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Photoaffinity Labeling of Bacteriorhodopsin†

Wei-Dong Ding,‡ Athanasios Tsipouras,‡ Hyun Ok,‡ Toshihiro Yamamoto,‡ Mary Ann Gawinowicz,§ and Koji Nakanishi*,‡

Department of Chemistry, Columbia University, New York, New York 10027, and Protein Core Facility, College of Physicians and Surgeons, Columbia University, New York, New York 10027

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ABSTRACT: ^{14}C-Labeled optically pure 3*S*- and 3*R*-(diazoacetoxy)-*all-trans*-retinals were incorporated separately into bacterioopsin to reconstitute functional bacteriorhodopsin (bR) analogues, 3*S*- and 3*R*-diazo-bRs. UV irradiation at 254 nm generated highly reactive carbenes, which cross-linked the radiolabeled retinals to amino acid residues in the vicinity of the β-ionone ring. The 3*S*- and 3*R*-diazo analogues were found to cross-link, respectively, to cyanogen bromide fragments CN 7/CN 9 and CN 8/CN 9. More specifically, Thr121 and Gly122 in fragment CN 7 were found to be cross-linked to the 3*S*-diazo analogue. The identification of cross-linked residues and fragments favors assignments of the seven helices A-G-F-E-D-C-B or B-C-D-E-F-G-A to helices 1-2-3-4-5-6-7 in the two-dimensional electron density map (Henderson et al., 1975, 1986; Mogi et al., 1987). The present results show that the chromophore chain is oriented with the ionone ring inclined toward the outside of the membrane (the 9-methyl group also faces the extracellular side of the membrane).

Bacteriorhodopsin (bR),[1] a light-driven proton pump, is the sole protein found in the purple membrane of *Halobacterium halobium* (Oesterhelt & Stoeckenius, 1971; Stoeckenius & Bogomolni, 1982). It consists of a polypeptide of 248 amino acids (Ovchinnikov et al., 1979; Khorana et al., 1979) and a chromophore, *all-trans*-retinal, covalently bound to Lys216 on helix G via a protonated Schiff base linkage (Bayley et al., 1981; Lemke & Oesterhelt, 1981; Mullen et al., 1981) (Figure 1). There are seven discrete hydrophobic segments in the bR

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* To whom correspondence should be addressed.
‡ Department of Chemistry.
§ Protein Core Facility.

[1] Abbreviations: bR, bacteriorhodopsin; CN *x*, fraction *x* obtained upon cyanogen bromide cleavage of bacteriorhodopsin; 3*R* (or 3*S*) bR, bacteriorhodopsin regenerated from 3*R* (or 3*S*)-(diazoacetoxy)-*all-trans*-retinal; PTH, phenylthiohydantoin; SE, size exclusion; bO, bacterioopsin.

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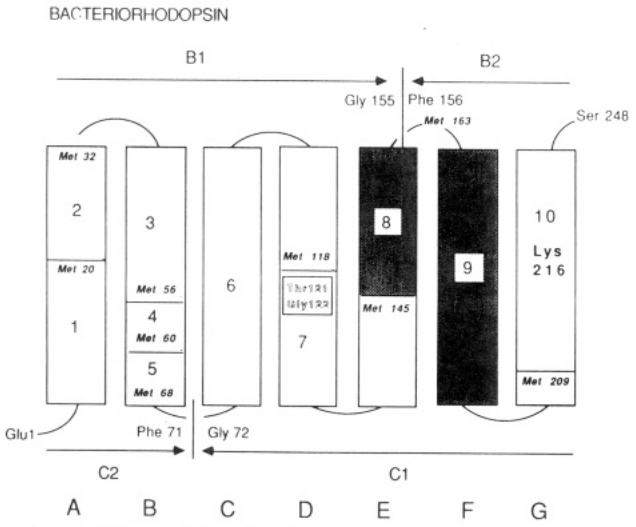


FIGURE 1: Schematic drawing of the amino acid sequence of bR and the seven α-helices, A–G. The cyanogen bromide cleavage sites (Met) are denoted by horizontal short lines. The numbers (1–10) on the sequence represent the 10 fragments due to CNBr cleavage. Chymotrypsin digestion site (Phe71/Gly72) and $NaBH_4$ cleavage site (Phe155/Gly156) are indicated with vertical lines. Thr121, Gly122, and CN 9 (shaded fragment) in 3*S*-diazo-bR and CN 8 and CN 9 fragments in 3*R*-diazo-bR contain the cross-linking sites.

sequence which are believed to form seven transmembrane α-helices (A–G, Figure 1) (Dunn et al., 1981; Engelman et al., 1980). Efforts to delineate the mechanism of proton translocation presuppose knowledge of the three-dimensional protein structure so that spatial relationships between chromophore and amino acid residues that are implicated in the process can be evaluated.

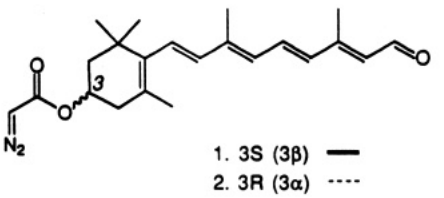
Two-dimensional electron density maps obtained by low-dose electron microscopy (Henderson et al., 1975) showed the existence of seven α-helices and the trimeric arrangement of bR in the purple membrane; the seven density rods that represent the seven helices are numbered 1–7 according to standard convention. A great deal of structural evidence concerning the assignment of the seven helices A–G to the seven rods and the position of the chromophore has been generated by further X-ray diffraction, neutron diffraction, and fluorescence energy transfer methods (Kouyama et al., 1981; Wallace & Henderson, 1982; Agard & Stroud 1982; Jubb et al., 1984; Engelman et al., 1986; Seiff et al., 1986; Heyn et al., 1988). Due to the low resolution (~3.5 Å) of the maps (Henderson, 1986), however, structural information on the folding of the seven helices, the position of the retinal chromophore, and the orientations of the individual amino acid residues cannot be extracted solely from the X-ray data.

Neutron diffraction of bR, reconstituted with deuterium-labeled C2 fragment (helices A and B), showed that the most probable assignments of A and B are positions 1 and 7, and to a lesser degree positions 6 and 7 (Trewhella et al., 1986). It was reported that helix G should be identified with helix 2 on the basis of the results from X-ray and electron diffractions (Wallace et al., 1982). Separate diffraction calculations (Trewhella et al., 1983), on the other hand, assigned helices F and G as helices 3 and 4, respectively. The amino acid segments comprising the seven α-helices have been proposed on the basis of the accessibility to proteolytic cleavage (Ovchinnikov et al., 1979), hydrophobicity considerations (Engelman et al., 1980), and photoaffinity labeling with (*m*-diazirinophenyl)retinal (Huang et al., 1982). The proposed secondary structures, while similar in general, are significantly different in some of the helices and remain to be verified.

The projected position of the retinal chromophore in the two-dimensional electron density map has been determined by neutron diffraction studies on bR regenerated from deuterated retinals (Heyn et al., 1986, 1988) and more recently by X-ray diffraction studies on bR analogues containing mercurio- and bromoretinals (Büldt et al., 1989). The retinal chromophore is fully elongated (Heyn et al., 1988) and tilted away from the membrane plane by 20–25° (Heyn et al., 1977; Earnest et al., 1986; Ikegami et al., 1987).

Photoaffinity labeling of bR with tritium-labeled (*m*-diazirinophenyl)retinal cross-linked Ser193 and Glu194 residues on helilx F (Huang et al., 1982). On the basis of the relative position of Lys216 with respect to the cross-linking sites, the chromophore was found to be inclined toward the outside of the membrane, and this led to the proposal of an amino acid composition for helix F, significantly different from those put forward earlier (Ovchinnikov et al., 1979; Engelman et al., 1980). Other evidence regarding the helical assignment came indirectly from the point charge models of both bR and halorhodopsin, in which Asp115 and helix D of bR are placed close to the β-ionone ring to provide the negative charge (Lanyi et al., 1988). Such evidence, combined with the knowledge gained from mutagenesis (Mogi et al., 1987, 1988; Hackett et al., 1987), will eventually lead to the understanding of spatial relationships between the side chains that undergo chemical and conformational changes during the proton translocation process.

We had previously shown that the bR analogue regenerated from 3-(diazoacetoxy)retinal is capable of pumping protons and that the binding site of the retinal analogue is not appreciably different from native retinal as judged from the nearly identical CD spectra of the two bR species (Sen et al., 1982). In the present photoaffinity labeling studies of bR, two optically pure (>95%) ^{14}C-labeled retinal analogues, 3*S*- and 3*R*-(diazoacetoxy)-*all-trans*-retinals (**1** and **2**) (Ok et al., 1988), have been incorporated into bR, and the amino acids and/or helices that become cross-linked to the β-ionone ring upon irradiation have been identified. This experimental evidence narrows the possible assignments of the seven α-helices to those depicted in Figure 6. Furthermore, the tilting direction of chromophore can be determined by inspecting the relative positions of Lys216 and cross-linking sites in the proposed secondary structures of bR. The orientation of the amino acids on the helices containing the cross-linked sites can be estimated under the assumption that the helix is rigid due to hydrogen bonding and, therefore, the relative orientations of the residues are fixed unless interrupted by amino acids such as proline. The findings resulting from the present photoaffinity studies, retinal analogues containing mercury (Büldt et al., 1989) and C-9 ω-sulfate chains (Park et al., 1989; showing distance of C-9 from membrane surface is ca. 15 Å), in combination with results from other laboratories lead to arrangements of helices and chromophores shown in Figures 5 and 6.



Materials and Methods

Reconstitution of Diazo-bR Analogues. 3*S*- and 3*R*-(diazoacetoxy)-*all-trans*-retinals were synthesized according to published procedures (Ok et al., 1988). bR was bleached

according to the method of Scherrer and Stoeckenius (1984). A 3 M excess of bacterioopsin suspended in distilled H_2O was incubated with diazo-*all-trans*-retinals in ethanol (final EtOH concentration: 0.5%) for 5 h to reconstitute diazo-bR analogues devoid of free retinal analogues, as ascertained by the absence of retinal UV absorption (reference cell: bO); the λ_{max}s of the dark-adapted 3*R*- and 3*S*-diazo-bR analogues were 535 and 528 nm, respectively.

White Membrane Vesicles. Retinal-deficient mutant cells of *H. halobium* (strain JW-2N) were cultured and the white membrane vesicles were prepared according to published procedures (Oesterhelt & Stoeckenius, 1982). The 3*R*- and 3*S*-diazoretinal analogues **1** and **2** and native retinals in ethanol (5 OD each at 380 nm, in 25 μL of ethanol) were added to white membrane (20 OD at 280 nm) suspended in 4 M NaCl (5 mL), and the reconstitution was allowed to proceed overnight. Proton pumping ability was qualitatively evaluated by irradiating the sample with a projector lamp and a 530-nm cutoff filter for 1 min and observing the pH changes in the media.

Low-Temperature UV Spectra of the Photointermediates. The absorption spectra of bR^{LA} analogues were obtained at room temperature by irradiating the dark-adapted bR analogues in water suspension at 530 nm with a projector lamp for 10 min. Hydrated films of the bR analogues were prepared by standard procedures (Dollinger et al., 1986). UV spectra were recorded on a Perkin-Elmer IF320 UV spectrophotometer. The K and M intermediates for both 3*S* and 3*R* analogues were obtained at 70 and 250 K, respectively, while L intermediates were not observed at low temperature. Low temperatures were achieved by compressor Model SC from CTI-CRYOGENICS and regulated by a Model 4025 cryogenic thermometer/controller. Once the temperature was stable, a spectrum was obtained and stored (light-adapted bR at 70 K, for example). The sample was then irradiated with >530-nm light for 20 min to give the spectrum of the resulting mixture of light-adapted bR and K intermediate.

Irradiation of the Diazoacetoxy Group and Cross-Linking Percentage. Activation of the 3-(diazoacetoxy) group can be monitored from the disappearance of its strong absorption around 250 nm (ε, 16 000 in ethanol). The diazo group is also dissociated by irradiation of its weak absorption at 300 nm, but at a very slow rate (Bayley, 1983). Diazo-bR was suspended in distilled water in the sample cell. bO of the same concentration was used as reference to cancel the 280-nm absorption from protein. Both sample and reference cells were irradiated with a low-pressure mercury lamp (New England Ultraviolet) with 254-nm narrow band emission under argon while stirred by a small magnetic bar. UV–vis absorption spectra were recorded at various intervals during irradiation (Figure 2a). The 250-nm band completely disappeared after 12 min. The percentage of cross-linking was calculated from the total radioactivity of bound retinal in 3 μg of protein as compared to the radioactivity associated with the protein after irradiation; the latter was determined by SDS gel electrophoresis (glycerol) of cross-linked samples (~3 μg loading) and radioactivity counting of protein bands, which are free of non-cross-linked retinals. The percentage of cross-linked retinals was 25–30% (Figure 2b).

SDS Gel Electrophoresis (Glycerol). Glycerol polyacrylamide gel (0.075 cm, analytical) electrophoreses were run according to published procedures (Swank, 1971). Under these conditions, the Schiff base bond between the protein and the retinal is completely cleaved, and therefore only cross-linked retinal migrates with the protein. bR sample bands were

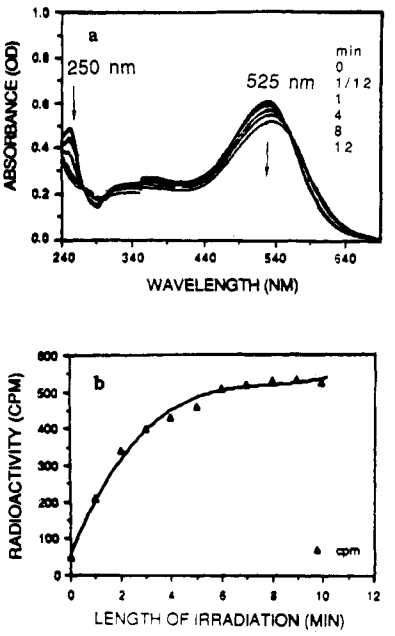


FIGURE 2: (a) UV–vis spectra of 3*S*-diazoacetoxy-bR in distilled water taken after various lengths of irradiation (bleached bR-opsin was used as reference). The decrease of the 250-nm diazo band followed first-order kinetics and stopped after 12 min of irradiation. (b) Radioactivity of bR versus irradiation time. bR samples were separated at different time intervals from un-cross-linked retinals by gel electrophoresis, and their radioactivities were counted.

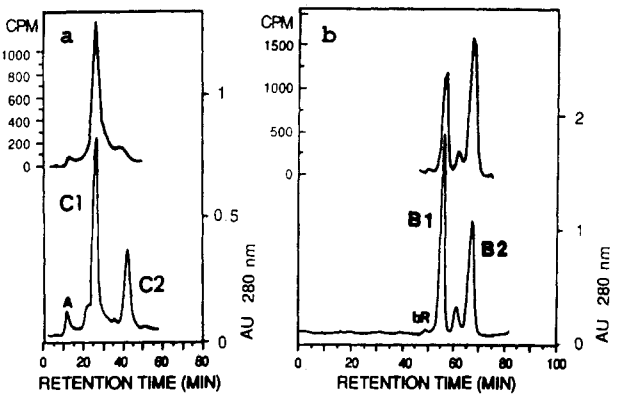


FIGURE 3: (a) HPLC profile of the chymotryptic fragments C1 and C2 (3*S*) and the radioactivity counting (in cpm) of HPLC fractions collected at 1-min intervals. (Peak A) Protein aggregation. Column: TSK G3000 + 2000SW in tandem arrangement. Eluent: 0.1% TFA, 70% EtOH in H_2O. Flow rate: 1 mL/min. Detection: 280 nm. (b) HPLC profile of the $NaBH_4$-digested fragments B1 and B2 (3*S*) and the radioactivity counting of HPLC fractions collected at 1-min intervals. Preparative TSK G3000SW column and an organic solvent system (0.1% TFA, $CHCl_3$/MeOH/H_2O, 2:5:2). Flow rate: 2 mL/min. Detection: 280 nm.

visualized by staining the gels with Coomasie blue and destaining with 10% aqueous acetic acid. The bR sample bands were sliced and dissolved in 0.5 mL of aqueous 30% H_2O_2 at 60 °C before they were mixed with 10 mL of Aquasol (from NEN) for radioactivity counting.

Chymotrypsin Digestion. The bleached diazo-bR analogues (4 mg) were digested with chymotrypsin (Sigma) (1/100 w/w) according to published procedures (Khorana et al., 1982) to yield the chymotryptic fragments C1 (72–248) and C2 (1–71) (Figure 1). C1 and C2 (0.8 mg) (only those from 3*S* are shown) were purified by size exclusion HPLC on a Tandem G3000/G2000 semipreparative column and 70% aqueous EtOH containing 0.1% TFA as solvent (Figure 3a). A preparative TSK G3000SW column and an organic solvent system (0.1% TFA, $CHCl_3$/MeOH/H_2O 2:5:2) were used for

the 3*R* analogue (figure not shown). Comparison of the integrated area for the C1 and C2 peaks, with that predicted from the known extinction coefficients of these fragments, suggested that cleavage proceeded to an extent greater than 95%. The un-cross-linked retinal analogues, retinal oximes, and lipids eluted later and were well separated from C1 and C2 under these conditions (retention time: ~75 min).

Radioactivity Counting. Fractions of 2-mL aliquots were collected. Radioactivities (cpm, 200 μL from each fraction) were plotted vs fraction numbers (Beckman LS 3801). The radioactivity counting showed that more than 95% radioactivity was associated with the C1 fragment while <4% was found on C2 (Figure 3a).

Sodium Borohydride Cleavage. The reported procedures (Huang et al., 1982) were followed for the sodium borohydride cleavage of the radioactive bR analogues except that they were preceded by bleaching with hydroxylamine. It was found that bleaching of the analogues prevented the reduction of the Schiff base linkage, which leads to covalent binding of retinal to Lys216. The fragments contained no retinal covalently bound to Lys216 as ascertained by the absence of 330-nm absorption (polyene absorption) in B2 and the absence of radioactivity on the fragment CN 10 (209–248) that contains Lys216. The cleavage occurred at the same site whether the bR was bleached or not, as confirmed by amino acid analyses of B1 (1–155) and B2 (156–248) fragments (Figure 1).

CNBr Cleavage. Purified bR fragments (C1, C2, B1, and B2) were further cleaved into smaller fragments (Gerber, 1982; Gross, 1980) at the methionine residues by incubating with cyanogen bromide (molar ratio: CNBr/Met 2500/1). The excess CNBr and the coproduct MeSCN were removed by lyophilization. Retinals were separated from the peptides due to the cleavage of the ester bond under these conditions.

Purification of CNBr Fragments. Reverse-phase (RP) HPLC was used to purify the CNBr fragments since the low specific radioactivity on the fragments made detection difficult on polyacrylamide electrophoresis gels. The Vydac C_{18} protein and peptide column showed low sample adsorption and good resolution (Tarr & Crabb, 1983). A YMC ODS of 300 Å was also used and found to be equally effective. Two solvent gradients were employed with RP HPLC: Gradient I was used for fragments with molecular weights (M_r) up to 5000, as in the case of CN 6 or CN 9; gradient II was used for fragments with M_r lower than 3000. (Gradient I: 10 min at 30% EtOH, 50 min at 30–100% EtOH, 10 min at 100% EtOH. Gradient II: 10 min at 15% EtOH, 50 min at 15–70% EtOH, 10 min at 70% EtOH.) CNBr fragments thus purified were identified by amino acid analysis.

Results

Diazo Analogue Formation. Reconstitutions of the diazo-bR analogues (3*S* and 3*R*) with excess opsin (3:1, molar) resulted in all the retinal analogue being bound to the opsin as indicated by the absence of retinal absorption in the UV–vis spectrum using opsin as reference (Figure 2a). Table I shows the absorption maxima of the photointermediates, bR^{LA}, bR^{DA}, K, and M. Proton pumping efficiencies for white membrane vesicles that are incubated with all-trans native, 3*R*, and 3*S*, and without retinals are listed in Table I. They were qualitatively measured as ΔpH after 1 min of irradiation.

Photointermediate. The difference absorption spectrum showed the presence of approximately 20% K intermediate, with λ_{max}s of 590 nm for 3*R* and 610 nm for 3*S*. As much as 90% of the bR^{LA} analogues were converted to the M intermediate at 250 K, with λ_{max} of 410 nm for 3*R* and 402 nm for 3*S* (Table I). L intermediates of neither 3*S* nor 3*R* can be trapped at 150 K, where native L is stable (550 nm for native L). A possible explanation is that the diazo L intermediate is too unstable; it quickly converts to M intermediate (a substantial amount of M was trapped at 150 K).

Table I: Absorption Maxima and Proton Pumping Abilities of Various bR Species

	native	*R*	*S*	control[a]
λ_{max} (DA) (nm)	558	535	525	
λ_{max} (LA) (nm)	570	545	538	
λ_{max} (K) (nm)	610	590	610	
λ_{max} (M) (nm)	412	410	402	
ΔpH[b]	0.030	0.030	0.032	0.00

[a] White membrane vesicles without retinal analogues. [b] pH change in 1 min in the medium containing white membrane vesicles incubated with *all-trans*-retinals or analogues. Control: white membrane without retinals.

Carbene Formation and Cross-Linking Percentage. The diazoacetoxy group in bR has a strong absorption at 250 nm in H_2O suspension. Irradiation with 254-nm light resulted in a decrease of the 250-nm band and the decrease in chromophore absorption (525 nm for 3*S*). The dissociation of the diazoacetoxy group followed first-order kinetics ($t_{1/2}$, 55 s). No further change of the intensity of the 250-nm band was observed by irradiation beyond 12 min (Figure 2a). The radioactivity associated with the protein versus length of irradiation is shown in Figure 2b. As can be seen, after 10 min of irradiation the protein radioactivity reached a plateau, where the percentage of cross-linked radioactivity amounted to 25–30% of the total.

Chymotryptic and $NaBH_4$ Fragments. The C1 (72–248) and C2 (1–71) fragments were well separated on size exclusion HPLC (Figure 3a, 3*S*). Some aggregation was observed with Tandem G3000/G2000 semipreparative columns and 70% aqueous EtOH containing 0.1% TFA as solvent. The C1 fragment from 3*S* contained 96% of the radioactivity of bR, and that from 3R contained 95% (data not shown). The C2 fragments from neither 3*R*- nor 3*S*-retinals contained appreciable amounts of radioactivity (Figure 1).

The B1 (1–155) and B2 (156–248) fragments from 3*S* were both found to be radioactive (Figure 1) and contained 36% and 60% of the radioactivity on the protein (Figure 3b) (only 3*S* is shown). The B1 and B2 from 3*R* were also both radioactive, their radioactivities accounting, respectively, for 34% and 66% of the total. Both 3*S* and 3*R* bRs should therefore contain at least two cross-linking sites (at least one each on B1 and B2). The use of chloroform-containing solvent and a preparative TSK Gel G3000SW column enabled the sodium borohydride B1 and B2 fragments to be purified with minimal aggregation and sample adsorption (Figure 3b). Large-scale fragment preparation and radioactivity counting can also be quickly achieved on size exclusion HPLC.

Cyanogen Bromide Cleavage. B2 (156–248) gives three fragments, CN 8″ (156–163), CN 9 (164–209), and CN 10 (210–248) (Figure 1). They were well separated by reverse-phase HPLC on a Vydac C_{18} protein and peptide column with 280-nm detection and solvent gradient I (retention times: CN 8″, 5.6 min; CN 9, 47 min; CN 10, 34 min). Only CN 9 was found to be radioactive both in 3*S* and 3*R*. The formic acid peak contained a significant amount of radioactivity, presumably due to the drop of the radioactive acetoxy group from the cross-linked residues, which is hydrophilic and would travel together with formic acid. Conceivably, the location of CN 8″ in the loop prevented it from being cross-linked.

Figure 4a shows the RP HPLC profile of CNBr fragments of radioactive B1 (3*S*) and radioactivity counting. Only ra-

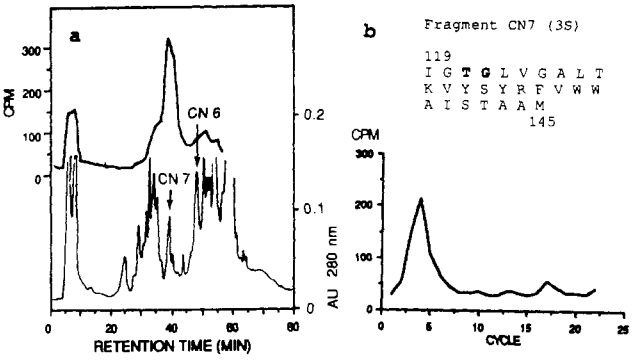


FIGURE 4: (a) HPLC profile of the CNBr fragments of B1 (3*S*) and the radioactivity counting of HPLC fractions collected at 2-min intervals. Column: Vydac C_{18}. Eluent: 0.5% HCO_2H, H_2O/EtOH gradient (10 min, 30% EtOH; 50 min, 30–100% EtOH; 10 min, 100% EtOH). Flow rate: 0.7 mL/min. Detection: 280 nm. (b) Edman degradation of CN-7 (3*S*) and the radioactivity counts (cpm) of the cycles. The bold letters denote the amino acids that contained radioactivity.

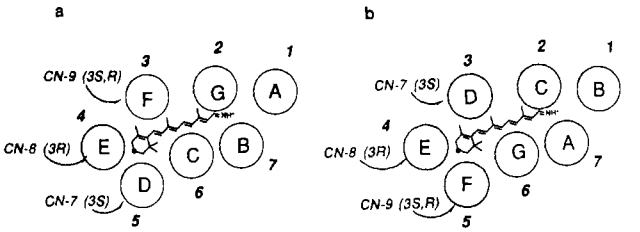


FIGURE 5: Schematic representations of the seven density rods in the 2D electron density map, viewed from the inside of the purple membrane (Henderson, 1975). The numbering of the seven rods is in the standard convention. The two favored assignments of the seven helices are based on the photoaffinity labeling of 3*S* and 3*R* bRs. The locations of the β-ionone ring and the nitrogen of Lys216 are adopted from X-ray and neutron diffraction results (Heyn, 1988; Büldt et al., 1989).

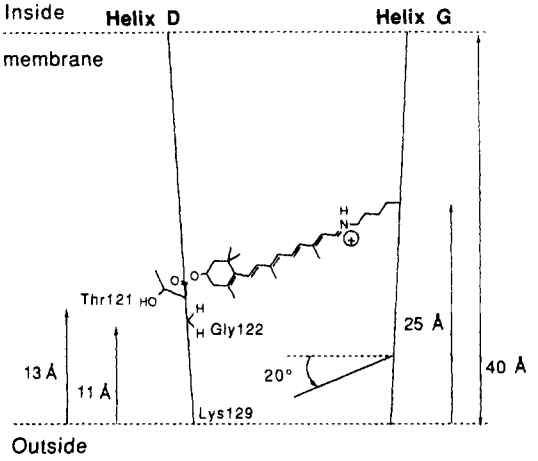


FIGURE 6: Chromophore orientation estimated from the tilt angle (Heyn, 1977; Rothschild, 1986; Ikegami, 1987), the position of Lys216 (Huang et al., 1982; Ovchinnikov et al., 1979), and the length of the diazo *all-trans*-retinal (23 Å) and by placing the carbene equidistant from Thr121 and Gly122. The 15-Å distance of C-9 from the membrane outside surface (Park et al., 1989) is also in accordance with this figure.

dioactive peaks were collected and analyzed. CN 7 (Figure 1) was well separated from other fragments and was found to have most of the radioactivity that was associated with B1. CN 6 did not contain radioactivity, thus being excluded as the cross-linking site. The radioactivity counting of the CNBr fragments of B1 derived from the corresponding 3*R* analogue indicated that the CN 7 was not labeled; in this case, most of the radioactivity was found among several peaks including CN 8 (146–163), which, however, were not resolved on RP HPLC. (3*R*)-CN 8 from the CNBr fragments of C1 was therefore first collected on SE HPLC, and the radioactive peak (CN 8) was further purified on RP HPLC with gradient II (data not shown).

Edman Degradation. Edman degradation of CN 7 from 3*S* bR was carried out on an Applied Biosystems Model 470A gas-phase sequencer equipped with the Model 120A PTH analyzer. Approximately 40% of each PTH amino acid in 20% acetonitrile was injected automatically into the PTH analyzer, and the remainder was collected in a fraction collector. The collected fractions (200 μL) were counted for radioactivity as described under Materials and Methods. Radioactivity at cycles 4 and 5 indicated cross-linking at Thr121 and Gly122 (Figure 4b). The radioactivity in cycles 3–5 accounts for about 25% of the total subjected to the Edman degradation analysis. The filter in the sample chamber also contained 12% of the total radioactivity. Although 3*S* bR labeled CN 8 (helix E), while 3*R* bR labeled CN 8 and CN 9 (helices E and F), Edman degradation of these fractions gave no clear-cut results because of the low level of radioactivity and presumably the amino acids were too far from the N terminus.

Discussion

Binding Site of the (Diazoacetoxy)retinals. The existence of a photocycle and efficient proton pumping of the 3*S*- and 3*R*-(diazoacetoxy)-bR analogues strongly suggests that the binding site of (diazoacetoxy)retinal is not significantly different from that of the native chromophore. This did not come as a surprise since the addition of the relatively small and linear diazoacetoxy group to the β-ionone ring was not expected to cause great steric interaction with the protein. In the following, the photoaffinity data will be interpreted by assuming the diazoretinal is in the same binding site as the native retinal.

Assignment of the Seven Helices. The cross-linking sites were found to be present in CN 7 and CN 9 in 3*S* bR and in CN 8 and CN 9 in 3*R* bR (Figure 1). Spatial consideration of the helices favors the assignment of helix G to 2, 3, or 6. Other assignments for helix G are excluded because they would put helices D, E, and F, in which CN 7, CN 8, and CN 9 are located, too far away to be cross-linked by the same chromophore. The two assignments (Figure 5) are supported by our photoaffinity labeling results and are in agreement with neutron and X-ray diffraction results of the chromophore position (Heyn, 1988; Büldt et al., 1989). The fact that multiple cross-linkings were observed for both the 3*S* and 3*R* isomers is probably caused by conformational flexibility of the apoprotein or the chromophore (Sheves et al., 1984).

Chromophore Orientation (Figure 6). As mentioned above, the 3*S* analogue of retinal 3-diazoacetate cross-linked to Thr121 and Gly122 on helix D. On the basis of the proposed secondary structures (Ovchinnikov et al., 1979; Engelman et al., 1980; Huang et al., 1983) this result suggests an orientation of the chromophore in which the β-ionone moiety should be inclined toward the outside of the purple membrane relative to the protonated Schiff base terminal (Figure 6). This is because Thr121/Gly122 is aligned closer to the outside surface of the purple membrane than Lys216. This inclination is in good agreement with that previously reported (Huang et al., 1982). Recent proton translocation results of bR reconstituted from a series of retinal analogues having ω-sulfate groups suggested that the 9-Me and 13-Me groups are directed toward the outside of the membrane and that C-9 is approximately 15 Å from the outer surface of the membrane (Park et al., 1989). The retinal moiety in Figure 6 is depicted in accordance with these results.

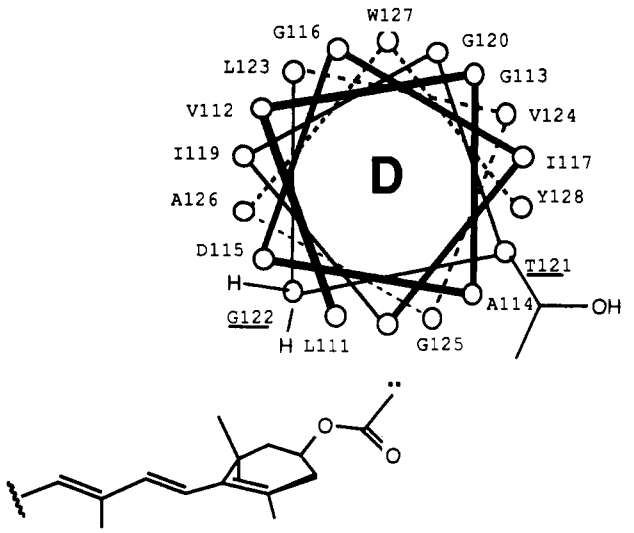


FIGURE 7: Amino acid orientations of helix D with respect to the chromophore carbene. The carbene is placed between the residues Thr121 and Gly122.

Secondary Structure of Helix D. Since both Thr121 and Gly122 were cross-linked, it can be proposed that the carbene is situated halfway on the line connecting the two residues. The distances between Thr121 and Gly122 and the surface of the outer membrane are determined to be 13 and 11 Å, respectively (Figure 6). They are not significantly different from what is calculated from the secondary structures of helix D proposed either by Khorana or by Engelman (about 13.5 and 12 Å for Thr and Gly, respectively). Lys129 instead of Val130 can be placed on the surface of the membrane.

Amino Acid Orientations on Helix D. The labeling of Thr121 and Gly122 by 3*S* bR suggests that the amino acid orientations in helix D, with respect to the carbene, are as depicted in Figure 7.

Present results with functional bRs radiolabeled with 3*R* (or 3*S*)-(diazoacetoxy)-*all-trans*-retinals have led to the identification of two specific amino acids and three other CNBr fragments and have contributed to our understanding of the tertiary structure of the pigment. However, the present study with the [^{14}C](diazoacetoxy)retinals suffered from the inherently low specific activity of ^{14}C (8.8 mCi/mmol) coupled with the loss of radioactivity accompanying each Edman degradation cycle; as a result, the cross-linked amino acid residues from only one of the four radiolabeled CNBr fragments could be characterized. The studies originally started as a collaboration with the group of Professor G. Khorana several years ago. The radiolabeled bR prepared from *dl*-[^{14}C]-3-(diazoacetoxy)retinal was sent to the MIT lab, but after a chymotrypsin digest and iodosobenzoic acid cleavage the radioactivity level became too low for amino acid characterization. The radiolabel was retained on a fragment corresponding to the CN 9 in the present studies. Current efforts are focused on developing photoaffinity probes of [^{3}H]retinals that can be more readily prepared (Boehm et al., 1990).

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Transmembrane Location of Retinal in Bacteriorhodopsin by Neutron Diffraction†

T. Hauss, S. Grzesiek, H. Otto, J. Westerhausen, and M. P. Heyn*
Biophysics Group, Department of Physics, Freie Universität Berlin, Arnimallee 14, D-1000 Berlin 33, Federal Republic of Germany

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ABSTRACT: The transmembrane location of the chromophore of bacteriorhodopsin was obtained by neutron diffraction on oriented stacks of purple membranes. Two selectively deuterated retinals were synthesized and incorporated in bacteriorhodopsin by using the retinal⁻ mutant JW5: retinal-d_{11} (D11) contained 11 deuterons in the cyclohexene ring, and retinal-d_5 (D5) had 5 deuterons as close as possible to the Schiff base end of the chromophore. The membrane stacks had a lamellar spacing of 53.1 Å at 86% relative humidity. Five orders were observed in the lamellar diffraction pattern of the D11, D5, and nondeuterated reference samples. The reflections were phased by D_2O–H_2O exchange. The absolute values of the structure factors were nonlinear functions of the D_2O content, suggesting that the coherently scattering domains consisted of asymmetric membrane stacks. The centers of deuteration were determined from the observed intensity differences between labeled and unlabeled samples by using model calculations and Fourier difference methods. With the origin of the coordinate system defined midway between consecutive intermembrane water layers, the coordinates of the center of deuteration of the D11 and D5 label are 10.5 ± 1.2 and 3.8 ± 1.5 Å, respectively. Alternatively, the label distance may be measured from the nearest membrane surface as defined by the maximum in the neutron scattering length density at the water/membrane interface. With respect to this point, the D11 and D5 labels are located at a depth of 9.9 ± 1.2 and 16.6 ± 1.5 Å, respectively. The chromophore is tilted with the Schiff base near the middle of the membrane and the ring closer to the membrane surface. The vector connecting the two label positions in the chromophore makes an angle of 40 ± 12° with the plane of the membrane. Of the two possible orientations of the plane of the chromophore, which is perpendicular to the membrane plane, only the one in which the N→H bond of the Schiff base points toward the same membrane surface as the vector from the Schiff base to the cyclohexene ring is compatible with the known tilt angle of the polyene chain.

Bacteriorhodopsin (BR)[1] is a membrane-bound protein from *Halobacterium halobium* which functions as a light-driven proton pump. The primary event of absorption occurs at the chromophore, which consists of retinal bound via a protonated Schiff base to lysine 216. Following absorption, a photocycle takes place in which the pigment goes through several spectral intermediates before returning to the ground state in about 10 ms. During the first part of the cycle the Schiff base is deprotonated and a proton is released to the extracellular medium. In the course of the second part a proton is taken up from the cytoplasm and the Schiff base is reprotonated. Recent optical and electrical measurements show that one proton is translocated per cycle (Drachev et al., 1984; Grzesiek & Dencher, 1986; Braun et al., 1988; Kouyama et al., 1988b), making it very likely that the Schiff base proton is directly involved in the proton translocation. Associated with the photocycle is a cyclic trans–cis isomerization at the 13–14 double bond of the chromophore. The interaction of the chromophore with charges in the protein environment and the protonation state of the Schiff base are the main factors involved in the color regulation of the pigment. In all of these

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[1] Abbreviations: PM, purple membrane; BR, bacteriorhodopsin; Pro-BR, D5-BR, and D11-BR, BR regenerated with native retinal, retinal-d_5, and retinal-d_{11}, respectively; D5, retinal-d_5; D11, retinal-d_{11}.

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