}H NMR ($CDCl_3$) δ 1.75 (s, 3H), 4.34 (d, 2H, J = 6.3 Hz), 5.30 (d, 1H, J = 16.3 Hz), 5.97 (t, 1H, J = 6.3 Hz), 7.01 (d, 1H, J = 16.4 Hz).

5-Cyano-3-methyl-2,4-pentadienyl *tert*-Butyldimethylsilyl Ether (18). To a solution of alcohol **17** (5.4 g, 44.1 mmol) and Et_4N (4.9 g, 48.4 mmol) in CH_2Cl_2 (100 mL) was added TBDMSCl (7.3 g, 48.4 mmol). The mixture was stirred at room temperature for 6 h and washed with water (2 × 30 mL) and then brine (30 mL). The organic phase was separated, dried over $MgSO_4$, filtered, concentrated, and purified by flash chromatography (SiO_2, 67% CH_2Cl_2–hexanes) to provide silyl ether **18** (9.0 g, 86%): CI-MS m/z 238 (M + 1); ^{1}H NMR ($CDCl_3$) δ 0.06 (s, 6H0, 0.88 (s, 9H), 1.72 (s, 3H), 4.34 (d, 2H, J = 5.9 Hz), 5.26 (d, 1H, J = 16.4 Hz), 5.91 (t, 1H, J = 5.7 Hz), 7.01 (d, 1H, J = 16.3 Hz).

4-Methyl-6-[(*tert*-butyldimethylsilyl)oxy]-2,4-hexadienal (19). To a solution of ether **18** (4.3 g, 18.1 mmol) in anhydrous diethyl ether (60 mL) at -78 °C was slowly added DIBAL-H (1.0 M in hexanes, 25 mL, 25 mmol). The mixture was stirred for 2 h at -20 °C to -30 °C, warmed gradually to 0 °C, poured onto ice-cold wet silica gel in ether (50 mL), and stirred for an additional 1 h. The mixture was dried over $MgSO_4$ and filtered. The filter cake was washed with EtOAc (20 mL). The solution was concentrated and chromatographed (SiO_2, 15% EtOAc–hexanes) to afford aldehyde **19** (3.8 g, 87%): CI-MS m/z 200 (M^+); ^{1}H NMR ($CDCl_3$) δ 0.07 (s, 6H), 0.89 (s, 9H), 1.78 (s, 3H), 4.38 (d, 2H, J = 5.8 hz), 6.06 (t, 1H, J = 6.2 Hz), 6.12 (dd, 1H, J = 7.5, 15.6 Hz), 7.10 (d, 1H, J = 15.7 Hz), 9.56 (d, 1H, J = 7.7 Hz).

2-(4'-Hydroxybutyl)-6-methyl-8-[(*tert*-butyldimethylsilyl)oxy]-2,4,6-octatrienal (20). To a suspension of NaH (80% in mineral oil, 0.6 g, 20.0 mmol) in dry THF (50 mL) at 0 °C was added dropwise triethyl 5-cyano-5-phosphonopentanoate (**8**; 5.4 g, 18.5 mmol). After the mixture was stirred at room temperature for 1.5 h, aldehyde **19** (3.7 g, 15.4 mmol) was added slowly at 0 °C. After being stirred at room temperature for an additional 1 h, the reaction solution was poured onto a mixture of ice, concentrated HCl (1.5 mL), and saturated aqueous NH_4Cl solution (20 mL). The organic layer was separated, dried over $MgSO_4$, filtered, concentrated, and purified by chromatography to give a mixture of nitrile isomers (4.9 g, 85%). To a solution of the nitriles (4.9 g, 13.0 mmol) in anhydrous diethyl ether (60 mL) at -78 °C was slowly added DIBAL-H (1.0 M in hexanes, 65 mL, 65 mmol). The mixture was stirred for 3 h, warmed gradually to 0 °C, poured onto ice-cold wet silica gel in ether (300 mL), and stirred for an additional 1 h. The mixture was dried over $MgSO_4$ and filtered. The filter cake was washed with EtOAc (20 mL). The solution was concentrated and chromatographed (SiO_2, 40% EtOAc–hexanes) to give aldehyde **20** as a mixture of 75% trans and 25% cis (2.6 g, 60%): CI-MS m/z 339 (M + 1); ^{1}H NMR ($CDCl_3$) δ 0.07 (s, 6H), 0.89 (s, 9H), 1.35–1.56 (m, 4H), 1.80 (s, 3H), 2.26 (t, 1.5H, trans, J = 7.1 Hz), 2.40 (t, 0.5H, cis, J = 7.1 Hz), 3.64 (t, 2H, J = 5.9 Hz), 4.35 (d, 2H, J = 6.1 Hz), 5.79 (t, 1H, J = 6.2 Hz), 6.48 (d, 0.75H, trans, J = 14.7 Hz), 6.58–7.15 (m, 2.25H, trans and cis), 9.40 (s, 0.25H, cis), 10.27 (s, 0.75H, trans).

7-(4'-Hydroxybutyl)-9-(2',6',6'-trimethyl-1'-cyclohexenyl)-3-methyl-2,4,6,8-nonatetraenyl *tert*-Butyldimethylsilyl Ether (21). To a suspension of β-cyclogeranyltriphenylphosphonium bromide (0.92 g, 1.92 mmol) in dry THF (20 mL) at -78 °C was added *n*-BuLi (2.5 M in hexanes, 1.5 mL, 3.75 mmol). After the mixture was stirred at room temperature for 0.5 h, aldehydes **20** (0.50 g, 1.48 mmol) in dry THF (5 mL) were added slowly at -78 °C. The reaction mixture was warmed to room temperature and stirred for an additional 12 h in the dark. The crude product was

washed with water (2 × 20 mL) and then brine (20 mL), and the aqueous layer was extracted with ether (2 × 20 mL). The combined organic solution was dried over $MgSO_4$, filtered, concentrated, and purified by chromatography (SiO_2, 25% EtOAc–hexanes) to provide silyl ether **21** as a mixture of four isomers (0.35 g, 52%): CI-MS *m/z* 458 (M^+); ^{1}H NMR ($CDCl_3$) δ 0.05 (s, 6H), 0.88 (s, 9H), 0.99 (s, 3H), 1.01 (s, 3H), 1.35–1.65 (m, 8H), 1.68 (s, 1.5H), 1.71 (s, 1.5H), 1.77 (s, 3H), 2.01 (t, 2H, *J* = 5.4 Hz), 2.31 (s, 1.5H, cis), 2.45 (s, 1.5H, trans), 3.64 (m, 2H), 4.32 (d, 2H, *J* = 6.3 Hz), 5.58 (t, 1H, *J* = 6.3 Hz), 5.95–6.70 (m, 5H).

7-(4′-Hydroxybutyl)-9-(2′,6′,6′-trimethyl-1′-cyclohexenyl)-3-methyl-2,4,6,8-nonatetraenal (22). To a solution of ethers **21** (0.10 g, 0.218 mmol) in dry THF (5 mL) was added *n*-$Bu_4N^+F^-$ (1.0 M in THF, 0.65 mL, 0.65 mmol) in the dark. After being stirred at room temperature for 2 h, the reaction mixture was washed with water (2 × 10 mL) and then brine (10 mL), and the aqueous layer was extracted with ether (2 × 10 mL). The combined organic solution was dried over $MgSO_4$, filtered, concentrated, and chromatographed (SiO_2, 25–50% EtOAc–hexanes) to give the deprotected product. To a solution of this product in dry CH_2Cl_2 (2 mL) was added MnO_2 (0.18 g, 2.07 mmol) in the dark. After being stirred at room temperature for 36 h, the reaction mixture was filtered through Celite, and the filter cake was washed with EtOAc (20 mL). The organic solution was concentrated and purified by chromatography (SiO_2, 33% EtOAc–hexanes) to give retinal analog **22** as a mixture of isomers (25 mg, 84%). The mixture was further separated by semipreparative HPLC (SiO_2, 10 mm × 250 mm, 50% EtOAc–hexane, flow rate 4 mL/min, detection 360 nm) to give all-trans retinal analog **22** (8 mg, 27%): CI-MS *m/z* 343 (M + 1); UV (hexane) 368 nm; ^{1}H NMR ($CDCl_3$) of the all-trans isomer δ 1.01 (s, 6H), 1.43–1.66 (m, 8H), 1.69 (s, 3H), 2.01 (t, 2H, *J* = 5.4 Hz), 2.30 (s, 3H), 2.52 (t, 2H, *J* = 7.0 Hz), 3.67 (t, 2H, *J* = 5.8 Hz), 5.95 (d, 1H, *J* = 8.2 Hz), 6.02 (d, 1H, *J* = 16.2 Hz), 6.14 (d, 1H, *J* = 11.5 Hz), 6.34 (d, 1H, *J* = 16.2 Hz), 6.36 (d, 1H, *J* = 15.1 Hz), 7.08 (dd, 1H, *J* = 11.5, 15.1 Hz), 10.08 (d, 1H, *J* = 8.2 Hz).

7-{4′-[(4″-Azido-3″-)[^{3}H])phenyl)acetoy]butyl}-9-(2′,6′,6′-trimethyl-1′-cyclohexenyl)-3-methyl-2,4,6,8-nonatetraenal (6). To a solution of all-trans retinal analog **22** (8 mg, 0.0234 mmol) in dry CH_2Cl_2 (2 mL) were added *p*-azidophenylacetic acid (6 mg, 0.0339 mmol) and DCC (7 mg, 0.0339 mmol) in the dark. After the mixture was stirred at room temperature for 2 h, DMAP (16 mg, 0.131 mmol) was added, and the reaction solution was stirred for an additional 1 h. One drop of HOAc was then added to quench the reaction. The solution was dried through a Pasteur pipet filled with $MgSO_4$, concentrated, and purified by a pipet column (SiO_2, 20% EtOAc–hexanes). The flash-chromatographed product was further purified by semipreparative HPLC (SiO_2, 10 mm × 250 mm, 20% EtOAc–hexane, flow rate 3 mL/min, detection 360 nm) to give all-trans azide retinal analog **6** (5.8 mg, 50%): FAB-HRMS *m/z* 501.2999 (M^+), calcd for $C_{31}H_{39}N_3O_3$ 501.2991; UV (MeOH) 380 nm; ^{1}H NMR ($CDCl_3$) δ 1.01 (s, 6H), 1.43–1.66 (m, 8H), 1.69 (s, 3H), 2.01 (t, 2H, *J* = 5.7 Hz), 2.28 (s, 3H), 2.49 (t, 2H, *J* = 7.4 Hz), 3.56 (s, 2H0, 4.11 (t, 2H, *J* = 6.3 Hz), 5.97 (d, 1H, *J* = 9.1 Hz), 6.02 (d, 1H, *J* = 16.4 Hz), 6.15 (d, 1H, *J* = 11.6 Hz), 6.30 (d, 1H, *J* = 16.4 Hz), 6.36 (d, 1H, *J* = 15.0 Hz), 6.94 (d, 2H, *J* = 8.4 Hz), 7.03 (dd, 1H, *J* = 11.5, 15.4 Hz), 7.22 (d, 2H, *J* = 8.6 Hz), 10.09 (d, 1H, *J* = 8.1 Hz). [^{3}H]-Azide retinal analog **6** was obtained by the reaction of retinal analog **22** with [^{3}H]-*p*-azidophenylacetic acid (2 Ci/mmol) under same conditions. The reaction mixture was chromatographed with a pipet column (SiO_2, 5% MeOH–CH_2Cl_2) prior to HPLC purification. The all-trans tritiated azide retinal analog was isolated on the basis of the HPLC retention time of the non-radioactive analog (26% radiochemical yield).

Incorporation of Retinal Analogs into Bacterioopsin. Bacteriorhodopsin was isolated from *H. halobium*[61] and bleached in the presence of hydroxylamine to remove endogenous retinal;[62] residual retinal oxime was removed by washing the membrane suspension with 5% w/v bovine serum albumin and then with water. To a suspension of the apomembrane in deionized water was added retinal analog **5** or **6** in an ethanol solution (final ethanol concentration: 0.5%). A molar excess of apoprotein was used (1/0.65) in order to avoid random cross-linking by unincorporated chromophore during photolysis. The reconstitution was carried out at room temperature in the dark and was monitored by UV/vis spectrophotometry using bacterioopsin as the blank. The reconstitution yields were estimated from the A_{503} and the A_{475}, respectively, by applying the ϵ_{568} of natural BR.

CD Spectrum and Proton Pumping of BR Analogs. The CD spectrum of an aqueous membrane suspension of monomeric BR reconstituted with analog **5** (0.5 mg of protein/mL, 35% reconstitution yield) was measured in a 1-cm quartz cell, scanning from 680 to 280 nm and subtracting the spectrum of the bacterioopsin blank.[46,47] Natural BR, which was also reconstituted to 35% yield but with *all-trans*-retinal, was measured under identical conditions. Functionality of the BR analogs was determined by measuring the proton-pumping ability of reconstituted pigments according to a modification of a published method.[39] For this purpose, equal concentrations of apoprotein were incubated with *all-trans*-retinal, with analog **5**, and with analog **6**; natural BR reconstituted to 85% yield, whereas BR analogs **5** and **6** reconstituted to 50% and 35%, respectively. The membrane suspensions were then irradiated for 1 min (BR analog **5**, 470-nm-cutoff filter; BR analog **6**, 430-nm filter; natural BR, 530-nm filter), and the ΔpH was recorded. The negative control was apoprotein (530-nm filter).

Photolysis of BR Analogs. To initiate cross-linking of the azido retinal analog to the polypeptide, the BR analog in suspension was irradiated in a quartz tube at 4 °C for 8 min with a low-pressure mercury lamp (New England Ultraviolet) having a 254-nm narrow-band emission. After the cross-linked pigment was bleached in the presence of hydroxylamine to hydrolyze the Schiff base,[62] the cross-linking yield was obtained by separating the cross-linked protein from free ligand by gel-filtration HPLC under denaturating conditions (preparative TSK Gel-G3000 SW column (600 mm × 21.5 mm); H_2O–MeOH–$CHCl_3$ (2/5/2, 0.1% TFA) solvent system; 4 mL/min[18]) and counting the radioactivity associated with the polypeptide.

Limited Cleavage by Chymotrypsin or Sodium Borohydride. The bleached polypeptide was cleaved into two fragments with either α-chymotrypsin or sodium borohydride (Figure 3) using published protocols with minor modifications.[4,41] Incubation of the cross-linked pigment with chymotrypsin (pigment/enzyme = 20/1 w/w) at 37 °C for 6 h generated fragments C1 (Gly-72/Ser-248) and C2 (Glu-1/Phe-71). Treatment of the labeled pigment with sodium borohydride (6% w/w) under Argon at 4 °C in the dark for 2 days gave fragments B1 (Glu-1/Gly-155) and B2 (Phe-156/Ser-248). The digested fragments were separated by size-exclusion HPLC.

Exhaustive Cleavage by Cyanogen Bromide. The purified chymotryptic C1 fragment was further cleaved at the methionine residues into five smaller fragments, numbered 6–10 (Figure 3), by treating with cyanogen bromide at 250–300-fold molar excess over methionine in 70% formic acid.[63] The cleavage was performed at room temperature in the dark for 24 h.

Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed with a Bio-Rad minigel apparatus on 0.75 mm thick gels consisting of 16.5% resolving gel, 10% spacer gel, and 4% stacking gel.[64] Protein bands were visualized by staining with coomassie blue R in 10% aqueous acetic acid and destaining with 10% aqueous acetic acid. Fluorography was carried out on Kodak X-Omat AR5 film after impregnation of gels with autoradiographic enhancer (NEN research products). For sequence analysis of individual peptide bands, the gels were blotted to PVDF membranes in 10 mM CAPS[1], pH 11, with 10% methanol, and the blots were stained with 0.1% coomassie blue R-250 in 40% methanol and destained with 45% methanol, 5% acetic acid.[65] The desired bands were cut and applied to the gas-phase sequencer.

Edman Degradation. Sequencing of cold peptide bands blotted from the polyacrylamide gel to a PVDF membrane was performed on an Applied Biosystems 470A gas-phase sequencer equipped with a Model 120A PTH[1] analyzer, using the program PTH. For radioactive sequencing, the cyanogen bromide-cleaved chymotryptic C1 fragment of BR was dissolved in 70% TFA, spotted onto a PVDF membrane, and sequenced on an Applied Biosystems model 477A sequencer using the ATZ-1 program.

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