13-Desmethyl Rhodopsin and 13-Desmethyl Isorhodopsin: Visual Pigment Analogues

Ralph Nelson, * J. Kim deRiel, † and Allen Kropf

DEPARTMENT OF CHEMISTRY, AMHERST COLLEGE, AMHERST, MASSACHUSETTS

Communicated by George Wald, January 13, 1970

Abstract. The preparation and properties of three geometric isomers of 13-desmethyl retinal (13-dmr) are described. They are analogous to the all-trans, 11-cis, and 9-cis isomers of retinal since two of the cis isomers combine with cattle opsin to form pigments which are spectrally indistinguishable from rhodopsin and isorhodopsin and the all-trans isomer is unreactive.

The pigment which resembles rhodopsin, 13-desmethyl (13-dm) rhodopsin, is formed at about one ninth the rate at which 11-cis retinal reacts with opsin at 20°C. The reaction with 13-dmr does not go to completion; and 0.05 M hydroxylamine, to which rhodopsin is stable, decomposes 13-dm rhodopsin. Irradiation of 13-dm rhodopsin results in a cis → trans isomerization of the chromophore; but the photosensitivity of 13-dm rhodopsin is only 40 per cent that of rhodopsin.

13-dm isorhodopsin, the 13-desmethyl analogue of isorhodopsin, is formed at approximately the same specific rate as 13-dm rhodopsin. The reaction goes to completion and the pigment is not decomposed by 0.03 M hydroxylamine. 13-dm isorhodopsin can also be photolyzed to the all-trans chromophore plus opsin.

Introduction. The remarkable discrimination for geometrical shape shown by the visual protein, opsin, in its reaction with certain cis isomers of retinal is one of the more spectacular examples of the stereospecificity of proteins. Of five geometric isomers of retinal (vitamin A aldehyde) only the 11-cis and 9-cis compounds react with opsin to form photopigments. Three other isomers that have been studied (all-trans, 13-cis, and 9,13-dicis) are completely unreactive.1 In addition to the selectivity shown by opsin in the synthesis of visual pigments, there is also a stereospecificity shown during photolysis. When either rhodopsin (11-cis) or isorhodopsin (9-cis) is irradiated, the all-trans isomer of retinal is formed exclusively.1

We might expect to learn something of the structure of visual pigments, specifically in the region where the 11-cis or 9-cis chromophore is bound to the protein, by the use of synthetic chromophores which differ in known ways from the natural chromophore. We can attempt to explore the stereochemical properties of the region around opsin’s chromophoric site by studying its reactions with compounds of known shape and by measuring and comparing the spectral properties and stabilities of such visual pigment analogues with rhodopsin and isorhodopsin. The present experiments examine in this way the reactions of 13-desmethyl retinal (13-dmr) with cattle opsin.
13-dmr differs from the natural chromophore of rhodopsin only by having an —H instead of —CH$_3$ at C—13. The 13-methyl group does not affect the planarity of the all-trans or 9-cis isomers, but does overlap the C—10 hydrogen atom in 11-cis retinal and causes a nonplanarity, or twist, in its side chain. Hence, we might expect to find close similarities between the all-trans and 9-cis isomers of retinal and the corresponding isomers of 13-dmr in their interactions with opsin. On the other hand the 11-cis isomers may behave differently.

**Methods and Materials.** 13-desmethyl retinal: 13-dmr was prepared in our laboratory by Lodwig$^2$ according to methods described by van den Tempel and Huisman.$^3$ Subsequently, we received a sample of 13-desmethyl vitamin A acid methyl ester from Prof. Huisman. In the experiments described in this paper we have used 13-dmr from both sources, with identical results.

13-dm retinol was prepared from the methyl ester by reduction with either lithium aluminum hydride or diisobutyl aluminum hydride. Without purification the alcohol was oxidized with activated MnO$_2$ in hexane.$^4$ The aldehyde formed in this last step was purified by thin-layer chromatography on Merck silica gel H by development in ether-hexane (1:4 v/v).

**Preparation and purification of geometric isomers of 13-dmr:** Solutions containing 10–100 μg or purified 13-dmr in 95% ethanol were irradiated with unfiltered white light from a 200-watt tungsten bulb for periods of 10 to 30 min. Most of the solvent was evaporated off, and the contents were chromatographed on silica gel H with ether-hexane (1:4 v/v) at 0°C. Three bands were clearly resolved: cis II: $R_f = 0.55$; cis I: $R_f = 0.48$; and trans: $R_f = 0.32$.

Opins: Opin was prepared from cattle retinas by a modification of methods already described$^6$ for the preparation of rhodopsin. The rod outer segments collected at a phosphate buffer/40% sucrose solution interface were successively washed with pH 6.5 phosphate buffer, water, and 4% alum solution and then treated with 0.05 M hydroxylamine,$^6$ to convert metarhodopsin and retinaldehyde to retinaldehyde oxime. The rod outer segments were then dried by lyophilization, extracted with petroleum ether, treated with 2% digitonin solution, and finally cleared by centrifugation.

**Spectrophotometry and kinetics:** All spectra were recorded with either a model 14 or model 15 Cary spectrophotometer. Kinetic runs were made in the thermostated cell compartments of the spectrophotometers. All temperatures were measured in the cell compartments.

**Results. Geometric isomers of 13-desmethyl retinal:** Spectra of the three chromatographically purified isomers of 13-dmr are shown in Figure 1. Irradiation of solutions of any of the isomers results in a new spectrum, identical in all three cases. The designations cis I, cis II, and all-trans were chosen because the chromatographic behavior of the 13-dmr isomers paralleled the behavior of other cis and trans isomers of polyenes.$^7$ Furthermore, both isomers designated cis, on irradiation with white light, showed increased absorbance near 375 nm and decreased absorbance near 250 nm. The all-trans isomer, besides having the highest molar absorptivity at $\lambda_{max}$, showed the opposite behavior on irradiation. The photochemical and chromatographic behavior, the similarity of the spectra of the 13-dmr isomers to the trans and monocis isomers of retinal, and the reactions with opsin described below, implied the proposed stereochemical designations.

**Reactions of 13-desmethyl retinal with opsin:** Cis II 13-dmr reacts with cattle opsin to form a pigment which we shall call 13-dm rhodopsin and whose main absorption band, as measured in the difference spectrum, is almost identical
Fig. 1.—Ultraviolet spectra of three chromatographically purified isomers of 13-dmr in ethanol. All three isomers, when irradiated in ethanol with white light, transformed to the same mixture of isomers (*—*). The heights of the curves for the all-trans, cis I, and cis II isomers were adjusted so as to give a common curve for the photoisomerate.

with the corresponding band in cattle rhodopsin. Its $\lambda_{\text{max}}$ is at 495 ± 3 nm, and its band width at half maximum is only about 2–3 nm larger than that of rhodopsin (Fig. 2).

Fig. 2.—Difference spectra obtained when rhodopsin (—) and 13-dmr rhodopsin (●) were photolyzed at 20°C. Each solution contained 0.01 M NH$_2$OH. Both spectra were brought to optical density = 1 at $\lambda_{\text{max}}$. Since cis II 13-dmr does not react completely with ops in, it is not possible to measure the absorption spectrum of 13-dmr rhodopsin directly without interference from unreacted 13-dmr.
These similarities in spectrum contrast with a striking difference in reactivity. At 20°C the desmethyl analogue reacts with cattle opsin at approximately one ninth the specific rate of 11-cis retinal. This lower rate of combination probably contributes to a second important difference in behavior, the thermodynamic instability of 13-dm rhodopsin. In contrast to the opsin system, where 11-cis retinal and opsin combine almost stoichiometrically to yield rhodopsin, the reaction between cis II 13-dmr and opsin does not go to completion. Incubating cis II 13-dmr and opsin together yields an equilibrium mixture of 13-dm rhodopsin, cis II 13-dmr, and opsin. We have measured the concentration of opsin, cis II 13-dmr, and 13-dm rhodopsin present in equilibrium mixtures which were formed from solutions originally containing a molar excess of aldehyde as well as those containing an excess of opsin.

We find for the equilibrium, cis II 13-dmr + opsin ⇌ 13-dm rhodopsin, that the molar extinction coefficients of cis II 13-dmr and 13-dm rhodopsin are, respectively, 3.2 × 10^4 at 380 nm and 3.8 × 10^4 at 500 nm and the equilibrium constant, K, is 5 × 10^6 liters/mole at 20°C. Furthermore, in the limited temperature range, 15–25°C, K remains constant within our experimental error. ΔH° for this association-dissociation equilibrium is thus close to zero. Substituting into ΔS° = ΔH°/RT ln K we find ΔS° = 26 entropy units.

Another unusual property of 13-dm rhodopsin is its lability toward hydroxylamine, a reagent which condenses with the aldehyde group. We find that, in the presence of 0.05 M hydroxylamine at 20°C, 13-dm rhodopsin forms 13-dm retinaldehyde and opsin at a rate approximately ten times faster than its spontaneous rate of decomposition, as calculated from k_decomposition = k_formation/K. Some other visual pigments are known to be decomposed by hydroxylamine: iodopsin and the "green rod" pigment of the frog are unstable in NH_2OH solutions of about the same concentrations as used here.

We have used this hydroxylamine sensitivity to demonstrate that cis II 13-dm retinal and 11-cis retinal compete for the same site on opsin. We first formed an equilibrium mixture by incubating opsin and excess cis II 13-dmr at 20°C in the dark for 36 hr. Then we added 11-cis retinal to the solution and removed aliquots periodically. The first sample contained pigment with λ_max at 498 nm which was totally decomposed by NH_2OH. This was 13-dm rhodopsin. Absorption spectra taken of subsequent aliquots showed spectral curves identical with the first sample, but when NH_2OH was added to the solution, only a portion of the pigment with λ_max at 498 nm reacted to form an aldoxime with λ_max at 367 nm. The pigment remaining with λ_max at 498 nm was photolabile and we assumed it was rhodopsin. The decomposition of 13-dm rhodopsin by 11-cis retinal follows first-order kinetics, although the rate constant is only about a seventh as large as that for the spontaneous first-order decomposition of 13-dm rhodopsin.

13-dm isorhodopsin: Cis I 13-dmr also reacts with cattle opsin to form a pigment indistinguishable from isorhodopsin, the photopigment formed from 9-cis retinal and opsin. The reaction with opsin is second order for cis I dmr, as it is for cis II dmr, and at 20°C the rate constants are almost the same for the two isomers: 4.4 l/mole-sec for cis I and 5.5 l/mole-sec for cis II 13-dmr.
The absorption spectrum of the 13-desmethyl analogue of isorhodopsin is shown in Figure 3 together with that of cattle isorhodopsin. In this case we have been able to plot the absorption spectrum of 13-dm isorhodopsin over the entire spectral range, because cis I 13-dmr reacts completely when opsin is present in slight excess. The absorption spectrum of 13-dm isorhodopsin is essentially the same as that of isorhodopsin. 13-dm isorhodopsin also is stable in the presence of 0.03 M hydroxylamine.

![Absorption spectra of isorhodopsin and 13-dm isorhodopsin](image)

**Photochemistry of the 13-dm pigments:** Like rhodopsin and isorhodopsin, the 13-desmethyl pigments are isomerized by light to form the all-trans 13-dm retinal plus opsin. Neither the products of this direct photolysis of the 13-desmethyl pigments nor chromatographically purified all-trans 13-dm retinal react with opsin to form a pigment. Because of the lability of 13-dm rhodopsin in hydroxylamine solutions, this photochemical isomerization can be demonstrated in a novel way.

Figure 4 shows the difference spectra resulting from the action of light on 13-dm rhodopsin and 13-dm isorhodopsin in the presence of hydroxylamine. The oxime formed appears identical in the two cases and is presumed to be all-trans 13-dm retinaldoxime. On the other hand, 13-dm rhodopsin is decomposed by NH₂OH in the dark and the difference spectrum can be measured. Since the initial state for 13-dm rhodopsin is the same whether it is subsequently photolyzed or decomposed by NH₂OH, the 13-dm rhodopsin spectra from each process can be superimposed. It is then seen that the 13-dm retinaldoxime formed in the dark has a lower molar absorption than the 13-dm retinaldoxime formed as a
result of photolysis. It seems reasonable to suppose that the oxime formed in the dark is cis II 13-dm retinaldoxime, whereas light had isomerized the chromophore to the all-trans isomer.

By superposing the 13-dm retinaldoxime curves from the photolysis of 13-dm rhodopsin and 13-dm isorhodopsin, as was done in Figure 4, we can find the ratio of their molar absorptions (at 497 and 486 nm, respectively). This ratio is 0.93. The corresponding ratio for cattle rhodopsin and isorhodopsin is 0.94.1

Another significant comparison between rhodopsin and its 13-dm analogue concerns their relative photosensitivities. When a solution of rhodopsin and another of equal concentration of 13-dm rhodopsin were irradiated at about 23°C with orange light (λ > 513 nm; Corning 3486), the solution of 13-dm rhodopsin was photolyzed at 0.4 the rate of the rhodopsin solution. Though we have not yet directly measured the quantum efficiency of photolysis of 13-dm rhodopsin, its rate of bleaching relative to rhodopsin places it close to isorhodopsin in photosensitivity.12

The absence of the methyl group on C—13 means that there is no steric hindrance of the polyene side chain in 13-dm rhodopsin. It is not surprising that this pigment is similar in photosensitivity to isorhodopsin, which also has an unhindered polyene chromophore. The lowered photosensitivity of 13-dm rhodopsin also tends to support the suggestion12 that the twist in the polyene chain resulting from the steric hindrance of the methyl group on C—13 with the hydrogen atom on C—10 in the 11-cis chromophore of rhodopsin is responsible for rhodopsin's high photosensitivity.
Conclusion. Other reports of pigments formed from opsin and retinal analogues\textsuperscript{15–16} have indicated that structural modifications in the ionone ring, such as formation of a 5,6-epoxide, saturation of the 5-6 double bond, or oxidation at the 4-position, do not interfere with pigment formation. These studies did not, however, examine the thermodynamics or kinetics of interaction between the chromophore and opsin, or the role of geometric isomerism in the synthesis and photolysis of the pigment analogues formed. After the present experiments were completed, a communication appeared reporting reactions of 13-dmr with opsin.\textsuperscript{16} The $\lambda_{\text{max}}$ for the pigments observed agree with ours, but the authors state that their pig mens do not react with hydroxylamine. This discrepancy in the behavior of 13-dm rhodopsin might be due to the use of different NH$_2$OH concentrations.

The use of 13-dmr as an analogue of retinal has established effects of the 13-methyl group on the binding of the 11-cis chromophore, its specific rate of combination with opsin, the stability of cattle rhodopsin towards hydroxylamine, and the photosensitivity of rhodopsin. On the other hand the 13-methyl group has no important effect on the absorption spectra of the visual pigments. We\textsuperscript{17} and Blaž et al.\textsuperscript{18} have shown that analogues of retinal lacking the 9-methyl group yield pigments that differ from rhodopsin and isorhodopsin in their absorption spectra.

One structural feature resulting from the C—13 methyl group which may account for our results is the large difference in twist in the polypene side chains of 11-cis retinal and cis II 13-dmr, which we take to have the 11-cis configuration. Another more direct effect of the absence of the methyl group on C—13 may be decreased van der Waals or "hydrophobic" interactions between the chromophore and protein in 13-dm rhodopsin and, hence, a decreased stability of this pigment relative to rhodopsin. A combination of these two and perhaps other factors may be involved; but it seems to us premature to try to decide now. It does seem clear, though, that studies of pigment formation by opsin and molecules related to retinal can increase our understanding of the properties of visual pigments, and may ultimately lead to a clearer understanding of the forces acting between their chromophores and the protein.

We wish to acknowledge the help of several Amherst students, Sigfried Ludwig, Michael Garrett, and John McDowell. We are particularly grateful to Prof. H. O. Huisman of the University of Amsterdam for the gift of synthetic samples of the retinal analogues, and to Dr. Jan L. Baas who generously shared his knowledge of the properties of these compounds with us. We also wish to thank Dr. Ruth Hubbard, Dr. George Wald, and Mrs. Rita Kropf whose comments helped to make this paper more readable. We wish also to thank the USPHS for supporting this work through grant NB-1923.

* Present address: Department of Biophysics, Johns Hopkins University, Baltimore, Md. 21218.
† Present address: 1846 Dwight Way, Berkeley, Calif. 94703.
‡ Requests for reprints may be addressed to Dr. Kropf, Department of Chemistry, Amherst College, Amherst, Mass. 01002.
BIOCHEMISTRY: NELSON, DERIEL, AND KROPF  PROC. N. A. S.

6 Bownds, D., private communication.
12 Hubbard, R., and A. Kropf, these PROCEEDINGS, 44, 130 (1958).
17 de Riel, K., R. Nelson, and A. Kropf, unpublished observations.