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Photoaffinity labeling of rhodopsin and bacteriorhodopsin

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Abstract

Photoaffinity labeling with bovine rhodopsin using a retinal with a fixed 11-*cis*-ene cross-linked exclusively to Trp-265/Leu-266 in helix F, showing that the β-ionone C-3 is close to helix F. Moreover, since these labeled amino acids are in the middle of helix F, while the Schiff-base linkage to Lys-296 at the other terminus of the chromophore is also in the middle of helix G, the chromophore lies horizontally near the center of the lipid bilayer. In bacteriorhodopsin, photoaffinity studies using a retinal with a C-10 tritiated phenylazide appended through a 13 Å spacer cross-linked to Arg-175/Asn-176 on the cytoplasmic side of helix F; this indicates that 9-Me points toward the extracellular space. This result agrees with our earlier studies with 9-sulfate analogs but is opposite to that deduced by biophysical measurements.

Keywords: Rhodopsin; Bacteriorhodopsin; Photoaffinity labeling; Rhodopsin (chromophore location); Locked retinal analog; Side-chain methyls

1. Rhodopsin

The tertiary structure of rhodopsin, including the site and orientation of the 11-*cis*-retinal chromophore **1** is essential to understand the visual transduction mechanism, but the stickiness of this membrane protein has eluded efforts to clarify this aspect by X-ray and NMR. Intensive biochemical, biological and biophysical studies have led to plausible overall tertiary structures for bovine rhodopsin [1] but location of the chromophore is still not clear.

Photoaffinity labeling of retinal proteins, particularly rhodopsin, has been hindered by serious factors: (i) the difficulty in separating the peptidic fragments obtained upon enzymatic or chemical cleavages, a factor that caused problems in the sequencing of the protein [2,3], and (ii) the scrambling of cross-linking upon photoactivation of the photolabel, due to isomerization of the *cis*-ene to the *trans*-ene. In 1982 we had prepared ^{14}C-labeled 9-*cis*-3-diazoacetate **2** (* denote ^{14}C; λ_{max} 248 nm) [4] and subsequently the 3*S* and 3*R* enantiomers [5]. Incorporation of 3*S* and 3*R*-**2** yielded a rhodopsin absorbing at 465 nm; the incorporation of the 3*S* enantiomer (3α in **2**) was high whereas that of 3*R* (3β in **2**) was very poor. Irradiation of the pigment at 254 nm under mildest

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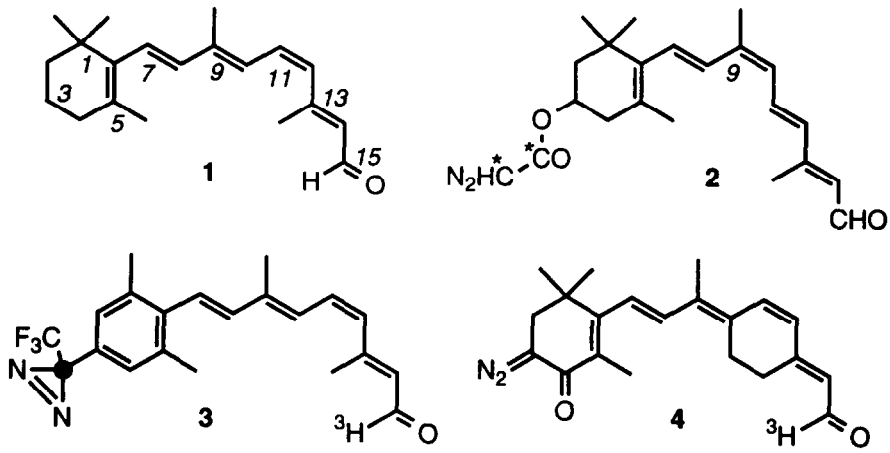


conditions, i.e., 6–10 min from a 10-cm distance with the lamp in use, that gave rise to the disappearance of the 248 nm diazoacetoxy band, also led to a 65% decrease in the 465 nm pigment band due to *cis/trans* isomerization and bleaching. However, despite very serious efforts we could not identify the cross-linked amino acids due to reasons given [6]. Nakayama and Khorana used the aromatic trifluoromethyldiazirene **3** and identified the amino acids in helices C and F to which the diazirene carbon (black dot in **3**) had cross-linked; other cross-linked sites were not identified [7] (Fig. 3, below). Their results show that the ionone ring is in the vicinity of helices C and F, but the multitude of cross-linked amino acids and the large distance between some labeled residues (13.5 Å) could be due to scrambling resulting from *cis/trans* isomerization [7].

We thus decided to examine the chromophore orientation by using analog **4**, [15-^{3}H]-3-diazo-4-oxo-10,13-ethano-11-*cis* retinal. The 11-*cis* → all *trans* isomerization is blocked in this tritiated retinal with a diazoketone moiety [8]; the analog with a fixed 6-membered ring has been reported to adopt a conformation similar to that of native 11-*cis* retinal within the protein binding pocket [9]. Incubation of analog **4** with bovine opsin for 1 day gave a pigment, λ_{max} 483 nm, reconstitution yield ca. 50% as determined spectrophotometrically; the opsin shift of 2300 cm^{-1} is 85% that of native rhodopsin.

Native rhodopsin exhibits two CD bands at 490 nm (α-band) and 340 nm (β-band). In contrast, the CD spectrum of the rhodopsin derived from analog **4**

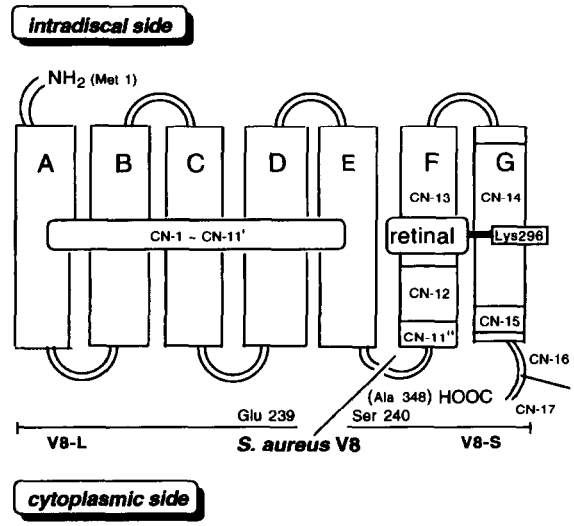


Fig. 1. Rhodopsin secondary structure showing CNBr cleavage sites at Met (represented by horizontal lines) in V8-S fragment. The seven transmembrane α-helices are depicted by rectangles A–G. Digestion with V8 protease cleaves between Glu-239 and Ser-240 residing in CNBr peptide-11 fragment to yield CN-11′ and CN-11″.

has only one extreme at 308 nm; this lack of the α-band is in line with the CD spectra of rhodopsins Rh5 and Rh6 reconstituted from analogs containing *planar* 5-membered [10] and 6-membered rings [11], respectively. Rh5 and Rh6 both lack the α-band, showing only the β-bands at 336 nm [10] and 340 nm [11], respectively; Ito and coworkers [10] have attributed the α-band arising from distortion around the 12–13 bond and the β-band due to distortion around the 6–7 bond. Irradiation of the reconstituted pigment at 254 nm (10 min, $-5°$ C) resulted in a 23% cross-link to the protein as estimated from the radioactivity of the peptide after separation from the unbound retina by size-exclusion (SE) HPLC. Although the CD 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.

The cross-linked apoprotein in membrane suspension was cleaved by V8 protease into large (V8-L) and small (V8-S) fragments; cleavage yield ca. 50% as judged by gel electrophoresis. After detergent solubilization and carboxymethylation of cysteine residues, the cleaved V8-L and V8-S fragments were

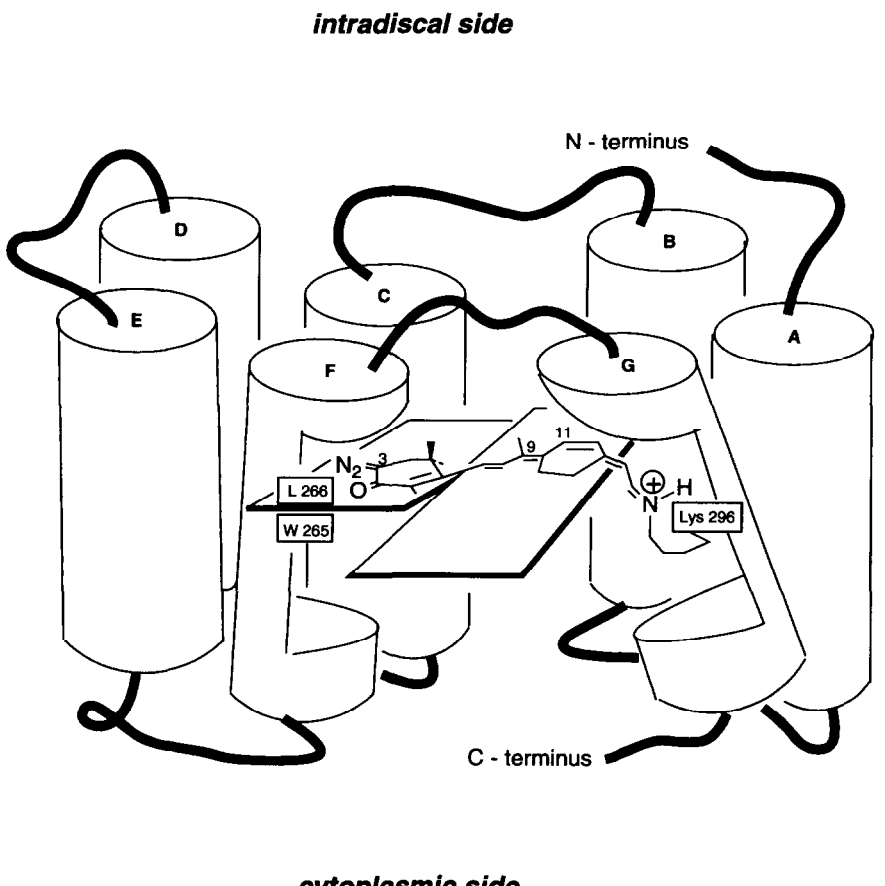


Fig. 2. Model of chromophore binding site in rhodopsin. Cylinders A–G represent the seven transmembrane α-helices. The squares denote two slightly offset planes of the chromophore. The main features of this model are: (i) C-3 of the β-ionone ring is in close contact with helix F and (ii) the entire chromophore resides near the center of the lipid bilayer.

separated by SDS-PAGE. All radioactivity resided in V8-S, while *no radioactivity was associated with the V8-L portion of rhodopsin*, indicating that the cross-linking site resided in helix F or G (Fig. 1). The V8-S polypeptide separated by SE-HPLC was further cleaved by CNBr into seven fragments. It was extremely fortunate that radioactivity was associated with the V8-S HPLC peak since this was the only peak base-line separated from the rest, which consisted of a mixture of V8-L, and uncleaved and aggregated rhodopsin. The CNBr-cleaved mixture was separated by SDS-PAGE and the labeled peptide was identified by autoradiography. The major radioactive band was characterized as CNBr peptide-13 (CN-13, see Fig. 1) by blotting on a PVDF membrane followed by sequencing. Edman degradation of this CN-13 fragment revealed that cycles 8 and 9, corresponding to Trp-265 and Leu-266, respectively, had the maximum radioactivity and accounted for ca. 20% of the total tritium recovered from the sample filter. The locked analog almost exclusively cross-linked to Trp-265 and Leu-266, thus yielding a most clear-cut result (Fig. 2). This result, in conjunction with previous labeling results [7], also indicates that isomerization moves C-3, which is close to helix F, towards helix C.

The helices in Fig. 2 are arranged on the basis of recent electron cryomicroscopic data [12] and a published theoretical assignment of the seven transmembrane helices [13]. A current transmembrane model of the rhodopsin helices places both the labeled Trp-265/Leu-266 residues, and the Schiff-base linkage to Lys-296 in the middle of the lipid bilayer [14]. Since the plane of the polyene is essentially parallel to the membrane plane [15], *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 [16].

Present studies show that Trp-265 is close to the ionone ring (Fig. 3). 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 [17]. Previous photoaffinity labeling with

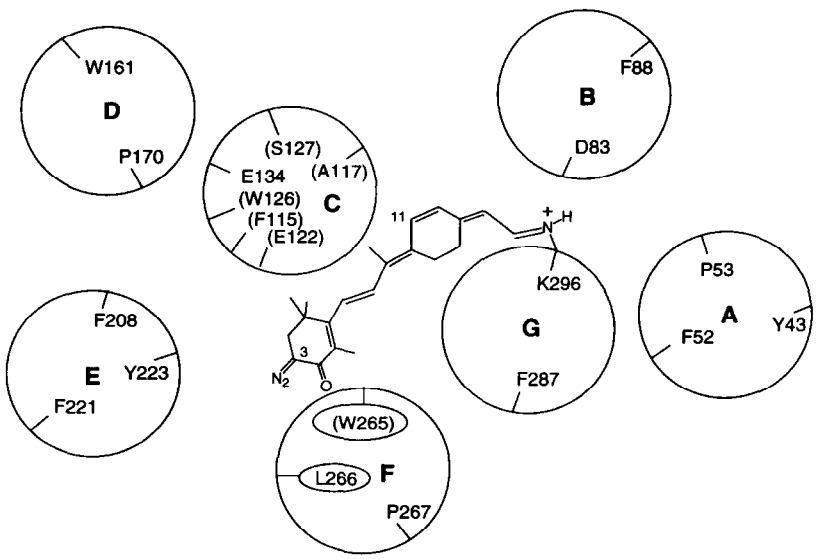


Fig. 3. Helical wheel projection model of rhodopsin (looking down from the intradiscal side of the membrane). The residues cross-linked in the present study are shown in ovals, while those from a previous photoaffinity labeling with **3** [7] are in parentheses. Coordinates of helices are based on electron cryomicroscopic data [12] and a theoretical assignment of the seven helices [13]. The amino acid residues on the helices are oriented according to Ref. [7]. The *anti* conformation of the Schiff base was shown by resonance Raman spectroscopy [23].

retinal analog **3** also resulted in cross-linking of Trp-265, along with several other amino acids on helices F and C (parenthesized in Fig. 3) [7]. In this projection the plane of the polyene is placed parallel to the membrane plane [15], and the amino acid residues on the helices are oriented according to [7]. Our exclusive labeling of helix F with analog **4** suggests that C-3 of the ionone ring is in van der Waals contact with helix F, the opposing gem-dimethyl group pointing away from helix F (Fig. 3). Light-induced isomerization could then move C-3 to come in contact with helix C. This conclusion is supported by FT-IR 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) [18].

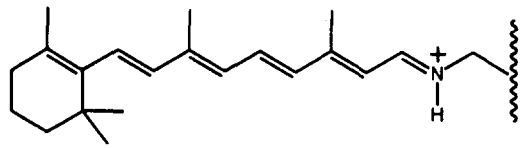
The rhodopsin structure shown in Figs. 2 and 3 indicates that the β-ionone ring is between helices C and F. Upon photoexcitation of the chromophore and redistribution of positive charge from the protonated nitrogen to the C-11–C-5 segment, this cationic moiety could form a transient salt bridge with 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 protein-assisted isomerization model is supported by site-specific mutagenesis of Glu-122 to Gln, in which the maximum of the neutralized mutant blue-shifted to 480 nm; transducin-stimulating activity was also reduced [17,19]. The Glu-122 assisted isomerization might explain why this residue, together with the Schiff-base counterion Glu-113, is the most highly conserved membrane-embedded carboxylate found in rhodopsins [17,20,21]; it also accounts for the high quantum yield of 0.67 in retinal isomerization, much higher than that in free solution (0.04–0.2).

Since the polypeptide reverts to the original conformation upon release of all-*trans* retinal after formation of the enzyme-activating *meta*-II species, the all-*trans* isomer appears to maintain (as well as generate) the active protein conformer. The electron density map [12] of rhodopsin reveals a binding pocket that lacks a straight portion to accommodate the all-*trans* chromophore (Fig. 3); thus only *cis* isomers (except for 13-*cis*) can be incorporated into the protein core which is formed predominantly by helices B, C, F, and G. Therefore, the *cis/trans* isomerization of the chromophore could push one or more helices away to generate a more accommodating transoid cavity. This could be the origin of the protein conformational changes leading to *meta*-II stage and which are reversed upon release of all-*trans* retinal [22].

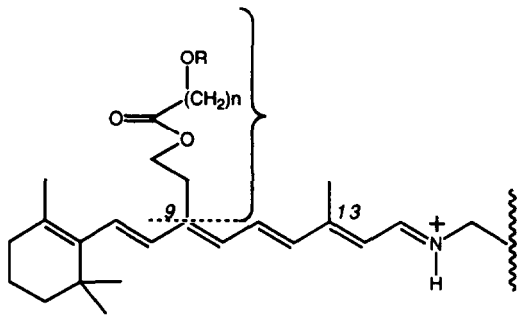
2. Bacteriorhodopsin

High-resolution electron cryomicroscopy has clarified the tertiary structure of the proton pumping bacteriorhodopsin (BR) [24], but the resolution was insufficient to define whether the C-9 and C-13 methyls were directed towards the extracellular surface or the cytoplasmic side of the membrane. This direction is important since it dictates the direction of the protonated Schiff-base proton (which is opposite to the methyls, see structure **5**), and therefore has a direct bearing on the mechanism of proton pumping.

Our past bioorganic studies with C-9 substituents containing ω-sulfate groups suggested that the methyls are directed to the exterior [25]. In contrast, measurements of linear dichroism [26], neutron diffraction [27] and solid-state deuterium NMR [28] suggested the opposite (see Fig. 6A below). The chain lengths from C-9 of alcohols **6** for $n = 3$, 5, 7, and 9 were 10 Å, 13 Å, 15 Å, 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 n-heptadecanyl branch at C-9 also absorbs at 450 nm. The series **6** analogs pumped protons with an efficiency of 7–40% relative to native BR. Sulfates **7** with branch lengths of 15 Å and 18 Å, respectively, for $n = 7$ and 9, also gave BR analogs, λ_{max} 475 nm, that pumped protons at 12% efficiency. However, sulfated retinals with branch lengths less than 13 Å (10 Å and 13 Å for $n = 3$ and 5, respectively, in **8**) did not regenerate BR, presumably because electrostatic interaction between the sulfate anions and charges on the membrane surface prevent the retinal to sink deeply into the binding site. Since BR analogs were reconstituted from intact lipid vesicles and



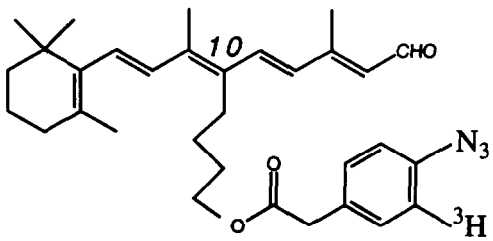
(5)



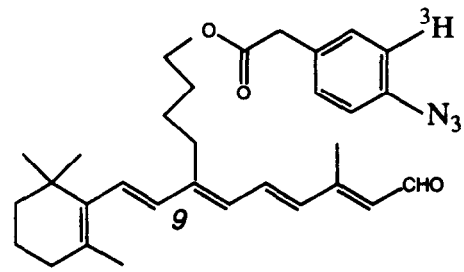
(6) R : H n = 3, 5, 7, 9; λmax 452 nm, functional

(7) R : OSO_3K n = 7, 9; λmax 475 nm, functional

(8) R : OSO_3K n = 3,5; no BR analog formation



(9) C-10 azidophenyl analog



(10) C-9 azidophenyl analog

since the direction of proton pumping was the same for native and artificial BRs, it was 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. The distance of 15 Å from C-9 to the external lipid surface also leads to a tilt angle of ca. 25° for the side chain [29] which agrees with other experiments giving angles of 15–25° [30,31].

We sought further evidence to solve this discrepancy between biophysical studies with azidophenyl analogs **9** and **10** with photoaffinity labels at C-9 or

C-10 extending in opposite directions. Unlike the three previous photoaffinity labeling studies which led to multiple labeling sites [29,32,33], the present experiments with analog **9** cross-linked to only two amino acids.

Incubation of bacterioopsin with analog **9** for 5–8 days yielded a pigment, λ_{max} 503 nm, 50% reconstitution yield, and with a proton pumping activity similar to that of BR. The 503 nm peak was blue-shifted relative to the 568 nm maximum of natural BR, the 2340 cm^{-1} opsin shift being less than half that of BR, 5100 cm^{-1}. Analog **10** gave a pigment with λ_{max} 475 nm, 35% reconstitution yield, in only 2 hours, but will not be discussed further because labeling resulted in cross-linking to many amino acids. The CD of the reconstituted BR with analog **9** showed strong positive and negative Cotton effects at 496 nm and 290 nm, respectively, which were similar in sign and intensity to the 544 nm and 320 nm peaks of BR reconstituted from all-*trans* retinal [34]. Photolysis with a 254 nm low-pressure Hg lamp, 4° C, 8 min, resulted in a decrease of the 253 nm *p*-azidophenylacetoxy band and a blue shift to 246 nm, indicating generation of the nitrene and cross-linking, yield ca. 7%.

Chymotrypsin cleaved, in almost quantitative yield, the labeled BR between Phe-71/Gly-72 (Fig. 4) to give C-2 and C-1 fragments, where 85% of the radioactivity was in C-1. Cyanogen bromide cleavage of HPLC-purified C-1 followed by SDS-PAGE indicated that the radioactivity resided in either fragment 6 or 9, which could not be separated because of similar molecular weight. However, sodium borohydride cleaves between Gly-155/Phe-156 [35] to yield B-1 and B − 2 fragments, yield ca. 60% (Fig. 4); the HPLC radioactivity profile revealed that B-2 contained ca. 86% of the total radioactivity. Thus it is *CNBr segment 9 that contains the cross-linked amino acid(s)*. The cross-linked amino acids of cyanogen bromide fragment 9 labeled by analog **9** were identified by subjecting the mixture of fragments 6–10 (of which only fragment 9 is radioactive) to Edman degradation. Since Edman degradation revealed that cycles 12, 13, and 14, which represent Arg-175,

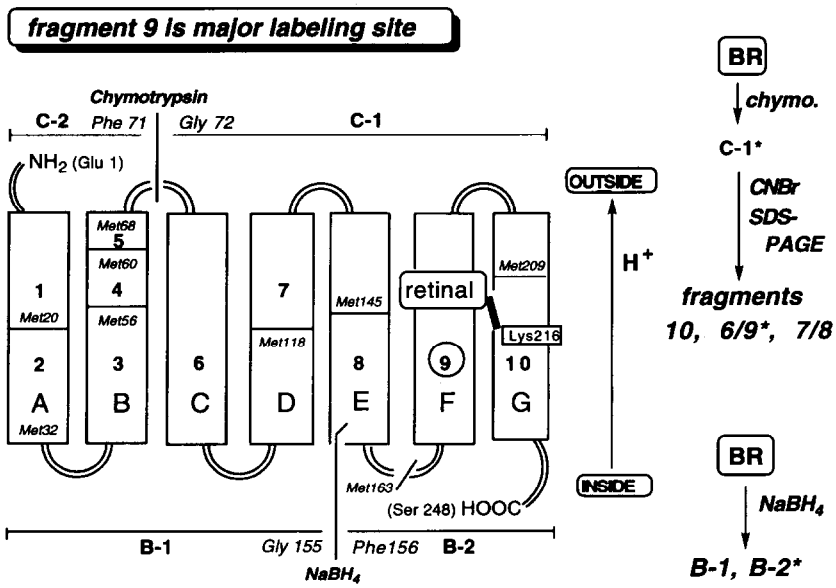


Fig. 4. BR secondary structure showing cleavage sites. The chymotryptic and sodium borohydride fragments are named C-1/C-2 and B-1/B-2, respectively. The CNBr cleavage sites are denoted by short horizontal lines. The ten CNBr fragments are numbered 1–10 from the amino terminus. The arrow labeled by H^+ shows the direction of proton pumping from the intracellular to the extracellular side of the membrane.

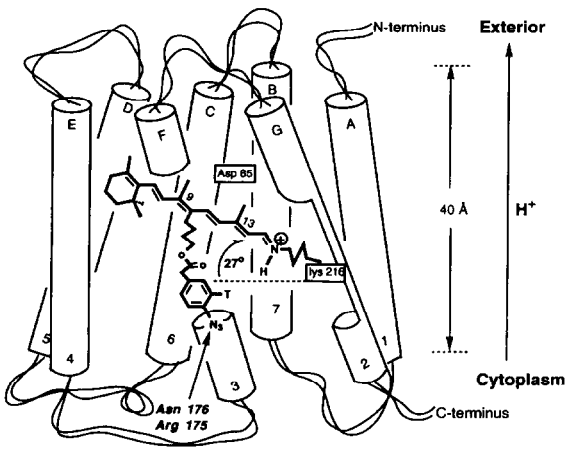


Fig. 5. Model of the retinal binding site in BR. Cylinders A–G represent the seven transmembrane α-helices. The arrow labeled by H^+ shows the direction of proton pumping toward the extracellular side.

Asn-176 and Val-177, respectively, contained the bulk of radioactivity (ca. 30% of total), Arg-175 and Asn-176 were determined to be the amino acids involved in the cross-linking. The radioactivity corresponding to Val-177 was probably due to incomplete cleavages in the preceding cycles, as this residue is known to be on the outer surface of helix F exposed to the lipid. The almost exclusive labeling of Arg-175/Asn-176 on the cytoplasmic side of helix F (Fig. 5) suggests that the 9-Me (and 13-Me) of retinal is directed toward the exterior [36].

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. Since these two amino acids are 12 Å from the cytoplasmic surface (see Fig. 6C) [24], the present study leads to a distance of 25 Å from C-10 to the intracellular surface. Since the membrane is ca. 40 Å

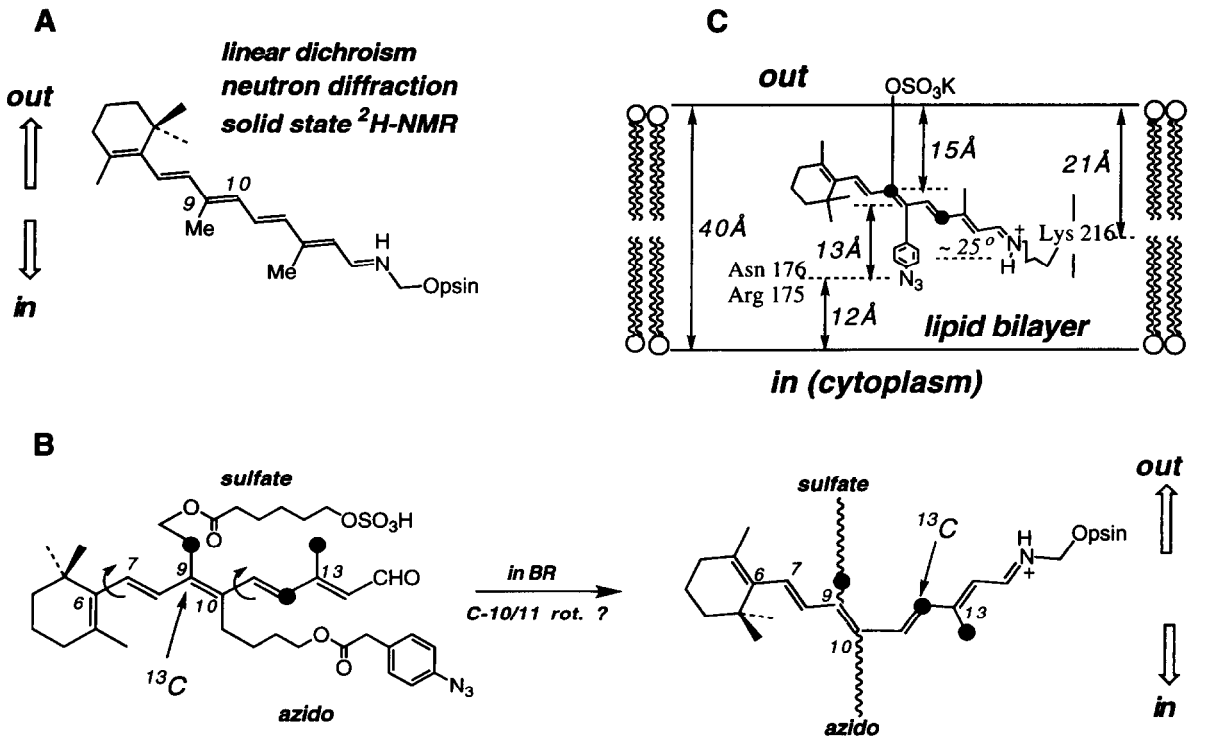


Fig. 6. (A) Orientation of retinal with side-chain methyls directed toward the cytoplasmic side. (B) Does a single bond rotation occur upon incorporation of the retinal analogs with 9-sulfate or 10-azidophenyl branching into bacterioopsin? Black dots denote ^{13}C. (C) Spatial relationships in BR binding site determined from two bioorganic studies.

thick, then C-10 is about 15 Å from the extracellular surface. This 15 Å distance is in good agreement with our earlier bioorganic studies using analogs with C-9 branches containing sulfate groups [25]. Helix G of BR contains residues 203–227 [24], meaning that Lys-216, which forms the Schiff base with retinal, is located approximately in the middle of the helix at ca. 21 Å from the extracellular surface (Fig. 6C). This leads to a chromophore tilt angle of ca. 27° since the labeling studies show that C-10 is ca. 15 Å from the extracellular surface; this again agrees with angles of 10–25° derived from several measurements [26,30,37] (Fig. 6C).

The reason for the discrepancy between the physical measurements and the two bioorganic studies is not clear. The polyene conformation of the chromophore in the protein binding pocket might be disturbed in the analogs with long branches so that the conformation becomes cisoid around some single bond(s). As depicted in Fig. 6B, 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 [38], upon incorporation into BR so that the chromophore becomes 10-*s*-*cis*. The possibility of a single bond twist in the chromophore of native BR, especially in the photocycle intermediates, has been raised to account for absence of a C14–C15/N–H coupling in the FT-IR [39]. Experiments to differentiate the occurrence or nonoccurrence of such side-chain single bond rotations, and hence solve the discrepancy are underway, e.g., measurements of the solid stated NMR ^{13}C chemical shifts of doubly ^{13}C-labeled retinals with the sulfate or azidophenyl branching before and after binding to the protein (Fig. 6B).

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