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PHOTOAFFINITY LABELING OF BOVINE RHODOPSIN

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Abstract—Photoaffinity labeled (3-diazoacetoxy)-9-*cis*-retinal (1) and (9-methylenediazoacetoxy)-9-*cis*-retinal (2) were synthesized and bound to bovine opsin to obtain visual pigment analogs having absorption maxima at 465 and 460 nm respectively. Binding studies established that synthetic retinals 1 and 2 bind to the natural binding site and that the integrity of the diazoacetoxy photoaffinity label is preserved in the process. Incorporation of 3-(O¹⁴COCHN₂)-labeled 9-*cis* retinal could be conveniently carried out in high yield using apomembrane solubilized in CHAPS as detergent to afford the pigment analog in a pure form. Photolysis of the diazoacetoxy group within the binding site led to 15–20% crosslinking of rhodopsin as estimated by using radiocarbon containing labeled retinal 1 thus showing that this synthetic retinal is suitable for photoaffinity labeling of the active site in rhodopsin. Subsequent experiments to establish the site(s) of crosslinking by sequencing studies will then contribute to our knowledge of the structure of rhodopsin.

Visual perception is initiated via absorption of light by visual pigments which are glycoproteins embedded in lipid bilayers of the photoreceiving rod and cone cell membranes. The binding of these proteins (opsins) with the chromophore 11-*cis* retinal¹ (and/or 3-dehydroretinal² in certain species, especially freshwater fish) by a protonated Schiff base linkage³ leads to visual pigments capable of capturing light of various wavelengths; for example, the rhodopsins, which are the visual pigments in the rod cells, have absorption maxima in the range of 345–575 nm depending on species.

Upon light absorption, rhodopsin is transformed into a high free energy photoproduct, which then thermally decays through several spectroscopically distinct intermediates, until the pigment is “bleached”, i.e. the SBH⁺ bond is hydrolyzed to give the isomerized all-*trans* retinal and apoprotein opsin. At some stage during the bleaching process, the permeability to sodium ions of the photoreceptor cell membrane is changed. The resulting potential changes in the receptor cell membranes are then processed by other retinal cells to produce the first integrated state of visual information which is then transmitted by the optic nerve to the brain.

Our knowledge of the molecular mechanism of processes occurring upon absorption of light by rhodopsins leading to visual transduction is very limited due to the paucity of structural information available on the system. Our aim for the past several years has been to synthesize various analogs of natural retinal, bind them to opsin (in most cases this has been confined to bovine opsin), and to contribute to our understanding of the rhodopsin structure and visual transduction process by studying the properties of such artificial visual pigments.⁴

Determination of the primary structure of rhodopsin (bovine) had eluded workers for many years, and it is only recently that this has been determined.^{5,6} Although it is known that the chromophore 11-*cis* retinal is bound to lysine-53,⁷ not much additional structural information is available for the crucial retinal binding site. Crystals suited for X-ray analysis of this integral membrane protein have as yet not been prepared. These are the reasons for carrying out photoaffinity labeling of visual pigments.⁸

Following its discovery two decades ago,⁹ the photoaffinity labeling technique is being applied to the study of the structure and properties of an increasing number and variety of biological targets.¹⁰ The visual pigments are well-suited for photoaffinity studies for several reasons:

(i) Unlike many enzyme/substrate pairs, the chromophore is covalently linked to the protein.

(ii) The opsin binding site is quite lenient in terms of its structural requirement, as shown by the fact that adamantyl allenic retinal yielded a visual pigment analog;¹¹ therefore, it is conceivable that a variety of photoaffinity labeling groups can be employed.

(iii) Binding of modified chromophores via the SBH⁺ linkage can usually be ascertained by the large bathochromic shift encountered in going from the SBH⁺ formed with *n*-butylamine to the pigment;¹² we have called this shift, which reflects the influence of the opsin binding site, the opsin shift.¹³ Comparison of opsin shifts formed from retinals having similar structures can conveniently be used as one of the criteria in judging that the chromophores occupy similar binding sites.

(iv) The shifted pigment maximum is accompanied by a correspondingly shifted extremum in the circular dichroic spectrum.⁴

(v) Whether the retinal analog occupies the natural binding site or not can be inferred by subsequent incubation of the artificial rhodopsin with the “natural” chromophore or reversing the order and observing the changes in absorption maxima, if any.¹⁴

(vi) Treatment of the pigment with CH₂Cl₂ will liberate the chromophore and hence HPLC analysis of the centrifuged CH₂Cl₂ extract enables one to check whether the chromophore, which is usually light- and heat-sensitive, has retained its original structure during the binding process.¹⁵

(vii) In certain cases, *in vivo* experiments have proven that the modified retinals bind to the natural receptor and perform the expected function. For example, administration of ³H-labeled 11,12-dihydro-chromophore¹⁶ and more recently the 11-*cis*-locked chromophore¹⁷ into the intestines of vitamin A deficient rats and retinal preparations of bullfrogs have led to decrease in eyesight; the labeled

chromophores were then isolated from the retina and properly identified.

Photoaffinity labeling of rhodopsin was carried out according to a sequence consisting of the following stages: (1) design and synthesis of retinals bearing the photolabile group; (2) binding of synthetic retinals to bovine opsin to obtain visual pigment analogs having a photolabile group inserted into the active site; and (3) activation of the photolabel to obtain crosslinked rhodopsin. Protein cleavage and sequencing will then be performed to locate the sites of crosslinking and thus provide information regarding the rhodopsin tertiary structure and location of the retinal moiety.

RESULTS AND DISCUSSION

Synthesis of retinals bearing a photoaffinity label

Retinal analogs designed to photoaffinity label rhodopsin should satisfy the following requirements: (1) the labeled retinal should be able to bind readily, i.e. should not cause conformational distortions of the opsin; (2) the photolabel bearing retinal should be reasonably stable in aqueous buffers and detergents, and to the conditions employed in binding studies; (3) the photolabile group should undergo facile photolysis at a wavelength where irradiation would result in the least protein damage; (4) the intermediate, e.g. carbene, generated by light should undergo little or no rearrangement but should react rapidly and indiscriminately with its immediate environment; (5) a reasonably simple route should be available not only for the synthesis of the photoaffinity-label-bearing retinal but also for its ^3H - or ^{14}C -labeled analog at high levels of radioactivity.

Three classes of photoaffinity labels are conceivable according to the generated reactive species upon photolysis, i.e. carbenes, nitrenes, or free radicals. Of these, the carbene- and nitrene-generating species have been the most widely used, in particular the former when hydrophobic regions of biological systems are concerned. Nitrenes are less seldom employed due to low reactivities of aryl nitrenes (the only species studied extensively) unless enhanced by electron-withdrawing groups, lower labeling yields, short singlet nitrene lifetimes and rapid intersystem crossing to the triplet species, and lack of well-documented precedents. Practical considerations such as the incompatibility of the usage of SH-

stabilizing reagents (e.g. dithiothreitol) with the aryl azide precursors and the steric bulk of the azide group led us to use a carbene-generating precursor.

A variety of photolabile ligands which generate carbenes¹⁰ upon irradiation, such as α -diazoketones, α -diazooesters (diazooacetyl, diazomalonyl, trifluoromethyldiazooacetyl, etc), aryldiazophosphonates, aryldiazomethanes, and more recently, aryl and alkyl diazirines, have been used for photoaffinity labeling in biological systems. Aryl derivatives where an aryl group replaces the trimethylcyclohexenyl ring of retinal were not considered appropriate because of the extensive structural modification and the possibility that the pigment may not be formed in high yield. Photolabels requiring activation at *ca* 350 nm, e.g. alkyl diazirines which have been useful for photolabeling intrinsic membrane proteins, are not suitable here because rhodopsin has a photochemically active β -band at *ca* 350 nm; the quantum yield of bleaching when irradiated close to the β -band is the same as that upon irradiation at 500 nm (α -band).¹⁸ Other photolabels such as the trifluoromethyldiazooacetyl group, which relative to the simple diazoacetyl group is less prone to undergo the Wolff rearrangement, the main side reaction in photoaffinity studies, was not selected as the prime candidate due to its steric bulk and this left us with the diazoacetate group.

The usage of the diazoacetyl group appeared to have several advantages. Namely, visual pigments are generated at neutral pH, a range in which diazoacetates are stable. The competing photochemical Wolff rearrangement is favored in polar protic media but the β -ionone ring binding site is assumed to be hydrophobic. The group introduced in the peptide by the diazoacetoxyl photoaffinity label after activation and hydrolysis is $-\text{CH}_2\text{COOH}$ (Fig. 1) which does not contain the retinal moiety that could lead to complications by isomerizations, etc, during the course of protein analysis.

The diazoacetoxyl group was first introduced at C-4 but this was abandoned when it was found that the affinity group is hydrolyzed to 4-hydroxyretinal during binding. Binding studies were thus carried out with 3-diazoacetoxylretinal 1, and 9-methylenediazoacetoxylretinal 2. The 9-*cis* rather than the 11-*cis* isomers were prepared as they are synthetically more accessible.¹⁹ 2-Hydroxy-*trans*-retinal 3 was syn-

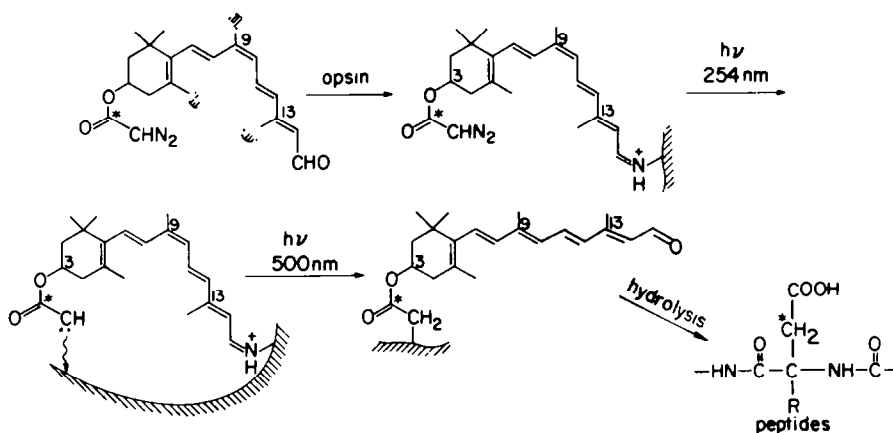
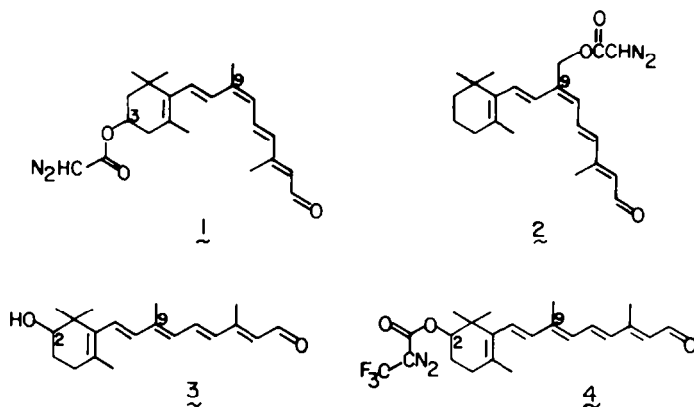


Fig. 1. Photoaffinity labeling of rhodopsin.



thesized and its 2-trifluoromethyldiazoacetate **4** was bound to bacteriorhodopsin²⁰ on an exploratory basis but has not been pursued further.

Since the synthesis of **1** was outlined previously⁸ and since it is currently being re-investigated to clarify the by-products and to increase the yield, it will not be described here.

To prepare the 9-methylenediazoacetate derivative **2** (Scheme 1), β -ionone was first oxidized with lead tetraacetate to the hydroxyketone in *ca* 30% yield.²¹ The silylated protected derivative **5** was then treated with silylated acetaldehyde *t*-butylimine to give the C₁₅ aldehydes **6** in 70–80% yield. The desired 9-*cis* isomer constituted 80% of the mixture and was separated from the *trans* compound by preparative tlc. A 5-carbon elongation of the polyene chain by Emmons reaction followed by reduction, oxidation and deprotection steps led to compound **8** composed of 9-*cis* and 9,13-*dicis* isomers (60/40). This mixture was esterified with the tosylhydrazone of glyoxylic acid following the same procedure as was used in the case of 3-hydroxyretinal.⁸ However, due to extreme instability of diazoester **2**, the yield of 9-*cis* isomer after HPLC purification was less than 1%. Synthesis of the radiolabeled compound was therefore abandoned until a better synthesis becomes available, and binding studies were carried out with the 3-diazoacetate.

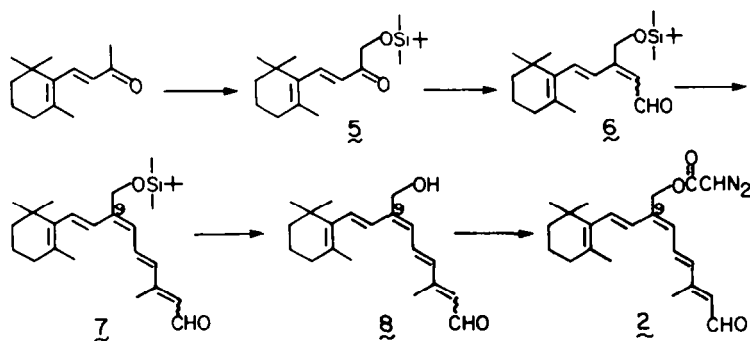
Binding studies; employment of CHAPS as detergent

Visual pigment analogs can be regenerated by combining an appropriate *cis* isomer of the retinal

with bleached rod outer segment (ROS) membranes.²² Either ROS in suspension is used, in which case regeneration is followed by purification procedures and solubilization in a detergent, or the ROS is solubilized in a detergent to which retinal is added; only a few of the known detergents allow reconstitution of rhodopsin in reasonable yields.²³

In the present studies the visual pigment analogs were regenerated either in ROS suspension, or more efficiently in the new detergent, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate ("CHAPS")²⁴ which we found to be especially suited for regeneration of both the natural²⁵ and artificial pigments. This zwitterionic detergent was found by Hjelmeland *et al.* to be the only detergent capable of solubilizing the opiate receptor in a state exhibiting reversible binding of opioids. Unlike digitonin, it is commercially available in a chemically pure form and has good optical transparency with a peak at 220 nm (amide) and 265 nm (very weak, ϵ 3).

We found that a 10 mM solution of CHAPS in 10 mM HEPES buffer at pH 7.0 solubilized bovine rhodopsin efficiently (1 mg/ml) to give solutions stable at room temperature for at least 24 hr. More importantly, regeneration of visual pigments proceeds in CHAPS with remarkable efficiency. Thus 94% of rhodopsin can be regenerated from bovine opsin and 11-*cis* retinal in 30 min at 36° by using only equimolar quantities of retinal and opsin, and regeneration can be achieved within 2 min at 25° by using 1.5 molar equivalent of retinal. In contrast, regeneration in suspension or in other detergents requires at least a 5-fold excess of the precious retinal analog,



Scheme 1.

which in most cases is available in only few mg quantities.

The analogs of visual pigments were also efficiently regenerated in CHAPS solution. Thus, isorhodopsin (or 9-*cis* rhodopsin), a rhodopsin analog containing a seven-membered retinal,¹⁷ and various aromatic analogs of rhodopsin²⁶ could all be prepared in high yields. The most dramatic improvement in the regeneration yield was encountered in the retinal analog containing an 8-membered ring; the corresponding rhodopsin could be formed in CHAPS solution but could not be formed at all in membrane suspension or by using other detergents.²⁷ It is interesting to note that while CHAPS was reported to be efficient in breaking protein-protein interactions as evidenced by the disaggregation of cytochrome P-450 into its monomeric form,²⁴ it does not dissociate the trimeric crystalline structure of bacteriorhodopsin. This was indicated by retention of the characteristic biphasic exciton-split CD in 10 mM CHAPS solution due to the hexagonally aggregated state of the protein. Such a state cannot be maintained in other detergents, such as Triton X-100 or octyl- β -D-glucoside, which leads to the formation of monomeric bacteriorhodopsin. Further, the spectrum in CHAPS does not contain interfering bands due to the detergent.

Despite the poor synthetic yield of retinal 2, 9-methylenediazoacetox-9-*cis*-rhodopsin was obtained in 90% yield when 2 was combined with a CHAPS solution of bleached ROS for 22 hr. Since the corresponding hydroxymethyl precursor 8 formed a pigment with absorption maximum at 473 nm within 10 min, this greatly reduced reaction rate represents the influence of the diazoacetox group. The pigment had absorption maximum at 460 nm and displayed CD extrema at 467 and 360 nm characteristic of visual pigments and visual pigment analogs (Fig. 2). As in the case of many pigment analogs and some natural pigments, the new pigment was not stable to NH_2OH in the dark and was sensitive to light; irradiation at > 510 nm bleached the pigment within 15 min. This highly modified retinal was

tightly bound to the natural binding site and could not be displaced by addition of 11-*cis*-retinal to the pigment. This system therefore should be suitable to investigate the region of the binding site surrounding the polyene chain. However, the synthesis has to be improved prior to such studies.

3-Diazoacetox 9-*cis* rhodopsin formed from retinal 1 in bleached ROS suspension absorbs at 465 nm (or $21,505\text{ cm}^{-1}$) in 2% digitonin solution. Since the SBH⁺ formed with *n*-butylamine absorbed at 425 nm (or $23,525\text{ cm}^{-1}$, in MeOH) the opsin shift¹³ is 2020 cm^{-1} . This is similar to the $2,110\text{ cm}^{-1}$ opsin shift of 9-*cis*-rhodopsin derived from the absorption maxima shifts between SBH⁺ (with *n*-butylamine, 440 nm or $22,730\text{ cm}^{-1}$) and the pigment (485 nm or $20,617\text{ cm}^{-1}$). It was previously shown that the chromophore of this pigment analog occupies the natural binding site, and that opsin preferentially bound to one of the optical antipodes at C-3.⁸ Preliminary studies also indicated that the diazoacetox group could be photolyzed efficiently within the binding site.⁸ However, in order to estimate the percentage of crosslinking resulting from activation of the label, we had to carry out studies with the pigment formed from 3-(O¹⁴COCHN₂)-labeled 9-*cis*-retinal.

In bleached ROS suspension, the regeneration of rhodopsin and visual pigment analogs requires the use of several-fold molar excess of retinal in order to obtain a high yield of pigment or to saturate most of the binding sites. This was not only very inconvenient as it requires larger quantities of the precious retinal models, but the excessive amount can lead to complications when radiolabeled chromophores are being investigated. In the case of retinal 1 having radio-carbon in the diazoacetox group, photolysis to activate the label would lead to random labeling outside the binding site, because the excess unbound chromophore would also be activated and react with the membrane. Additional random labeling may result from light activation of retinal decomposition product(s) which may be formed during the regen-

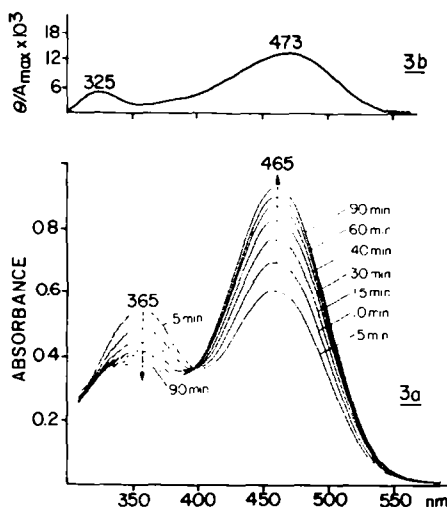
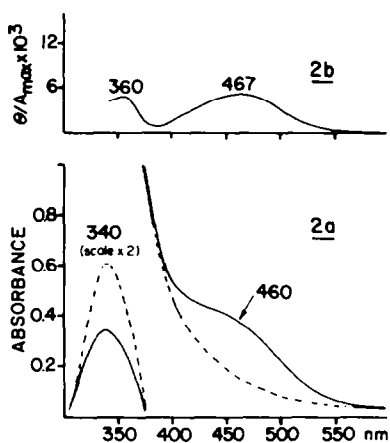


Fig. 2(a) Absorption curve of 9-methylenediazoacetox-9-*cis*-rhodopsin before (—) and after (---) bleaching. In 10 mM CHAPS/10 mM HEPES buffer, pH 7.0 at rt. (b) Circular dichroism curve of 9-methylenediazoacetox-9-*cis*-rhodopsin in 10 mM CHAPS/10 mM HEPES buffer, pH 7.0, dark, at rt.

Fig. 3(a) Formation of 3-diazoacetox-9-*cis*-rhodopsin in 10 mM CHAPS/10 mM HEPES buffer, pH 7.0, dark, rt. (b) Circular dichroism curve of 3-diazoacetox-9-*cis*-rhodopsin in 10 mM CHAPS/10 mM HEPES buffer, pH 7.0, dark, rt.

eration experiment. It is therefore necessary to remove excess unbound chromophore and decomposition product(s) from the membrane prior to photolysis experiments. This was accomplished either by repeated washing of the pigment in suspension with *n*-hexane or with fatty-acid-free bovine serum albumin (BSA).²⁸

However, when the pigment was regenerated in the apomembrane CHAPS solution (Fig. 3), it could be obtained in a pure state in high yield by using approximately equimolar amounts of chromophore and opsin, thus making the purification steps unnecessary. Moreover, regeneration in CHAPS proceeded much faster than in membrane suspension (1.5 hr as compared to 23 hr). The pigment thus formed displayed a CD spectrum similar to that resulting from solubilization in 2% digitonin, and could be stored at -20° for at least 24 hr without any decomposition. The apomembrane, however, was much less stable in CHAPS solution, and had to be used immediately following solubilization in order to obtain high regeneration yields.

Activation of the photolabel to obtain crosslinked rhodopsin

Efficient photolabeling requires the activation of the diazoacetoxyl group to form a carbene by irradiation close to its absorption maximum. Conditions for irradiation must also be chosen so as to minimize pigment destruction arising from concomitant absorption of light by the aromatic amino acid residues of opsin (Trp and Tyr), which may then undergo singlet-singlet energy transfer to the chromophore and hence cause bleaching of the pigment. Various conditions were tested to activate the label on purified pigment preparations formed in membrane suspension. Thus, irradiation experiments at rt, 254 nm, using narrow band interference filter did not succeed when a medium pressure Hg lamp (450W) was used or when the irradiation source was an excimer laser tuned to emit at 249 nm; indeed extensive protein damage was caused even by short periods of irradiation thought to be insufficient to activate all diazoacetoxyl groups.

It was then found that the diazoacetoxyl group in 9-*cis*-retinal 1 could be activated by irradiation at 4° with a low-pressure Hg lamp (90% emission at 254 nm) of low intensity (4W). Namely, in apolar solvents, such as *n*-hexane and cyclohexane, the diazoacetoxyl absorption band was destroyed in 4 hr but in the more polar medium of 2% digitonin/EtOH, the activation was complete in 2 hr. When the pigment was irradiated with this low intensity source, only ca 10% decrease in its 465 nm maximum was observed after 4 hr (Fig. 4b, curve 3), thus showing that no significant damage of the active site accompanied label activation. By using the CH_2Cl_2 extraction procedure to monitor the extent of crosslinking as a function of irradiation time it was found that considerable amount of crosslinking must have taken place after the 4 hr period, as the amount of extractable chromophore was only 15–30% of the non-irradiated control. A further indication of crosslinking was observed by comparing the rates of bleaching of the pigment by >500 nm light in 2% digitonin solution. As shown in Fig. 4(a), the pigment which was not pre-irradiated at 254 nm required 4 hr

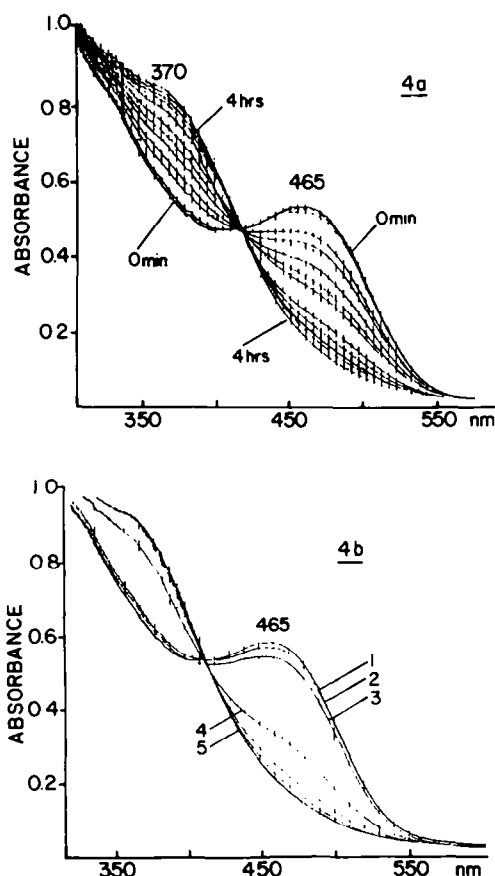


Fig. 4. All curves are measured in 2% digitonin/67 mM phosphate buffer, pH 7.0, rt. (a) Bleaching of 3-diazoacetoxyl-9-*cis*-rhodopsin by irradiation of >500 nm light. (b) Curve 1. 3-diazoacetoxyl-9-*cis*-rhodopsin, dark, rt. Curve 2 and 3. Pre-irradiated at 254 nm for 2 hr and 4 hr, respectively. Curve 4 and 5. Bleaching of pigment (curve 3) by irradiation at >500 nm for 1 min and 5 min, respectively.

to be completely bleached. In contrast, when the pigment was first crosslinked by the 254 nm irradiation, it was bleached after 4 min (Fig. 4b, curve 5).

Similar conditions of irradiation were adequate to activate the label when the pigment was prepared in a pure state in CHAPS solution at a concentration of ca 1.8 OD/ml. Thus, 8W low-pressure Hg lamp (98% emission at 254 nm) combined with a narrow-band interference filter was used for irradiation of the pigment solution in CHAPS at 4° . When the retinal is irradiated in CHAPS solution under these conditions the diazo band is photolyzed within 12 min, indicating that a shorter irradiation time than that used in suspension may be adequate to activate the label of the pigment prepared in CHAPS solution.

The extent of crosslinking was estimated more accurately by experiments carried out on pigment formed from radiocarbon containing labeled retinal as follows. The UV/VIS spectra were recorded after various irradiation times to monitor the extent of protein degradation, and concomitantly aliquots of the pigment were removed to be analyzed by electrophoresis. It was found that after 36 min irradiation only ca 10% loss of pigment absorption occurred, and

that this time was sufficient to activate the label. After electrophoresis on 9% polyacrylamide SDS gels of the irradiated pigment, thin slices of the gel were solubilized and were counted for radioactivity. After subtraction of background counts, the amount of radioactivity retained in the rhodopsin band amounted to approx. 15–20% of the loaded radioactivity. There was no radioactivity associated with the protein band when unphotolyzed rhodopsin was analyzed in a similar manner, thus showing that the 15–20% radioactivity is not due to noncovalently associated retinal.

Preliminary experiments conducted in CHAPS solution indicated that proteolytic cleavage of rhodopsin by chymotrypsin follows a different course from that observed in cleavage experiments on non-solubilized protein. Therefore, in order to determine the sites of crosslinking on the primary sequence of rhodopsin, we need to establish procedures to recover crosslinked rhodopsin from its CHAPS solution and/or to establish conditions for proteolytic cleavage in CHAPS. These experiments followed by sequencing work should lead to establishment of the crosslinking sites and thus contribute to our knowledge of the structure of rhodopsin.

EXPERIMENTAL

¹H-NMR spectra were measured on a Bruker WM-250 spectrometer in CDCl₃, CD spectra on a Jasco J-40 spectropolarimeter, and UV/VIS spectra on Jasco UVIDEQ or Perkin-Elmer 320 spectrometers. Radioactive counting was done on a Beckman LS 3133P liquid scintillation counter using Liquescent (National Diagnostics Ltd.) for aqueous samples and Betafluor for organic samples. Gel electrophoresis was carried out on a Savant 10 × 15 cm slab gel system powered by an LKB power supply (# 2103). Gels were stained with 0.1% Coomassie blue (G-250) in MeOH-AcOH-water (4.5:1:4.5) by heating at 60° for 1 hr followed by destaining in MeOH-AcOH-water (2.3:0.7:7) by heating at 60° for 2 hr followed by a change in the destaining soln and stirring overnight at rt. Preparative TLC was performed on Analtech silica gel GF plates (0.25 cm); Flash column chromatography²⁹ was carried out with silica gel (Merck No. 9385, 40–63 μm) and high performance liquid chromatography with a Waters HPLC system in the isocratic mode. Fresh cattle eyes were obtained from M. I. Cohen, Inc. and dark-adapted frozen retinæ from Hormel Corp. Commercial digitonin (Sigma) was processed according to the method of Bridges.³⁰ CHAPS was purchased from Calbiochem-Behring Corp. or Pierce Chem. Co. and was used without purification. Bovine serum albumin (essentially fatty acid free) was obtained from Sigma Chem. Corp. Buffer solutions were made in deionized water and were degassed by bubbling argon for 1 hr. All procedures involving synthesis of retinals and rhodopsins were conducted under dim red light and when required under argon.

(*t*-Butyldimethylsiloxy)-β-ionone (5). β-Ionone (9.01 g, 47 mM) was refluxed in 100 ml benzene with lead tetraacetate (13.88 g, 30 mM) for 12 hr. The cooled mixture was washed with water, then with NaHCO₃ aq followed by water again to give after evaporation of the solvent a yellow oil. Column chromatography on silica gel eluted with 20% ether in hexane afforded 5.8 g (49%) of monoacetate. ¹H-NMR δ 1.10 (s, 6H), 1.80 (s, 3H), 2.20 (s, 3H), 4.80 (s, 2H), 7.5 (d, J = 15 Hz, 1H), 6.20 (d, J = 15 Hz, 1H). Hydrolysis with 1% Na₂CO₃ in MeOH/H₂O (9:1) at –20° for 15 min and subsequent extraction with ether, washing of the extract with 2% H₂SO₄ and water and drying over Na₂SO₄ gave the hydroxy derivative after preparative TLC on silica gel, 30% ether in hexane (yield 60%). ¹H-NMR δ 1.10 (s, 3H), 1.50–2.12 (m, 6H), 1.82 (s, 3H), 4.47 (d, J = 4 Hz, 2H), 7.48 (d, J = 16 Hz,

1H), 6.18 (d, J = 16 Hz, 1H). The alcohol (1.66 g, 8 mM) in 20 ml dry DMF in presence of imidazole (2.18 g, 32 mM) was stirred with *t*-butyldimethylsilyl chloride³¹ (2.41 g, 16 mM) for 3 hr under argon at rt. The mixture was taken up in 2% ether in hexane (200 ml), washed with water three times and the organic layer was dried over MgSO₄. Purification by flash column chromatography on silica gel in 20% ether in hexane gave 1.8 g (75% yield) of 5. ¹H-NMR δ 0.11 (s, 6H), 0.95 (s, 9H), 1.09 (s, 6H), 1.81 (s, 3H), 4.31 (s, 2H), 7.52 (d, J = 16 Hz, 1H), 6.54 (d, J = 16 Hz, 1H).

(*t*-Butyldimethylsiloxy)-C-15-aldehydes (6). To lithium diisopropylamide (0.46 mM) in 1.5 ml dry THF under argon, silylated acetaldehyde *t*-butylimine³² (0.08 g, 0.46 mM) was added and the mixture was stirred at 0° for 15 min. The mixture was then cooled to –78° and ketone 5 (0.1 g, 0.3 mM) was introduced. The mixture was allowed to reach –20° over a period of 2.5 hr when it was quenched with 1 ml of water. The pH was adjusted to 4.5 by addition of solid oxalic acid and stirring was continued for another 30 min. 10 ml of brine was then added to the mixture before it was extracted with ether. The extract was washed with NaHCO₃ aq, dried over K₂CO₃ and concentrated under reduced pressure to obtain 6. The yield of 6 after preparative TLC on silica gel (30% ether in hexane) was 70%, and consisted of ca. 80:20 mixture of 9-*cis* and *trans* isomers. ¹H-NMR (9-*cis* isomer): δ 0.11 (s, 6H), 0.94 (s, 9H), 1.06 (s, 6H), 1.50–2.05 (m, 6H), 1.75 (s, 3H), 4.50 (s, 2H), 6.22 (d, J = 7.5 Hz, C₁₀-H), 6.50 (d, J = 16 Hz, C₇-H), 6.80 (d, J = 16 Hz, C₈-H), 10.17 (d, J = 7.5 Hz, –CHO); *trans* isomer: δ 0.04 (s, 6H), 0.94 (s, 9H), 1.06 (s, 6H), 1.50–2.05 (m, 6H), 1.75 (s, 3H), 4.80 (s, 2H), 5.98 (d, J = 8 Hz, C₁₀-H), 6.14 (d, J = 15.5 Hz, C₈-H), 6.85 (d, J = 15.5 Hz, C₇-H), 10.26 (d, J = 8 Hz, –CHO).

(9-Methylene-*t*-butyldimethylsiloxy)-retinals (7) and (9-hydroxymethylene)-retinals (8). To a slurry of NaH (0.005 g, 0.21 mM) in 1 ml dry THF triethylphosphonosenecioate (0.06 g, 0.21 mM) was added under argon at 0°, and the mixture was stirred at rt for 25 min. A soln of the 9-*cis* isomer of 6 (0.05 g, 0.14 mM) in 1 ml dry THF was then added and the mixture was stirred at 0° for 1 hr; after work-up and column chromatography of the crude product on silica gel using hexane 60 mg of the crude ester (90% yield), a mixture of 9-*cis* and 9, 13-*dicis* isomers, was obtained. Reduction with diisobutylaluminum hydride in dry THF followed by oxidation with MnO₂ in benzene, 30 min at 0°, led to the aldehyde 7 (ca 80% overall yield). The protective group was removed by treating 7 in dry THF with tetra-*n*-butylammonium fluoride³³ for 20 min at 0° to give 8 in ca 50% yield after purification by preparative TLC on silica gel (30% EtOAc in CHCl₃). The mixture of 9-*cis* and 9, 13-*dicis* isomers was separated by HPLC on a μ-Porasil column in 15% EtOAc in hexane. λ_{max} (hexane) 356 nm (9-*cis*), 349 nm (9, 13-*dicis*). The Schiff base with *n*-butylamine in MeOH absorbed at 327 nm and the protonated Schiff base at 427 nm. ¹H-NMR (9-*cis* isomer): δ 1.90 (s, 3H), 1.79 (s, 3H), 2.34 (s, 3H), 4.47 (br, 2H), 6.00 (d, J = 8 Hz, C₁₄-H), 6.36 (d, J = 11.5 Hz, C₁₀-H), 6.40 (d, J = 15 Hz, C₁₂-H), 6.45 (d, J = 16 Hz, C₇-H), 6.54 (d, J = 16 Hz, C₈-H), 7.20 (dd, J = 11.5, 15 Hz, C₁₁-H), 10.12 (d, J = 8 Hz, –CHO).

(9-Methylenediazoacetoxyl)-9-*cis*-retinal (2). Due to its lability even under argon at –70°, only minute amounts (ca 20 μg) of compound 2 could be isolated by treatment of 8 with the tosylhydrazone of glyoxylic acid chloride in presence of Et₃N.⁸ Retinal 2 after purification by HPLC on a Partisil column eluted with Me₂CN had UV in hexane at 356, 275 sh and 245 (diazo) nm.

3-[1-¹⁴C] Diazoacetoxyl)-9-*cis*-retinal. Exactly the same procedure⁸ was followed as was used for the synthesis of 1 but radioactive glyoxylic acid was employed to prepare the tosylhydrazone required for esterification of 3-hydroxyretinal. Thus, commercially available (Amersham) glyoxylic acid (specific activity 11.1 μCi/μM, total activity 250 μCi) was diluted with 16.5 mg of unlabeled glyoxylic acid and

reacted with 45 mg of tosylhydrazine and the reaction worked up after 2 hr of stirring by extraction with EtOAc. The yield of radioactive tosylhydrazone of glyoxalic acid was 70%. Half the material was then esterified with 3-hydroxy-9-*cis*-retinal. The mixture was initially purified by flash chromatography of SiO₂ and then by HPLC (solvent system 16% EtOAc/hexane). The authenticity of labeled 1 was established by coinjection in HPLC with a known sample of 3-diazoacetoxy-9-*cis*-retinal. The specific activity of labeled 1 obtained was *ca* 1 μ Ci/ μ M.

Binding of bovine opsin to retinals 1 and 2. Rod outer segments (ROS) were prepared either from freshly dissected retinæ or from frozen retinæ and were purified according to the procedure of Papermaster and Dreyer.²² For regeneration of pigments, opsin was freshly prepared each time by bleaching ROS in the presence of NH₂OH followed by repeated washing with buffer. The opsin suspension in 10 mM HEPES or 67 mM phosphate buffer (pH 7.0) was mixed with an EtOH soln (<2%) of the retinal and incubated at rt with stirring under argon. Aliquots of the incubation mixture were centrifuged, the pellets were solubilized by stirring in 2% digitonin/67 mM phosphate buffer (pH 7.0) 1 hr at rt followed by centrifugation to obtain a clear soln. The UV/VIS spectrum was then measured in each case to determine the extent of regeneration, and incubation was continued until no more increase at the absorption maximum of the pigment was observed. Alternatively, regeneration was also carried out in detergent solns as follows: 1 ml of 10 mM CHAPS in 10 mM HEPES buffer pH 7.0 was used to solubilize 1 OD of opsin by stirring 10 min at rt followed by centrifugation at 5000 rpm for 5 min (1 OD opsin is the amount which regenerates 1 OD of rhodopsin from 11-*cis* retinal). The opsin solution in CHAPS was then mixed with an EtOH soln of the retinal (<2% by volume) and the incubation was carried out in the dark under argon. Pigment formation was monitored by UV/VIS spectra. It was also possible to solubilize up to 2 OD of freshly made opsin in CHAPS and thus to obtain a more concentrated solution of the 3-diazoacetoxy-9-*cis*-rhodopsin (e.g. 1.8 OD/ml). It was, however, found that if freshly prepared opsin was not used or if the opsin was not used immediately following its solubilization in CHAPS, the yield of regeneration was considerably lower.

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