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Retinal migration during dark reduction of bacteriorhodopsin

(cyanoborohydride/Lys-41/proton pump/reductive amination/Schiff base)

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ABSTRACT **When the retinal Schiff base in chymotryptically cleaved bacteriorhodopsin is reduced to a secondary retinylamine by prolonged exposure to 10% (wt/vol) sodium cyanoborohydride, at pH 10, in the absence of light, ≈45% of the retinal is found linked to Lys-41 and 22% to Lys-40, and the remainder is scattered over various sites on the large chymotryptic fragment, including the physiological site at Lys-216. The retinal-binding site is destroyed or blocked by the reduction conditions, but the bacteriorhodopsin lattice remains intact. The results demonstrate that artifactual linkage to Lys-40/41 is possible under special conditions. Under these conditions, the ε-amino groups of Lys-40/41 show an enhanced ability to form retinylidene linkages with the retinal released by the physiological linkage site at Lys-216, due to some combination of close proximity to the normal linkage site, and increased reactivity with respect to other lysine ε-amino groups. The results are of interest for the characterization of the two newly discovered rhodopsin-like proteins, halorhodopsin and slow rhodopsin.**

The determination of the primary protein (1, 2) and structural gene (3) sequences of bacteriorhodopsin (bR), the light-driven proton pump from the purple membrane (pm) of *Halobacterium halobium*, has led to a reinvestigation of the retinal-binding site by chemical reduction and peptide analysis. This resulted in a reassignment for the binding site from the ε-amino group of Lys-41 (4) to the ε-amino group of Lys-216 (5–9). Additional chemical modification (10) and resonance Raman (11) studies have demonstrated conclusively that the binding site does not change during the photoreaction cycle as originally postulated by Ovchinnikov's group (12). The reason for the original misassignment of the binding site by Bridgen and Walker is obvious in retrospect (7). However, the same arguments cannot explain the more recent observations of reductive retinal linkage to Lys-40/41 (7, 9, 12). Lemke and Oesterhelt (6) consider these to be in error due to a previously unnoticed proteolytic cleavage of bR by $NaBH_4$. We show here that this explanation does not hold, at least for our results, and that under some conditions of reduction, two-thirds of the retinal are indeed found bound to Lys-40/41.

More important, we have previously argued that while binding to Lys-40/41 is a preparation artifact, it might still be used to obtain important information about the tertiary structure of bR. We have, therefore, repeated and extended our earlier experiments. Unfortunately, the new observations considerably weaken the structural argument, but they demonstrate an unusually high reactivity of Lys-40/41 for Schiff base formation and/or reduction and are of considerable interest for the interpretation of experiments trying to identify the two additional retinal pigments recently discovered in *H. halobium* (13–15).

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MATERIALS AND METHODS

Materials. Bovine serum albumin (fraction V, essentially fatty acid free), 2-(*N*-cyclohexylamino)ethanesulfonic acid (Ches), chymotrypsin (treated with *N*-tosyl-L-lysine chloromethyl ketone), all-*trans* retinol, all-*trans* retinal, LH-20, and LH-60 were purchased from Sigma and used without further purification. Sodium cyanoborohydride ($NaBH_3CN$, Alfa recrystallized grade) was stored in a desiccator, under argon. Formic acid (Aldrich, 95–97%) was distilled at reduced pressure before use. All-*trans* retinal, tritiated at the aldehyde carbon (78.2 mCi/mmol; 1 Ci = 37 GBq), was synthesized from all-*trans* [15-^{3}H]retinol (New England Nuclear, 14.3 Ci/mol), after 1:100 dilution with unlabeled retinol, by MnO_2 oxidation in CH_2Cl_2 (5), and purified by preparative thin-layer chromatography on silica gel G (Analtech, Newark, DE) with 15% acetone in petroleum ether. Retinal stock solutions in ethanol were stored under argon, in blackened containers, at −120°C.

Purple membrane was prepared from *H. halobium* (JW-3 strain; formerly ET1001) by standard procedures (16). The pm was stored suspended in 4 M NaCl solution at 4°C.

Regenerated, Chymotryptically Cut, [^{3}H]Retinal-Labeled pm (^{3}H-RG-CT-pm). RG-CT-pm was prepared essentially as described (7, 17), except that hydroxylamine-bleached pm was cut at a concentration of 0.125 mg/ml, in the presence of 50 mM $CaCl_2$, with 10 μg of chymotrypsin per ml. The product was washed with 75 mM EDTA at pH 7 to remove membrane-bound Ca^{2+}. Aliquots of stock solutions of all-*trans* [15-^{3}H]retinal (0.53 mM in ethanol) were concentrated by evaporation with a stream of argon in the dark prior to regeneration of bleached, cleaved pm (CT-pm).

Reduction of RG-CT-pm. Eight milligrams of ^{3}H-RG-CT-pm in 20 ml of 4 M NaCl was dark adapted for at least 1 hr under argon. Argon-saturated 200 mM Ches (pH 10) containing 20% (wt/vol) $NaBH_3CN$ was centrifuged (15,000 rpm, 20 min, Sorvall SS-34 rotor) to remove solid contaminants, and 20 ml was added to the ^{3}H-RG-CT-pm suspension under dim red light. The reaction mixture was placed in a black beaker, under argon, for 6 days, with magnetic stirring. In some cases, the beaker also held a control mixture, which contained all reaction components except $NaBH_3CN$. The reduced product was washed three times with distilled water and stored under argon, in the dark, at 4°C.

Reduction After pH Jump. Two-hundred fifty micrograms of ^{3}H-RG-CT-pm in 263 μl of water was added to 263 μl of 0.1 M sodium acetate (pH 5.0) or 263 μl of 0.1 M Ches (pH 10.0) and left at room temperature in the dark overnight. Next, 526 μl of ethanol was added while spinning in a vortex, followed immediately with an addition of 150 μl of 1 M HCl.

Abbreviations: bR, bacteriorhodopsin; CT, chymotryptically cut; pm, purple membrane; RG, regenerated; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

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The sample was then placed on ice and, after it had cooled, 100 μl of 20% (wt/vol) $NaBH_3CN$ was added. After 15 min, each sample was centrifuged, the supernatants were discarded, and the pellets were prepared for $NaDodSO_4$/polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE). Samples were electrophoresed on 10–20% linear gradient gels (18); the gels were stained, the stained bands were excised and dissolved with H_2O_2, and the radioactivity migrating with each band was measured by liquid scintillation counting.

Chromatography and Sequence Analysis. Unreduced retinylidene linkages in reduced RG-CT-pm (RD-RG-CT-pm) samples were broken by addition of an equal volume of ethanol to a concentrated (>1 mg/ml) sample, followed by addition of 1 M hydroxylamine (pH 7.0) to a final concentration of 50 mM. The sample was then centrifuged (15,000 rpm, 40 min, Sorval SS-34), and the pellet was suspended in formic acid. LH-60 chromatography to separate the chymotryptic fragments, CT-I and CT-II, and cyanogen bromide cleavage of the fragments were performed as described (7, 19). Column conditions for fractionation of the cyanogen bromide fragments are discussed in the legends of Figs. 2 and 3.

High-performance liquid chromatography (HPLC) was performed as described in the legend of Fig. 4. Amino acid sequence analysis was performed with a Beckman 890C sequencer as described (20) in the laboratory of S. C. Rall, Jr.

RESULTS

Reductive Linkage to CT-II. Fig. 1 shows the initial fractionation of ^{3}H-RG-CT-pm into the large (CT-I) and small (CT-II) fragments after the CT sample had been reduced with sodium cyanoborohydride in the dark, for 6 days, at room temperature. The fractionation pattern is clearly different from that of a sample reduced with sodium borohydride in the light, at pH 10 and 0°C (7); in the light-reduced sample, all of the radioactivity migrates with CT-I, whereas in the dark-reduced sample, 36% of the bound radioactivity migrates with CT-I (fractions 10–18) and 64% with CT-II (fractions 22–32). $NaDodSO_4$/PAGE of this and similar samples on 12–20% polyacrylamide gradients showed no evidence of proteolysis at other than the chymotrypsin cleavage site. Both the CT-I and CT-II fragments were fluorescent. Bands from other samples of cyanoborohydride-reduced ^{3}H-RG-CT-pm, cut from gels and dissolved in H_2O_2, gave similar labeling ratios upon subsequent liquid scintillation counting.

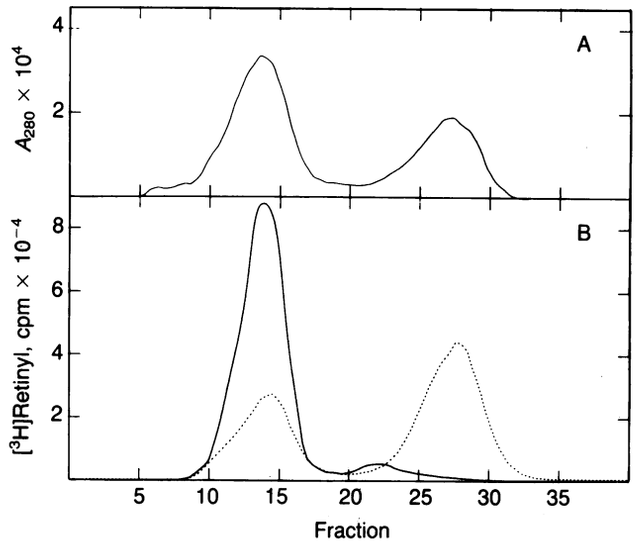

FIG. 1. Fractionation of reduced ^{3}H-RG-CT-pm on an LH-60 column. The column (2.5 cm in diameter × 50 cm) was eluted at 0.5 ml/min with formic acid/ethanol, 1:2.8 (vol/vol). The first 40 ml of eluant was discarded; 3-ml fractions were then collected. (*A*) UV absorbance at 280 nm; (*B*) ^{3}H activity as a function of fraction number for a sample reduced at 0°C in the light with $NaBH_4$ (—) and a sample reduced in the dark with $NaBH_3CN$ (····).

The absorption, fluorescence excitation, and fluorescence emission spectra of cyanoborohydride-reduced samples of RG-CT-pm are essentially the same as for samples reduced in the dark with borohydride (7). They differ from the properties of control samples reduced with borohydride in the light, at 0°C, in three important ways. First, their absorption maximum is at 335 nm, blue shifted 25 nm from the maximum in the control, and is similar to a solution retinyl spectrum (i.e., the control spectra are atypical). Second, the cyanoborohydride-reduced samples do not photoisomerize under near UV irradiation to give a retroretinyl (structured) absorption spectrum (21); the control samples do. Finally, their fluorescence in the gels is 5–10 times weaker than that of the control.

Reductive Linkage to Lys-41 and Lys-40. Linkage of retinal to CT-II can potentially take place at three sites: Lys-30,

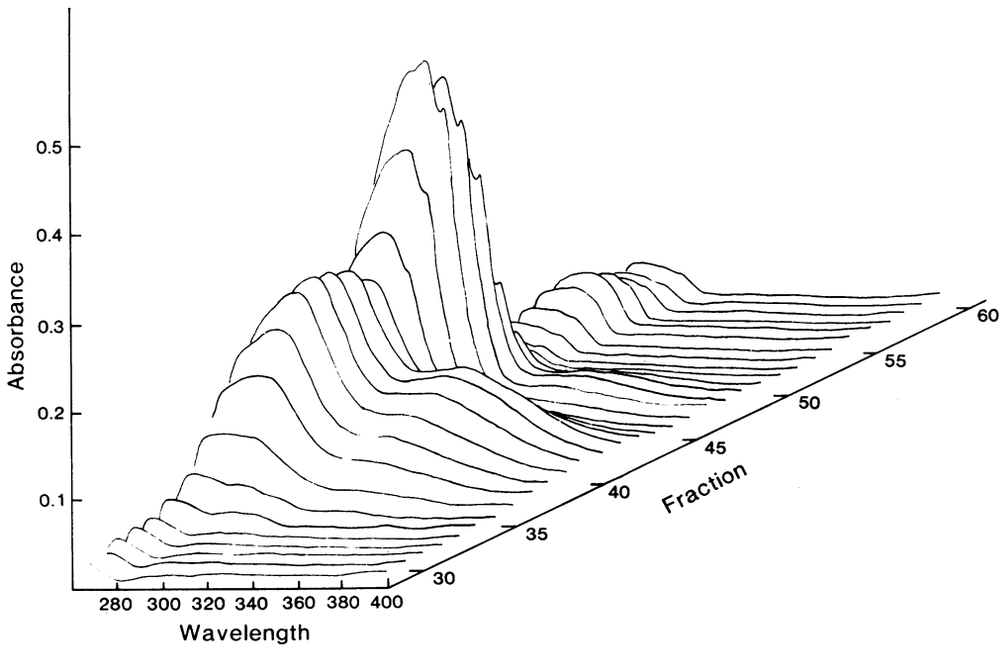

FIG. 2. Fractionation of CNBr fragments of CT-II from cyanoborohydride (dark) reduced ^{3}H-RG-CT-pm on an LH-20 column. The column (0.9 cm in diameter × 50 cm) was eluted with formic acid/ethanol, 1:2.8, at 2.7 ml/hr. After 80 min, collection of 0.33-ml fractions was initiated. A three-dimensional representation of the absorption spectra (260–400 nm) of fractions 30–60 is shown.

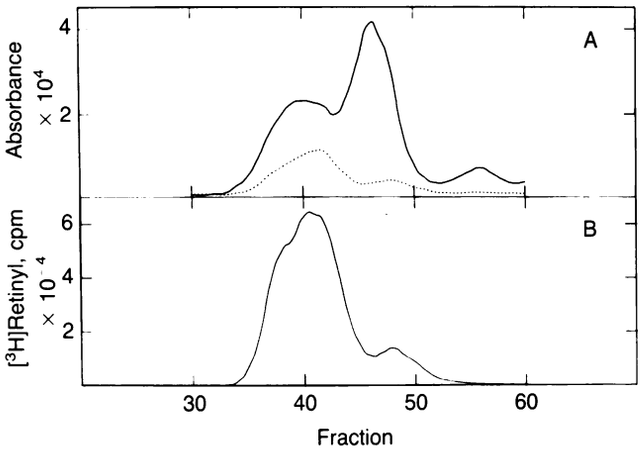

FIG. 3. Retinyl labeling of CNBr fragments of CT-II. The column eluant fractions are the same as in Fig. 2. (*A*) A_{280} (—) and A_{335} (····) are plotted as a function of fraction number. (*B*) [^{3}H]Retinyl activity is shown for the same fractions.

Lys-40, and Lys-41. These sites lie on cyanogen bromide fragments CNBr-3 (amino acids 21–32) and CNBr-1 (amino acids 33–56), respectively (notation of ref. 19). Therefore, these fragments must be distinguished to identify the linkage site.

The fractionation of CNBr fragments from cyanoborohydride-reduced CT-II in Figs. 2 and 3 shows that the retinyl absorbance at 335 nm comigrates with the first A_{280} peak eluted. This peak is normally due to fragment CNBr-1 (19). Little, if any, retinyl absorbance comigrates with the only tryptophan-containing fragment, CNBr-2 (amino acids 1–20), identified by the characteristic shoulder near 290 nm (Fig. 2). Therefore, the labeled fragment cannot be CNBr-2, but a labeled fragment CNBr-3 could still comigrate with fragment CNBr-1. Although this possibility is unlikely, because the peak 1/peak 2 A_{280} ratio is nearly identical to that in unlabeled samples, we have further purified the labeled component of the first peak via HPLC and obtained a partial sequence of the fragment to confirm our identification.

The gradient elution of peak 1 from a Waters C_{18} reverse-phase column is shown in Fig. 4. The radioactivity and A_{365} comigrate as a double peak (such double peaks are characteristic of CNBr fragments due to a homoserine–homoserine lactone equilibrium). The first four amino acids of the purified fragment were Gly-Val-Ser-Asp (>70%), with a contaminant of Gly-Leu-Gly-Thr (<30%). Therefore, the purified sample was mainly CNBr-1, with a contaminant of CNBr-3. The sequence analysis run was continued out to 10 cycles, and the ^{3}H activity released is shown in Fig. 5. Release should occur from Lys-40 at cycle 8, Lys-41 at cycle 9, and Lys-30 at cycle 10. The observed release is consistent with a fraction of labeled residues cleaved at cycles 8–10 of 0.5, with 32% label at Lys-40 and 68% at Lys-41. Apparently, cleavage at Pro-37 is poor and causes carry-over.

Recovery of radioactivity from the sequence analysis run was poor (*ca.* 2%, uncorrected for quenching), indicating that solubilization in the acetic acid used to load the sample was poor or that peptide or label loss (or both) during washes occurred. Such loss is indicated by the initial release at cycle 1 and the high background release at cycles 2–7 (Fig. 5). Poor count recovery could also be due to poor cleavage of labeled lysine; this would also rationalize the large release at cycle 10 as carry-over.

Linkage to CT-I. CT-I after reduction with cyanoborohydride, CNBr cleavage, and fractionation on LH-60 (2) shows label on most of the peptides from the experimental sample (Fig. 6). Reductive linkage to one site on each peptide is possible, since each peptide except CNBr-10 (amino acids 72–

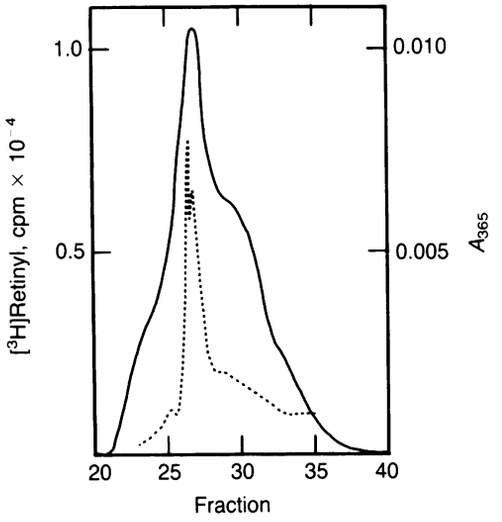

FIG. 4. Purification of the labeled CT-II CNBr fragment by reverse-phase HPLC. A Waters C_{18} column (μBondapak, 4 mm in diameter × 25 cm) was eluted (Spectra Physics model SP8700 gradient elution system) with a gradient from 10% B (5% formic acid in ethanol) to 40% B in 30 min, followed by 40% B to 100% B in 40 min. Solvent A was 5% formic acid in water. Absorbance at 365 nm was monitored by a Waters model 440 UV absorbance monitor. Flow was 1 ml/min; 2-ml fractions were collected. ^{3}H activity (—) and the continuously monitored A_{365} (····) are shown as a function of fraction number; no ^{3}H activity was detected in any other part of the elution profile.

118) contains one lysine, and CNBr-10 has a free amino terminus, due to the chymotrypsin treatment. The interpretation is complicated by the possible presence of partially cleaved peptides (22). However, the presence of radioactivity in fractions 70–80 indicates at least one site of labeling in addition to Lys-216, probably on fragment CNBr-11 (amino acids 146–163), at Lys-159. The structure in the elution pattern in fractions 30–52 from the experimental sample is also suggestive of labeling at additional sites, and the possibility of labeling at all possible sites cannot be excluded.

We have carried out additional experiments to determine the properties of the pm reduced with cyanoborohydride in

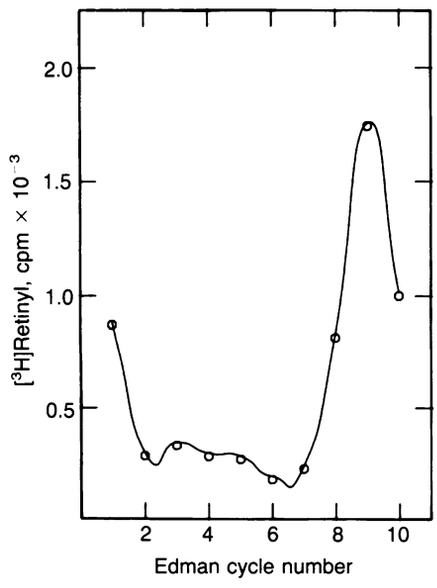

FIG. 5. Release of ^{3}H activity from the HPLC-purified fragment of Fig. 4 during Edman degradation, as a function of Edman cycle number.

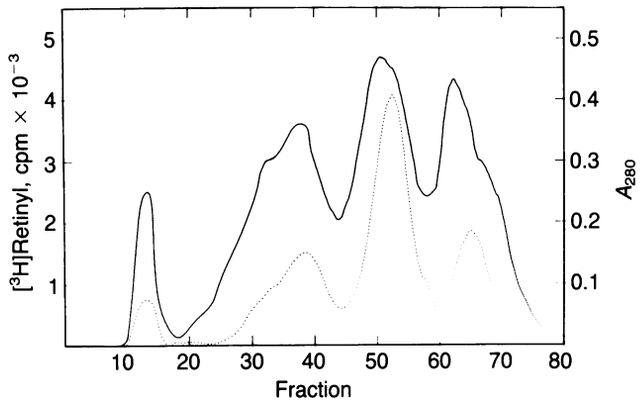

FIG. 6. Fractionation of CNBr fragments of labeled CT-I (dark, cyanoborohydride-reduced ^{3}H-RG-CT-pm) on an LH-60 column. Conditions were as in Fig. 1, except that the flow was 0.22 ml/min, and 2.2-ml fractions were collected. ^{3}H activity (—) and A_{280} (····) are plotted as a function of fraction number.

the dark. No new absorbance at 570 nm is generated by addition of one equivalent of all-*trans* retinal to a reduced sample. A RG-CT-pm sample containing all components of the reduction mixture except cyanoborohydride lost no absorbance at 570 nm after 6 days at room temperature, in the dark, and bleached but unregenerated CT-pm, after the same treatment, could still be regenerated by addition of retinal. However, bleached CT-pm, after the same treatment plus cyanoborohydride, could not be regenerated with retinal. The loss of regenerability was not coupled to any appreciable change in the retinal oxime absorption spectrum, and the dull orange fluorescence of retinal oxime was still present, indicating that the oxime had not been reduced to a new species. We conclude that retinal oxime may still occupy its noncovalent binding site in the protein or block access to it.

CT-pm shows little change functionally or structurally by most criteria we and others have examined. It shows the same lattice structure in x-ray diffraction patterns as native pm at both pH 7 and pH 10 (N. Katre and R. Stroud, personal communication). The reduced species still retains the lattice, as shown in Fig. 7. Cleaved pm photocycles with unchanged time constants for M-decay and bR recovery; a 75%

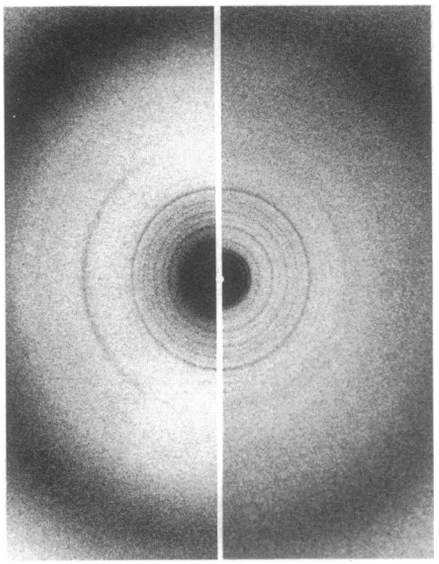

FIG. 7. X-ray diffraction patterns for cyanoborohydride, dark-reduced RG-CT-pm (*Left*) and native pm (*Right*).

regenerated sample has *ca.* 60% of the CD signal associated with the 570 nm absorbance of bR. Chymotryptically cut, regenerated, reconstituted bR is also known to still pump protons (23). The most striking difference we have seen from native pm is that the high-pH-induced changes in the absorption spectrum of dark-adapted pm (24) occur at lower pH in RG-CT-pm and are already pronounced at pH 10.

To trap the retinylidene residue at its location we utilized a combined denaturation and jump to a pH <1, followed by reduction with cyanoborohydride at 0°C. Similar techniques have been used successfully with bovine rhodopsin, and retinylidene linkages have been shown not to hydrolyse or transiminate at very low pH (25, 26). In all experiments, ^{3}H-RG-CT-pm was used, and labeling was assayed by NaDodSO$_4$/PAGE of the reduced samples, followed by excision, dissolution, and liquid scintillation counting of the CT-I and

Table 1. Location of retinyl residue under various reducing conditions

bR preparation*	Temperature, °C	Reducing agent	Time of reduction, min	pH	Location of retinyl residue	
					CT-I	CT-II
		Illuminated during reduction				
RG-RD-CT(LA)	0	BH_4^-	30	10	1.00	0.00
RD-CT(LA)	0	BH_4^-	60	8.6–9.1	+	−
RG-CT-RD(LA)	0	BH_4^-	30	10	1.00	0.00
CT-RG-RD(LA)	0	BH_4^-	30	10	1.00	0.00
RD-CT(LA)	25	BH_3CN^-	150	3	+	−
		Reduced in the dark				
CT-RD(DA)†	25	BH_3CN^-	3×10^3	5	+	−
CT-RD(DA)	25	BH_3CN^-	8.6×10^3	10	+	++
CT-RG-RD(DA)	25	BH_3CN^-	8.6×10^3	10	0.34	0.66
CT-RG-RD(DA)‡	0	BH_3CN^-	15	7, 10	+§	Trace§
CT-RG-RD(LA)‡	0	BH_3CN^-	15	7, 10	+§	Trace§

All reductant concentrations were 1% (wt/vol), and all reactions were run to completion, unless otherwise noted. Location fractions noted by + or − were measured by fluorescence only, unless otherwise noted.

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

†Solubilized in Triton X-100, 1.25% (wt/vol), after CT, before RD.

‡pH jumped to <1, in presence of ethanol, before reduction.

§Measured both by fluorescence and comigration with [^{3}H]retinyl.

CT-II bands. The results of various treatments are summarized in Table 1. Clearly, only CT-I is labeled.

In addition, RG-CT-pm was solubilized in Triton X-100 (27) and reduced at pH 5 in the dark over a period of 2 days by addition of 1% sodium cyanoborohydride. When the reduced material was fractionated on polyacrylamide gels, fluorescence was observed only from CT-I (Table 1).

Externally added retinal does not exchange with pm over a period of several days (unpublished data). Therefore, the following experiment was performed: 0.5 equivalents of [^{3}H]retinal were added to unlabeled RG-CT-pm, and the resulting sample was immediately reduced with cyanoborohydride under the usual conditions. The sample was subsequently fractionated on LH-60, after treatment with NH_2OH. The elution pattern observed was essentially the same as that shown for $NaBH_3CN$-reduced ^{3}H-RG-CT-pm in Fig. 1. The bound counts accounted for 22% (CT-I) and 35% (CT-II) of the radioactivity added. More important, the fractions of bound counts on CT-I (38%) and CT-II (62%) were quite similar to the fractions observed upon reduction of ^{3}H-RG-CT-pm with cyanoborohydride.

DISCUSSION

There is no doubt that the physiological binding site for retinal is at Lys-216 and that the other binding sites are artifacts. The results presented here, especially the dark reduction of Triton X-100-solubilized membrane and the dark reduction at low pH, confirm this conclusion. However, preferential binding of retinal at Lys-40/41 occurs and needs to be explained.

Two extreme interpretations are possible. First, the labeling may indicate that Lys-41 and Lys-40 lie close to the physiological retinylidene linkage in the tertiary structure of bR, so that, whenever conditions allow occasional breakage of the physiological linkage, the alternative linkages most easily formed are with these lysines. This possibility was first suggested by Katre *et al.* (7). Alternatively, the observed linkage may only be determined by the relative reactivity of the different amino groups, without any weighting by their proximity to Lys-216. The well-preserved lattice structure and lack of regenerability of 570 nm absorbance are arguments in favor of the first explanation; the binding pattern of added retinal to native pm strongly supports the second explanation.

The evidence supporting the proximity argument is comparatively weak. The reduction conditions may have destroyed the site, and a comparison of the absorption spectra of dark- and light-reduced pm shows a narrower, red-shifted spectrum with indications of fine structure for the light-reduced product where retinal is bound at the physiological site. These observations and the facilitated isomerization by light to a retroretinyl structure demonstrate that the light-reduced chromophore interacts more strongly with the protein. However, any change in protein structure in the dark-reduced product must be subtle because the reduced species still possesses a lattice.

Our results therefore cannot answer the question of whether Lys-41 and Lys-40 lie close to Lys-216. We can, however, reach some unequivocal conclusions from this work. First, Lys-41 and Lys-40 are special in their ability to reductively aminate retinal in the presence of cyanoborohydride. This could be due to proximity to a protein-binding site for retinal or proximity to the surface of the protein where it would be accessible to free retinal dissolved in the lipid. In either case, the ε-amino groups of these lysines must also be unusually accessible to aqueous anions. Second, simultaneous pH jump to pH <1 and denaturation, followed by reduction with cyanoborohydride, is the method of choice for localization of retinylidene linkages in retinal-containing proteins. The method has the advantages of yielding quantitative linkage in a short period of time with no retinylidene migration and of allowing easy subsequent recovery of the labeled protein, especially if ethanol is used as the denaturant. This has obvious implications for the ongoing investigations of halorhodopsin (13) and the newly discovered "slow rhodopsin" (14) of the extreme halophiles.

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