Attachment site(s) of retinal in bacteriorhodopsin
(halobacteria/purple membrane/tertiary structure/chromophore/borohydride reduction)

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ABSTRACT  After chemical reduction of the retinylidene-lysine Schiff base linkage in bacteriorhodopsin, the retinyl residue is covalently attached to Lys-216 (with a possible minor fraction on Lys-172) or to both Lys-216(172) and Lys-40(41). The linkage site (up to 100% on Lys-216; up to 70% on Lys-40(41)) depends on whether the sample is reduced in the light or dark, whether the sample is light or dark adapted, and on temperature. Absorbance and circular dichroism spectra indicate that the retinyl residue is in its original binding site after reduction in the light. Thus, the different attachment sites may reflect changes that occur during the photoreaction cycle or during light/dark adaptation, or the reduction of accidental physiologically irrelevant Schiff base linkages to lysines close to the normal linkage in the structure of bacteriorhodopsin. In either case, the retinal does not leave its binding site. This last point severely limits the possible arrangements of the amino acid sequence in the bacteriorhodopsin tertiary structure and clearly distinguishes two models that are consistent with all criteria.

A crucial feature of any detailed model of bacteriorhodopsin (bR) is the location of the retinylidene chromophore and its interaction with the surrounding amino acid residues. Bridgen and Walker determined an attachment site for retinal in bR (1). However, later investigations appeared to conflict with their assignment (unpublished). We have therefore reinvestigated this question. We have sought to identify the residue(s) to which the retinyl moiety is attached, and to quantitate the degree of labeling, using both fluorescence from the unmodified peptides and radioactivity from [3H]retinal incorporated in regenerated bR.

MATERIALS AND METHODS

Materials. Hydroxylamine hydrochloride (Matheson), all-trans-retinal (Eastman), cyanogen bromide (Eastman), sodium borohydride, bovine serum albumin ("albumin"; fraction V, fatty acid free), chymotrypsin (CT; type 1-S from bovine pancreas), glycylglycine, Tris, 2-(N-cyclohexylamino)ethanesulfonic acid (Ches), Hepes, Sephadex LH-60 (all from Sigma), and sodium cyanoborohydride (Alfa recrystallized grade) were used as purchased. Retinal stock solutions were stored in argon-saturated solvents (methanol or ethanol) at -90°C. Tritiated retinal (isomeric mixture), labeled at the methyl groups attached to C9 and C13 by base-catalyzed exchange, was the generous gift of W. Hubbell. The specific activity of the 1.8 mM ethanolic stock solution was 0.68 Ci/mmol. (1 Ci = 3.7 x 10^{10} becquerels).

Purple membrane (pm) suspensions were prepared from *Halobacterium halobium* [Rf strain, or JW-3 (formerly ET1001), a ruberin-free strain provided by J. Weber] by standard procedures (2). The suspensions were stored either at 4°C in the presence of 0.1% NaNO_{3} or as flash-frozen suspensions in 40% aqueous sucrose solution (which prevented aggregation).

Preparation of [3H]-Labeled pm. pm was bleached with hydroxylamine (3) and was either regenerated immediately or cleaved with CT prior to regeneration (4). In a typical regeneration, 1.7 μmol of the apomembrane (still containing retinal oxime) was suspended to a volume of 22 ml with distilled water, 197 μl (360 nmol) of [3H]retinal stock solution was added, and the system was allowed to regenerate for 1 hr, in the dark, at room temperature. Next, 253 μl (1.43 μmol) of nonradioactive all-trans-retinal in methanol was added, and regeneration was continued in the dark, overnight, at 4°C.

Unreacted retinal and retinal oxime were removed by washing the regenerated pm four times with 30 ml of 2% (wt/vol) albumin in 100 mM Hepes, pH 7.4, or in distilled water. Residual albumin was removed by washing two to four times with the same volume of buffer or distilled water. The final sample showed no retinal oxime absorbance at 370 nm. Control washes with albumin prior to regeneration did not affect the retinal oxime absorbance. Final regeneration of uncleaved samples was 95%, based upon the ratio of absorbances at 280 and 568 nm. Regeneration efficiencies for CT-cleaved samples were lower, typically 60–70%.

Liquid scintillation counting of the final suspension showed that 53% of the [3H] initially added was present in the final sample. This is a reasonable recovery, given that the wash supernatants contained small amounts of un pelleted material, and that the labeled retinal contained some isomers that do not regenerate the apomembrane (5).

The final labeled samples were prepared by diluting the labeled stock with unlabeled pm prepared by a parallel procedure.

Reduction of pm. Reduction in the light was performed with 1% (wt/vol) sodium borohydride essentially as described (6), but using 0.1 M Ches buffer, pH 10 (neutralized with NaOH). For reduction in the dark, pm was cleaved with CT before or after regeneration and was dark adapted for at least 1 hr at 37°C. Enough sodium borohydride was added to give a 2% (wt/vol) solution, and the reaction was allowed to proceed in the dark. The product was washed once with distilled water in dim red light. After a second wash, residual bR (typically 30%) was bleached with hydroxylamine.

To reduce light-adapted CT-cleaved pm in the dark, a distilled water suspension was light adapted for at least 15 min prior to addition of Ches buffer and borohydride. The reaction was stopped after 2 hr by centrifuging and resuspending the pellets in 0.2 M Hepes, pH 7.5, to decompose any remaining borohydride. The samples were washed twice with distilled water, and

Abbreviations: bR, bacteriorhodopsin; Ches, 2-(N-cyclohexylamino)ethanesulfonic acid; CT, chymotrypsin; pm, purple membrane; RP, retinyl protein.

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Sodium Cyanoborohydride Reduction. Native pm (2 mg) was suspended in 10 ml of 0.2 M glycylglycine, pH 3, and 162 mg of sodium cyanoborohydride was added. The reaction was allowed to proceed, with stirring and illumination, for 3 hr. The reaction product was washed twice with distilled water.

Measurement of Photocycle Intermediate M. The steady-state concentration of M was measured under 0°C reduction conditions (omitting BH4-) as absorbance change at 412 nm (7), using extinction coefficients of 4.5 × 10^4 M^-1 cm^-1 for M (at 412 nm) (8) and 6.3 × 10^4 M^-1 cm^-1 for Br (at 568 nm) (9).

Papain Cleavage (10). pm, regenerated from CT-cleaved apomembrane with [3H]retinal and reduced in the light at 0°C, was resuspended in 10 ml of 10 mM EDTA at pH 7, 0.1 ml of 70 mM 2-mercaptoethanol, and 10 ml of 50 mM cysteine-HCl (freshly prepared), and the pH was adjusted to 6.5 with 1 M NaOH.

Papain (Sigma, activity 12 units/mg), dissolved at 0.1 mg/ml in the same buffer, was added at 140 µl/ml of membrane suspension, and the preparation was incubated at 37°C. After 2 hr, phenylmethylsulfonyl fluoride was added to the preparation (10 µM final concentration), and the product was washed four times with distilled water.

RESULTS

Chymotryptic cleavage of hydroxylamine-bleached pm yielded two fragments migrating with apparent molecular weights of 19,000 (CT I, amino acids 72-248) and 7000 (CT II, amino acids 1-71) (numbering of ref. 11), as determined by NaDodSO4/polyacrylamide gel electrophoresis. The separation of these two fragments on a Sephadex LH-60 column is shown in Fig. 1A. In the light at 0°C (pH 10), reduction of pm (native or CT-cleaved) links radioactivity and fluorescence to CT I only (Table 1 and Fig. 1B). The photo-steady-state fraction of M was 10% under these conditions. At 25°C the M intermediate is present at a much lower photo-steady-state concentration, and the fraction of retinyl on CT I is only 60%; 40% is attached to CT II.

Reduction of pm in the dark (to eliminate photocycling) was possible only after CT cleavage. In dark-adapted pm, most of the retinyl (70%) was linked to CT II, with only 30% on CT I. There was no dependence on temperature (0°C or 25°C) or on whether the membranes were CT cleaved before or after regeneration (data at 25°C only).

Light-adapted pm, reduced in the dark at 25°C, gave essen-

![Diagram](image.png)

**Fig. 1.** Sephadex LH-60 fractionation of reduced CT-cleaved pm. Fractionation was as described for pm (4), except 96% (vol/vol) formic acid and a formic acid to ethanol ratio of 2.5:7 (vol/vol) were used. Thirty milligrams of protein was fractionated; 2 ml fractions were collected. (A) Elution profile of the material absorbing at 280 nm; (B) ^3H radioactivity (Beckman LS-233 scintillation counter) for light-reduced (0°C) pm (----), and dark-adapted dark-reduced pm (-----).

**Table 1.** Location of retinyl under various reducing conditions

<table>
<thead>
<tr>
<th>bR type*</th>
<th>Temp., °C</th>
<th>Reducing agent</th>
<th>Time of reduction, min</th>
<th>pH</th>
<th>Location of retinyl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CT I</td>
</tr>
<tr>
<td><strong>Illuminated during reduction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RG-RD-CT(LA)</td>
<td>0</td>
<td>BH4^-</td>
<td>30</td>
<td>10</td>
<td>1.00</td>
</tr>
<tr>
<td>RD-CT(LA)</td>
<td>0</td>
<td>BH4^-</td>
<td>60</td>
<td>8.6-9.1</td>
<td>+^</td>
</tr>
<tr>
<td>RG-CT-RD(LA)</td>
<td>0</td>
<td>BH4^-</td>
<td>30</td>
<td>10</td>
<td>1.00</td>
</tr>
<tr>
<td>CT-RG-RD(LA)</td>
<td>0</td>
<td>BH4^-</td>
<td>30</td>
<td>10</td>
<td>1.00</td>
</tr>
<tr>
<td>RG-RD-CT(LA)</td>
<td>25</td>
<td>BH4^-</td>
<td>150</td>
<td>9.0</td>
<td>0.60</td>
</tr>
<tr>
<td>RD-CT(LA)</td>
<td>25</td>
<td>BH4^-CN^-</td>
<td>150</td>
<td>3</td>
<td>+^</td>
</tr>
<tr>
<td><strong>Reduced in the dark</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT-RG-RD(LA)</td>
<td>25</td>
<td>BH4^-</td>
<td>2 × 120</td>
<td>10</td>
<td>0.55</td>
</tr>
<tr>
<td>CT-RG-RD(DA)</td>
<td>0</td>
<td>BH4^-</td>
<td>1.2 × 10^4</td>
<td>10</td>
<td>0.33</td>
</tr>
<tr>
<td>RG-CT-RD(DA)</td>
<td>25</td>
<td>BH4^-</td>
<td>4 × 10^5</td>
<td>10</td>
<td>0.33</td>
</tr>
<tr>
<td>CT-RG-RD(DA)</td>
<td>25</td>
<td>BH4^-</td>
<td>4 × 10^5</td>
<td>10</td>
<td>0.30</td>
</tr>
</tbody>
</table>

* Order of abbreviations indicates order of operations: chymotrypsin (CT), regeneration (RG), and reduction (RD); LA and DA indicate light-adapted and dark-adapted.

^ Checked only with fluorescence, because sample not regenerated.

the entire procedure was repeated. As before, the samples were bleached with hydroxylamine prior to subsequent manipulations.
lution, and in a slice of polyacrylamide gel (data not shown), and for CT I from RP<sub>350</sub> contained in a gel slice show the same maxima (Fig. 2B). Therefore, the fluorescence associated with peaks on gels derives from the retinyl moiety.

The Attachment Site(s). The CT-RG-RD sample, reduced in the light at 0°C (Table 1, entry 4), which resulted in linkage of the retinyl residue to CT I only, was used to determine the position of the retinyl linkage in CT I. CNBr treatment of CT I produced fragments (4) CNBr I–V, starting at the NH<sub>2</sub> terminus. Separation of the radioactive components of these fragments on Sephadex LH-60 is shown in Fig. 3. The radioactivity in the void volume can be attributed to aggregated peptides. Therefore, the retinyl residue is predominantly in one fraction, labeled V in Fig. 3. The amino acid composition of this fraction is most compatible with assignment to amino acids 210–246 (CNBr V; α = ±1.14 amino acids) or to amino acids 164–209 (CNBr IV; α = ±1.53 amino acids) (Table 2, data columns 1 and 2). The high standard deviations indicate that the radioactive fraction is contaminated with other peptides. To resolve this ambiguity, the 18 COOH-terminal amino acids of bR were removed with papain prior to separation of the CT fragments. The retention volume of the resulting radioactive CNBr fraction was increased (V' in Fig. 3). The amino acid composition of this fraction, reported as residues found (expected), agrees satisfactorily only with the predicted sequence of the modified CNBr V; α = ±0.47 amino acids (Table 2, data column 3).

Both observations are consistent only with assignment of this labeled peptide to CNBr V. The retinyl residue in RP<sub>350</sub> is linked to the ε-amino group of a lysine residue (13, 14); the site of linkage therefore can only be Lys-216.

The minor radioactive CNBr fraction (Fig. 3) is insensitive to papain treatment, and its amino acid composition suggests that there may also be minor retinal attachment to Lys-172.

### Table 2. Amino acid analyses* of retinyl-containing CNBr fragments

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>CT I major</th>
<th>CT I minor</th>
<th>CT II major</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No papain</td>
<td>No papain</td>
<td>Papain</td>
</tr>
<tr>
<td></td>
<td>No papain</td>
<td>No papain</td>
<td>No papain</td>
</tr>
<tr>
<td>Lys</td>
<td>0.4 (1)</td>
<td>0.3 (1)</td>
<td>0.6 (1)</td>
</tr>
<tr>
<td>His</td>
<td>0.0 (0)</td>
<td>0.0 (0)</td>
<td>0.0 (0)</td>
</tr>
<tr>
<td>Arg</td>
<td>1.5 (2)</td>
<td>1.2 (2)</td>
<td>1.5 (2)</td>
</tr>
<tr>
<td>Asp</td>
<td>3.2 (2)</td>
<td>2.6 (2)</td>
<td>1.2 (1)</td>
</tr>
<tr>
<td>Thr</td>
<td>2.3 (1)</td>
<td>1.8 (3)</td>
<td>0.7 (0)</td>
</tr>
<tr>
<td>Ser</td>
<td>2.0 (4)</td>
<td>1.6 (3)</td>
<td>1.6 (2)</td>
</tr>
<tr>
<td>Glu</td>
<td>2.5 (3)</td>
<td>2.0 (2)</td>
<td>0.3 (0)</td>
</tr>
<tr>
<td>Pro</td>
<td>2.2 (3)</td>
<td>1.8 (3)</td>
<td>0.4 (0)</td>
</tr>
<tr>
<td>Gly</td>
<td>4.3 (5)</td>
<td>3.5 (3)</td>
<td>1.8 (2)</td>
</tr>
<tr>
<td>Ala</td>
<td>5.7 (8)</td>
<td>4.6 (3)</td>
<td>2.6 (2)</td>
</tr>
<tr>
<td>Val</td>
<td>3.2 (3)</td>
<td>2.6 (8)</td>
<td>2.7 (3)</td>
</tr>
<tr>
<td>Leu</td>
<td>2.3 (2)</td>
<td>1.9 (3)</td>
<td>0.8 (1)</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.2 (0)</td>
<td>1.0 (1)</td>
<td>0.7 (0)</td>
</tr>
<tr>
<td>Phe</td>
<td>1.7 (2)</td>
<td>1.3 (2)</td>
<td>1.6 (1)</td>
</tr>
</tbody>
</table>

* Beckman 121M analyzer, after 24-hr hydrolysis in 12 M HCl. Expected values are given in parentheses

† See ref. 11.

‡ (χ²/212/2, Lys and His were not used to calculate χ².

Fig. 2. Excitation (below 400 nm) and emission (above 400 nm) spectra of reduced bR. (A) Membrane suspensions. Excitation spectra: RP<sub>350</sub>, emission at 439 nm (•—•); RP<sub>350</sub>, emission at 510 nm (★—★); Emission spectra: RP<sub>350</sub> and RP<sub>350</sub>, excitation at 360 nm (—); RP<sub>335</sub>, excitation at 337 nm (—); NaDodSO<sub>4</sub>-solubilized membranes: RP<sub>350</sub>, emission at 515 nm (—); RP<sub>360</sub>, excitation at 372 nm (—); CT I from RP<sub>350</sub> in NaDodSO<sub>4</sub>/polyacrylamide gel (gel fluorescence subtracted), emission at 560 nm (—). All spectra were measured at 25°C, pH 7.6. Spectra were obtained with an Aminco SP-500 fluorimeter and are not corrected for excitation lamp shape or photomultiplier response. All spectra are scaled to the same maximum.

Retinyl Spectroscopy. The excitation and emission spectra of borohydride-reduced retinyl proteins (RP) are a sensitive diagnostic of the retinyl environment (see Discussion). Reduction in the light produces a faintly structured absorption spectrum with a maximum at 360 nm (RP<sub>350</sub>). Subsequent illumination of all RP<sub>350</sub> samples at 360 nm photoisomerizes the retinyl moiety (S) and produces the highly structured excitation spectra (RP<sub>350</sub> shown in Fig. 2A). Spectra of all samples reduced in the dark are blue-shifted with respect to RP<sub>350</sub> (RP<sub>335</sub>, Fig. 2A) and are comparable to spectra of detergent-treated RP<sub>350</sub>. Illumination of detergent-treated RP<sub>350</sub> or of RP<sub>335</sub> at 350 nm causes only photodestruction of the chromophore.

The excitation spectra for NaDodSO<sub>4</sub>-solubilized RP<sub>350</sub> in so-
DISCUSSION

Our results can be interpreted to show that the Schiff base linkage site changes during the photocycle, during light/dark adaptation, or both. In that case the 13-cis isomer would most likely be attached to Lys-216 and the all-trans isomer to Lys-40/41. Reduction at 0°C in the light accumulates M, which contains 13-cis isomer (15–17), and links retinal to Lys-216. At higher temperature, [M] decreases, the reduction time increases, and some retinol is found on Lys-40/41. If M is the main reactive species, other species may compete significantly for reduction when [M] decreases. In dark-adapted, reduced pm, the ratio of retinyl-Lys-40/41 to retinyl-Lys-216 is similar to the ratio of all-trans to 13-cis retinal. The data for light-adapted pm reduced in the dark, believed to contain mainly all-trans retinal, do not fit this model (Table 1). However, the additional treatment involved makes interpretation of this result uncertain.

Alternatively, some of the observed linkage may be due to the unphysiological reaction conditions. An example of such linkage occurs in rhodopsin, in which, under certain conditions, retinal is found linked to phosphatidylethanolamine (18). In bR, Lys-216 is most likely the physiological site, because reduction at this site is most rapid and unambiguous (Table 1). However, the observation by Sigrist and Zahnler (19) that phenylisothiocyanate selectively reacts with Lys-216, with little effect on the spectral properties of pm, is difficult to reconcile with a Schiff base exclusively on Lys-216.

The view that Lys-41 forms the Schiff base is based on the work of Bridgen and Walker (1). They showed that one lysine accessible for succinylation in native pm is blocked by reduction with BH$_4^-$, and they concluded that this residue, later identified as Lys-41, must bear the Schiff base. However, it is now known that the site of retinyl attachment in pm reduced under comparable conditions is not blocked by succinylation prior to reduction (20).

Previous work from this laboratory (unpublished) produced a retinyl peptide of the composition Lys$_3$Gly$_2$Ala$_4$Val$_1$-Thr$_1$Leu$_1$Phe$_1$ from pm reduced in the light at 0°C and pH 10. Edman degradation was blocked after the first amino acid, alanine, had been split off. When the amino acid sequence of bR became available (11, 21), it was obvious that this peptide closely matched the sequence around Lys-216. This confirms our conclusion that under these conditions Lys-216 is the main attachment site.

While the work presented here was in progress, Ovchinnikov et al. (21) published data similar to ours. However, after reduction of both light-adapted and dark-adapted pm in the dark, they found the retinyl residue attached only to CT II, and concluded that during the photoreaction cycle, retinal transiently forms a Schiff base with another, unidentified, lysine residue in CT I, and then moves back to Lys-41. They used only fluorescence to locate the retinyl residue. This method of detection is less sensitive than radioisotopic labeling and is not quantitative. The retinyl moeity is photolabile in NaDodSO$_4$ and the relative quantum yields for retinyl attached to CT I and CT II are not known.

Neither our nor our data warrant the conclusion that retinal moves during the photocycle. Chemical reduction cannot conclusively demonstrate the physiological retinal attachment site, for the reasons stated above. Regardless of the physiological relevance of the observed linkage sites, our results do have important structural implications for bR.

Reduction in the light apparently preserves the noncovalent retinal binding site in bR. The spectral structure of RP$_{360}$ and the photoisomerization of RP$_{360}$ to a retroretinyl structure (RP$_{360}^\beta$) indicate that bR forces the β-ionone ring to lie more nearly coplanar with the side-chain polyene system [as also seen for M (22) and retinol bound to apo-pm (23)]. RP$_{360}^\beta$ is formed at 0°C, and also at 25°C, where retinal is found linked to both Lys-216 and Lys-40/41 (Table 1). Circular dichroism spectra and fluorescence polarization studies (unpublished) show that, in light-reduced samples, the retinyl residue occupies a chiral binding site and subdents the same angle to the membrane plane as that in native pm. X-ray diffraction studies show that the bR lattice is intact in light-reduced pm (unpublished) and in unreduced CT-cleaved pm (data not shown). Therefore, reductions yielding retinyl-Lys-216 and retinyl-Lys-40/41 do not significantly perturb the location of retinal, and these two residues must be close together in the bR tertiary structure.

In their low-resolution structure of bR, Henderson and Unwin (24) identified seven dense rods as α-helices spanning the membrane. Agard and Stroud (25) recalculated this model with corrections for the data missing due to limited tilting of the specimen. New densities appeared on both surfaces of the membrane, which they interpreted as four of the six links between helices and the NH$_2$-terminal sequence. The amino acid sequence can be arranged into seven membrane-spanning helical segments, which we label A−G, beginning with the NH$_2$ terminus, located on the external surface (21, 25–27). In all published schemes, Lys-41 lies near the cytoplasmic surface on helix B and Lys-216 occurs in the center of helix G. The links seen in the Agard and Stroud map allow five different assignments of helices A−G to the helical rods in the density map (Fig. 4).

King et al. (28) located retinal at the position indicated in Fig. 4. The least restrictive interpretation of our results requires that the two helices farthest from the retinal should not include B or G; this eliminates three of the five models of Agard and

![Fig. 4](image-url)
Stroud, and 27 of the 41 most favored schemes of Engelman et al. (26). The two remaining schemes of Agard and Stroud (Fig. 4, schemes 1 and 2) are also the two most favored schemes of Engelman et al. Thus, the two linkage schemes numbered 1 and 2 in Fig. 4 must be considered as the most probable by all three sets of independent criteria.

Note Added in Proof. While this manuscript was in press, Bayley et al. (29) published experiments in which they had obtained linkage only to Lys-216 after reduction both in the light and in the dark. They used detergent-solubilized pm for their dark reduction experiments. We also received the preprint of a manuscript by Lemke and Oesterhelt (30) that demonstrates linkage to Lys-216 after light reduction and further shows that peptide bond cleavage can occur during reduction. Lemke and Oesterhelt suggest that one of the resulting fragments containing Lys-216 may migrate with CT II during peptide separation. This raises the possibility that assignment of label to Lys-40/41 is in error. Additional results show that entry 5 in Table 1 is especially suspect. In that case, scheme 4 in Fig. 4 would also be allowed.

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