Analogue Pigment Studies of Chromophore–Protein Interactions in Metarhodopsins†

Geoffrey Renk and Rosalie K. Crouch*

Departments of Ophthalmology and Biochemistry, Medical University of South Carolina, Charleston, South Carolina 29425

Received April 15, 1988; Revised Manuscript Received August 23, 1988

ABSTRACT: Several analogue pigments have been prepared containing retinals altered at the cyclohexyl ring or proximal to the aldehyde group in order to examine the role of the chromophore in the formation of the metarhodopsin I and II states of visual pigments. Deletion of the 13-methyl group on the isoprenoid chain did not affect metarhodopsin formation. However, analogue pigments containing chromophores with modified rings did not show the typical absorption changes associated with the metarhodopsin transitions of native or regenerated rhodopsins. In particular, 4-hydroxyretinal pigments did not show clear transitions between the metarhodopsin I and metarhodopsin II states. Pigment formed with an acyclic retinal showed no evidence by absorption spectroscopy of metarhodopsin formation. A retinal altered by substitution of a five-membered ring containing a nitroxide required a more acidic pH than the native pigment for formation of the metarhodopsin II state. ESR data suggest that the ring remains buried within the protein through the metarhodopsin II state. However, the Schiff base linkage is susceptible to hydrolysis of hydroxylamine in the metarhodopsin II state. These data indicate that (1), in the transition from rhodopsin to metarhodopsin II, major protein conformational changes are occurring near the lysine–retinal linkage whereas the ring portion of the chromophore remains deeply buried within the protein and (2) pigment absorptions characteristic of the metarhodopsin I and II states may be due to specific protein–chromophore interactions near the region of the chromophore ring.

Rhodopsin is the primary photosensitive pigment in the mammalian retina. This transmembrane glycolipoprotein comprises the majority of the protein in the rod outer segment disk membrane and contains one molecule of retinal (vitamin A aldehyde; Wald, 1967) covalently attached via a protonated Schiff base linkage to the ϵ-amino group of lysine (Bownds, 1967). The absorption of light by the protein initiates the sequence of events which triggers an electrical response at the plasmalemma of the rod cell. The full mechanism by which a single photoexcited rhodopsin can affect this response is not yet fully elucidated but involves the interaction of G protein with the surface of rhodopsin as a signal mechanism [for a recent review, see Hurley (1987)].

The electrophysiological events at the plasmalemma are related to the late intermediates metarhodopsin (meta) I and II (Matthews et al., 1963). Evidence has been presented that these intermediates are able to activate cytoplasmic enzymes (Emeis et al., 1982; Bennett et al., 1982; Yamamoto & Schichi, 1983) and that conformational changes in rhodopsin have occurred in this transition (Kuhn et al., 1982; de Grip et al., 1985). To further define these conformational changes in the rhodopsin to meta I or meta II transition, we have investigated the chromophore–protein interactions using pigments containing structural derivatives of the chromophore.

Several groups have used synthetic structural analogues of retinal to define the specific requirements of the retinal binding site for pigment formation and photobleaching of the resulting pigments [see, for example, Balogh-Nair and Nakanishi (1984) and Crouch (1986)]. Activation of phosphodiesterase by analogue pigments has been previously documented (Ebrey et al., 1980; Fukada et al., 1982). We have extended this approach to the study of the protein dynamics of the late photoproducts of rhodopsin. In previous work, we have shown that the ring of the chromophore is well sequestered within the protein (Renk et al., 1987) and that a stable, bleachable pigment can be formed with an acyclic chromophore (Crouch & Or, 1983). We have now undertaken to determine if rhodopsin pigment analogues undergo photochemical changes similar to rhodopsin when under conditions which favor the formation of the meta intermediates in the native pigment. We present here our findings on the absorption characteristics of several analogue pigments and the corresponding changes which occur in one pigment at the chromophore binding site as revealed by electron spin resonance (ESR) spectroscopy. These data suggest that the meta I–meta II transition involves protein conformational changes in the region of the protein–chromophore Schiff base linkage and that the absorption characteristics of these states are strongly dependent on the interaction of the cyclohexyl ring of the chromophore with the protein.

Materials and Methods

All experiments were performed under dim red light except where noted. Absorption spectra were recorded on a Varian 2200 spectrometer.

Retinals (Figure 1). 11-*cis*-Retinal (**1**) was the generous gift of Hoffmann-La Roche. 9-*cis*-Retinal was purchased from Sigma Chemical Co. 11-*cis*-13-Desmethylretinal (**2**) was the generous gift of Dr. Walter Waddell and was purified before use by thin-layer chromatography (TLC) in 10% ether/hexanes. 9-*cis*-**2** was prepared and purified according to the method of Kropf et al. (1973). 11-*cis*-4-Hydroxyretinal (**3**) was synthesized from 11-*cis*-retinal by refluxing in dry tetrahydrofuran at 80 °C in the presence of selenium dioxide for 90 min and was stirred at room temperature overnight (Renk

† This work was supported by NIH Grant RO1 EY04939. G.R. was a recipient of an NIH Training Grant in Biotechnology Resources at the National Biomedical ESR Center.

* To whom correspondence should be addressed.

0006-2960/89/0428-0907$01.50/0

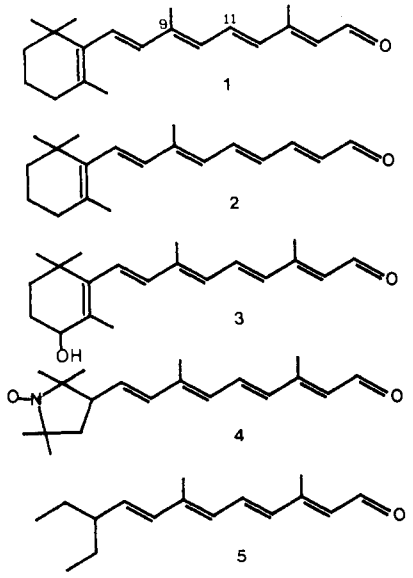

FIGURE 1: Structures of retinal analogues. Compounds are numbered to compare with the analogous atom in the native retinal molecule.

et al., 1981). The product was filtered, solvent removed under reduced pressure, and the pure 11-cis product purified by flash chromatography on silica gel in 10% ether/hexanes. 9-*cis*-**3** was likewise formed from 9-*cis*-retinal. The preparation and purification of the spin-labeled 9-*cis*-retinal (**4**) has been described (Renk et al., 1987). The 9-cis acyclic analogue **5** was prepared as described previously (Crouch & Or, 1983). The molecular weights of all retinals were confirmed by mass spectral analysis and the isomeric forms by nuclear magnetic resonance spectroscopy. All analogues were stored at −70 °C under argon and repurified before use by thin-layer chromatography.

Pigments. Rod outer segments (ROS) were isolated from bovine retinae (Hormel) essentially as described by Papermaster and Dreyer (1974), except all solutions were buffered with potassium phosphate (67 mM, pH 7.0, containing 2 mM $MgCl_2$). ROS were stored frozen at −70 °C. For use in analogue studies, an aliquot of ROS was bleached in white light (50 mW/cm^2) for 90 s in hydroxylamine (20 mM in phosphate buffer, pH 7.0) and centrifuged (12000*g*, 3 min). The pellet was resuspended in 2% bovine serum albumin (BSA) in the phosphate buffer, pH 7.4, or Tris–acetate buffer (10 mM, pH 7.4, containing 65 mM KCl, 2 mM $MgCl_2$) and centrifuged (three times) and washed (3 times) with buffer alone. The final pellet was resuspended in the buffer, and pigment was formed by stirring with 2-fold molar excess of the retinal analogue in ethanol (final concentration <1%) in the dark (4 °C, 12–18 h). Excess analogue was removed by washing with 20 mM hydroxylamine in phosphate buffer followed by 1% BSA in phosphate buffer.

Final suspension was in one of the following buffers: potassium phosphate, 67 mM, at pH 7.4; 10 mM Tris–acetate, 10 mM, 65 mM KCl, 0.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, at pH 8.5; NaOAc/HOAc, 10 mM, with the same salts at pH 5.1 or 4.5 (Kuhn et al., 1982; de Grip et al., 1985). Irradiation in the sample cells in the respective instrument cavities was with white light via fiber optics to isolate the sample from the heat of the light source. No differences were seen between filtered (No. 2A, <415 nm) and unfiltered light. When the protocol required changing the pH of the suspension, the sample was withdrawn from the sample cell and placed in a cold microcentrifuge tube, pelleted at 4 °C in the dark, and resuspended in cold buffer at the new pH.

ESR Measurements. ESR experiments were performed on a Varian E-4 spectrometer. Spectra were digitized and stored in a minicomputer (DEC PDP-11). ESR experiments were performed with the suspensions in a quartz flat cell. Additions to suspensions in the cavity were made with a mixing chamber/flow cell.

Saturation-transfer ESR (ST-ESR) measurements were made on a Varian Century series spectrometer, National Biomedical ESR Center at the Medical College of Wisconsin, Milwaukee. Rotational correlation time was estimated by the measurement of the peak height ratios (Thomas et al., 1976) and comparison to standard curves of correlation time versus spectral parameters of spin-labeled proteins of known hydrodynamic radius in solutions of known viscosity (Kusumi et al., 1980). Cavity temperature was monitored with a copper–constan thermocouple and controlled with a standard Varian variable-temperature controller. After centrifugation with an Eppendorf microcentrifuge for 30 s, ROS samples were introduced into the ESR cavity in a gas-permeable TPX (methyl pentane polyenes) capillary. At saturating power (100 mW), the sample was allowed to equilibrate with the nitrogen stream to remove all dissolved oxygen until no further increase in the ST-ESR signal amplitude was seen (Popp & Hyde, 1981). Modulation frequency was 100 kHz, and detection frequency was 50 kHz, 90° out of phase, to give the V'_2 display (second harmonic). The detector phase was checked immediately before and after each spectrum and shown to be less than 1% of the in-phase signal at 0.5 mW. Microwave power settings were such that the field intensity (H_1) at the sample was kept constant over all temperatures used as described by Kusumi et al. (1980). Magnetic field sweep was calibrated with Fremy's salt in saturated sodium carbonate. ROS samples were irradiated when appropriate prior to introduction of the pelleted membranes into the TPX capillary.

RESULTS

Retinals were purified and fully characterized before use. All the pigments have been previously reported except for the 9-*cis*- and 11-*cis*-4-hydroxy pigments (Kropf et al., 1973; Ebrey et al., 1975; Crouch & Or, 1983; Renk et al., 1987). Pigments were stable to the addition 11-*cis*-retinal as determined by the lack of decrease or shift in the absorption spectra over 60 min at 22 °C. Pigments were moderately stable to hydroxylamine under the conditions of the experiments, as judged by the above criteria. However, at 37 °C some decomposition was noted over a period of hours as has been previously reported by Kropf et al. (1973) for the 13-desmethyl pigments.

Rhodopsins. When rhodopsin is irradiated at pH 8.5 (2 °C), the bleaching sequence proceeds to an equilibrium of meta I and meta II favoring meta I. When irradiated at pH 5.1 (2 °C), the equilibrium favors the meta II intermediate. The meta I mixture at pH 8.5 will shift to favor meta II if resuspended in buffer at pH 5.1 (Kuhn et al., 1982; de Grip et al., 1985). This shift can then be reversed again by a return to alkaline pH. The addition of hydroxylamine to a meta equilibrium mixture in the dark results in the bleaching of the pigment with formation of the retinyl oxime (Ratner et al., 1981). Regenerated rhodopsins formed from opsin and 9- or 11-*cis*-retinal showed identical behavior under these conditions (Table I).

13-Desmethyl Pigments. When the 11-*cis*-13-desmethyl (**2**) pigment is irradiated at pH 5.1 at 2 °C, the λ_{max} of the absorbtion spectrum shifts to 380 nm, favoring meta II in the same proportion as in the native pigment (Figure 2a). Bringing the pH to 8.5 at 2 °C in the dark causes the equi-

Table I: Metarhodopsin States of Analogue Pigments[a]

chromophore	absorption maxima (nm) rhodopsin state	meta I state	meta II state
9-*cis*-retinal (**1**)	485	478	380
11-*cis*-retinal (**1**)	500	478	380
9-*cis*-13-desmethylretinal (**2**)	490	485	380
11-*cis*-13-desmethylretinal (**2**)	497	485	380
9-*cis*-4-hydroxyretinal (**3**)	460	ca. 450	ca. 390
11-*cis*-hydroxyretinal (**3**)	470	ca. 450	ca. 390
spin-labeled 9-*cis*-retinal (**4**)	448	440	380[b]
acyclic 9-cis-retinal (**5**)	454		

[a] Rhodopsin state in 10 mM potassium phosphate, pH 7.4, 24 °C; meta II state in 10 mM NaOAc/HOAc at pH 5.1, 2.0 °C. All buffers contained 5 mm KCl, 0.2 mM $CaCl_2$, and 0.2 mM $MgCl_2$. All absorption maxima are ±2 nm. The values for the 4-hydroxy intermediates are only approximate (±10–15 nm). [b] pH 4.5.

librium to favor meta I as in rhodopsin, and the shift is freely reversible. Exposure of the equilibrium mixture to 20 mM hydroxylamine abolishes the visible absorbance at the λ_{max} of the meta intermediates and forms the retinyl oxime. Thus the photochemistry of the 11-*cis*-13-desmethyl pigment mimics that of rhodopsin. The 9-*cis*-13-desmethylretinal pigment likewise formed stable meta I and meta II states with absorption maxima identical with those of the 11-cis chromophore (Table I), and these photoproducts likewise are labile to hydroxylamine.

4-Hydroxy Pigments. The 11-*cis*-4-hydroxy (**3**) pigment undergoes changes in the absorption spectrum which suggest that it may be able to form meta intermediates (Table I) although the transitions are not as clear (Figure 2b) as for the retinal and 13-desmethylretinal pigments. Illumination at pH 5.1 at 2 °C for 6 min caused a slow shift of the λ_{max} to ca. 390 nm with a broad shoulder at longer wavelengths (curves B and C). Changing the pH to alkaline (8.5) resulted in a shift toward longer wavelengths (curve D). Thus, these two species showed behavior typical of meta states although the contributing absorbance spectra were not well resolved. The 9-*cis*-4-hydroxy (**3**) pigment likewise shows spectral changes under the same conditions which suggest the formation of a meta I and meta II state (Table I), the spectral changes again not being well resolved. On extended experiments with the 4-hydroxy pigments, a small amount of the 3,4-didehydroretinal pigment was observed as evidenced by the formation of a species at longer wavelengths. In Figure 2b (curves C and D), the shoulders at 500–520 nm are most likely due to the formation of this pigment. The dehydro pigment results from the loss of the 4-OH group which, as an allylic substituent, is particularly labile. The 9-*cis*- and 11-*cis*-3,4-didehydro pigments have been previously reported by Azuma et al. (1973).

Acyclic Pigment. Manipulation of the pigment formed from the acyclic compound **5** failed to produce a bona fide meta intermediate identifiable by absorption spectroscopy. The manipulations included variation in pH from 4.0 to 9.0 and various permutations of temperature, time, and illumination. Decreases obtained in the absorption were unstable and irreversible with pH and were thus considered to be probably a result of degeneration of the pigment (Figure 2c).

Spin-Labeled Pigment. The meta II state with SLR **4** pigment forms only if the pH is lowered to 4.5. Irradiation at 0 °C then resulted in formation of a meta II state absorbing at 380 nm (Renk et al., 1988; Table I). Resuspension in pH 8.5 at 2 °C brought the λ_{max} to 440 nm, corresponding to that of the meta I state. Returning the pH to 4.5 reversed the mixture to the meta II state, thus demonstrating that an equilibrium had been established between two meta states, with

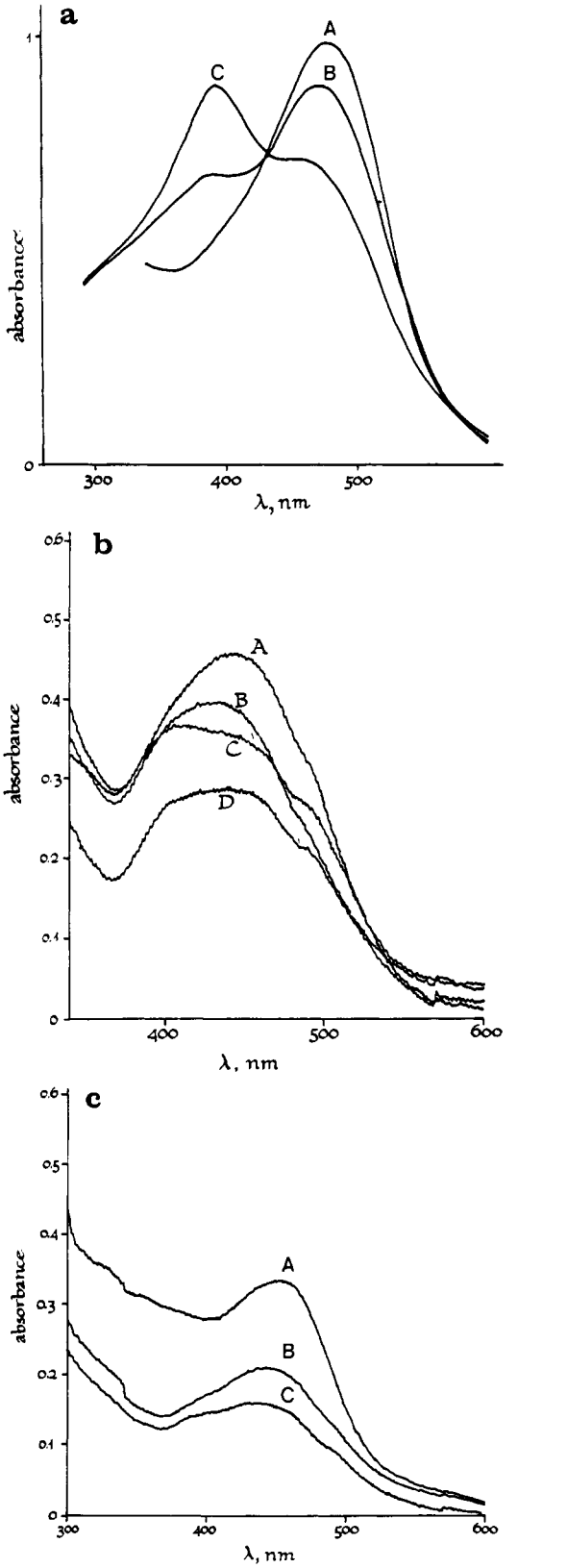

FIGURE 2: Absorption spectra of metarhodopsin intermediates of analogue pigments. Rhodopsin states at pH 7.4 in 70 mM potassium phosphate buffer, 22 °C; meta I states at pH 8.5, 2 °C, in 10 mM Tris–acetate buffer; meta II states at pH 4.5 or 5.1, 2 °C, in 10 mM NaOAc/HOAc buffer. (a) 11-*cis*-13-Desmethylretinal (**2**) pigment: (curve A) rhodopsin state at pH 7.4; (curve B) meta I state after illumination at pH 8.5, 2 °C; (curve C) meta II state after pH adjusted to 5.1, 2 °C. (b) 11-*cis*-4-Hydroxyretinal (**3**) pigment: (curve A) rhodopsin state at pH 7.4; (curve B) after illumination at pH 5.1, 2 °C, for 1 min; (curve C) after illumination for 6 min at pH 5.1; (curve D) after pH adjusted to 8.5. (c) Acyclic 9-*cis*-retinal (**5**) pigment: (curve A) rhodopsin state at pH 7.4; (curve B) after illumination at pH 4.5 at 2 °C for 2 min; (curve C) after a further 15 min at 20 °C.

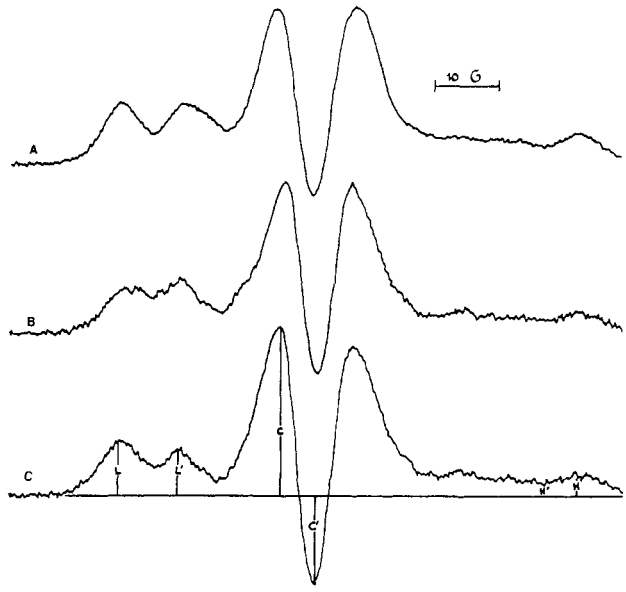

FIGURE 3: Saturation transfer ESR spectra of SLR (**4**) pigment: (A) dark adapted at 2 °C; (B) meta II formed at pH 4.5, 2 °C; (C) meta I formed at pH 8.5, 2 °C.

a pH dependence of the meta I/meta II transition similar to that of the meta equilibrium in rhodopsin. Both photoproducts were abolished by the addition of hydroxylamine in the dark, whereas the unilluminated SLR pigment is stable to hydroxylamine. The meta I species could also be formed directly by irradiation of the pigment at pH 8.5 at 0 °C. These absorbance spectra were broad and covered both the meta I and meta II absorbance maxima, underscoring the fact that either state is an equilibrium of both species. Some loss in intensity was observed over two cycles with concomitant increases in the absorbance near the SLR λ_{max} indicating that some of the meta I/meta II equilibrium mixture was escaping into the late photoproducts, free chromophore, and bleached opsin.

The conventional ESR spectrum of the SLR pigment in the dark at 0 °C is typical of a nitroxide which is strongly immobilized with a $2A_{ZZ}$ value of 64.1 G (Renk et al., 1987). When the pigment is irradiated at pH 4.5 at 0 °C no significant change in the ESR spectrum is produced. Under conditions that lead to the meta II state changes, there is a slight decrease in the separation of the hyperfine extrema as well as some minor line-shape changes (Renk et al., 1988).

The ST-ESR spectrum of the dark-adapted pigment showed strong evidence for a highly anisotropic motion of the SLR (Figure 3, curve A). The correlation times for the dark-adapted SLR pigment derived from each of the three ratios L'/L, C'/C, and H'/H (defined in Figure 3) are significantly different and cover a range of 2 orders of magnitude. The C'/C ratio yields a much faster estimate than the other two measurements. The H'/H measurement would place the rate of rotation at longer than 5×10^{-4} s, which is slower than the rate at which the calibration curves are accurate. The L'/L measurement is in the range of previous estimates of the rotational correlation time by transient dichroism measurements (Cone, 1972) and other ESR studies (Baroin et al., 1977; Kusumi et al., 1978, 1982). These discrepancies suggest a probable high degree of anisotropy of the motion of the SLR nitroxide (Kusumi et al., 1978; Marsh, 1980; Delmelle et al., 1980) and raise the issue of which value, if any, best approximates the actual rate of motion of the spin-labeled retinal within the rhodopsin molecule.

Under the conditions described above which favor the formation of the meta II state, the ST-ESR spectrum showed a clear change in line shape (Figure 3, curve B) relative to that of the dark-adapted pigment. These changes in line width and splitting constants indicate an increase in mobility in the meta II state.

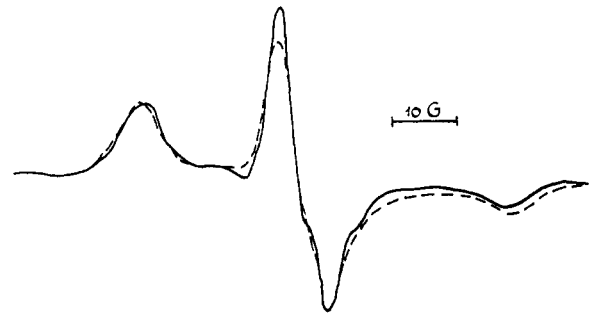

FIGURE 4: Low-temperature ESR (V^1) spectra of SLR (**4**) pigment: (a) dark adapted at pH 4.5, 2 °C (--); (b) meta II formed by illumination at 4.5, 2 °C (—). Both spectra at −40 °C in 30% glycerol/10 mM NaOAc/HOAc.

To investigate by conventional ESR changes in polarity between the rhodopsin and meta II states, samples were brought to −40 °C before and after formation of the meta II state to remove the effects of motional differences. Under these circumstances, the primary physical property of the system responsible for splitting of the outer hyperfine extrema is the relative polarity of the solvent in which the spin-label is dissolved (Knowles et al., 1976) or, in this case where it is surrounded by the protein in the binding site, the microenvironment of the binding site. The unilluminated pigment at −40 °C had a spectrum which is that of a completely immobilized nitroxide in a fairly nonpolar milieu (Figure 4, curve a). When the meta II state of the SLR pigment was brought to −40 °C, there was a small but significant decrease in the separation of the hyperfine extrema, suggestive of a more polar environment around the spin-label (Figure 4, curve b).

Given the above evidence for a more polar microenvironment at the chromophore ring end of the binding site, it is of interest whether this effect reflects changes in the protein–chromophore interactios or an increased exposure of the chromophore to the aqueous environment, i.e., an "opening up" of the normally "tightly sealed" binding site. To address this, small hydrophilic agents capable of absorbing the ESR signal of the nitroxide, either by reduction of the nitroxide (ascorbate; Seigneuret et al., 1984) or by spin–spin interactions (nickel chloride; Rousselet et al., 1984), were studied. When the meta II pigment is exposed to nickel chloride, the bound signal arising from the pigment chromophore remains unchanged. Similarly, ascorbate is able to reduce the ESR signal of the bound SLR under these conditions only at a rate paralleling the stability of the pigment. For comparison, when free SLR is bound to BSA in solution, ascorbate is able to rapidly reduce the bound SLR.

Discussion

Analogues were selected for these studies on the basis of specific structural changes from the native 11-*cis*-retinal (Figure 1). 13-Desmethylretinal (**2**) lacks the side-chain methyl group at the 13-position. 4-Hydroxylretinal (**3**) contains the moderately electronegative hydroxyl group on the ring. The acyclic derivative **5** lacks an intact ring but does contain a portion of the ring skeleton. In the spin-labeled retinal (SLR) analogue **4**, the cyclohexenyl ring has been replaced with the five-membered pyrrole ring containing the nitroxide group, a highly electronegative species. Thus, there is a range of steric modifications as well as electronegative

substitutions that affect either the ring end or the side chain of the chromophore and can be used to define the physiochemical constraints on chromophore–protein interaction in the photochemical functioning of mammalian rhodopsin. In addition, the SLR analogue provides a direct physical measurement of changes in the interaction as the process occurs.

All analogues in this study have been shown to form stable photosensitive pigments when combined with bovine opsin (Kropf et al., 1973; Ebrey et al., 1975; Crouch & Or, 1983; Renk et al., 1987). Regenerated rhodopsin was found to behave identically with the native pigment in the formation and interconversion of meta intermediates. Similarly, 9-*cis*-rhodopsin is known to proceed through the same bleaching pathway as native rhodopsin via the common intermediate bathorhodopsin (Mao et al., 1980). Thus, results obtained with the 9-cis analogues are directly comparable to those of the 11-cis isomers.

In this study, we have varied both the cyclohexyl ring and the isoprenoid side chain of the retinal chromophore and examined the formation of the late photoproducts meta I and meta II, which are implicated in the activation of the transduction process (Abrahamson & Fager, 1973). Our results here show that while the acyclic analogue fulfills the structural requirements for formation of a pigment, a meta II intermediate is not formed by this pigment as assessed by absorption spectroscopy. The SLR and 4-hydroxy pigments, which have modified but intact ring structures, form meta I and meta II intermediates. However, the 4-hydroxy pigment does not have clearly defined spectral shifts, and the markedly altered ring structure of the SLR analogue requires more acidic conditions than retinal itself. These results indicate the importance of an intact ring to the formation of the meta intermediates and that modification to the ring can also affect meta formation. The ease with which the 13-desmethylretinal pigment is able to form the meta intermediates suggests that this side-chain methyl group is not limiting in the function of the pigment.

Further evidence for the role of the chromophore ring comes from the ESR results which demonstrate a difference in accessibility to the two ends of the retinal chromophore. Neither ascorbate nor nickel has the ability to affect the ESR signal of the SLR pigment, indicating that these species are excluded from the ring end of the binding site where the nitroxide reporter group is located. This inability persists in light under conditions which favor the formation of stable meta intermediates. However, the Schiff base at the opposite end of the molecule becomes susceptible to nucleophilic attack by hydroxylamine with destruction of the pigment even before formation of the meta intermediates (Ratner et al., 1981). Thus these results show that while the ring remains shielded from the aqueous environment, the Schiff base is exposed on meta formation.

The conventional ESR results had shown that the nitroxide at the ring end of the chromophore does not experience a major change in its interaction with the surrounding protein on the formation of the later photointermediates (Renk et al., 1988). The Fourier transform infrared spectroscopy data of de Grip et al. (1985) provide strong evidence for protein rearrangement at the lumi-meta I–meta II transitions involving significant alterations in the relationship of different transmembrane helices to one another. It is possible that the ring of the chromophore is anchored firmly in its binding site formed by several of the transmembrane helices and that this portion of the retinal–protein complex acts as a relatively fixed point. Cis–trans isomerization may then force the Schiff base end with its attached helix (connected to the carboxy-terminal domain) to undergo most of the motion involved in presenting new conformations to the cytoplasmic surface (Hargrave, 1982). In such a scheme the spin-label would not be expected to "report" large-scale changes in the protein conformation on photoexcitation or to display significant motional modulations.

In summary, we have demonstrated that the structure of the ring portion of the chromophore strongly affects the ability of resulting pigment to form meta intermediates detectable by absorption spectroscopy. However, pigments formed from chromophores with alterations proximal to the aldehyde end of the polyene chain show typical metarhodopsin transitions. The conventional and ST-ESR data on the SLR suggest that the chromophore ring remains deeply buried within the protein through the meta II state although significant conformational changes may be occurring in other portions of the protein. These conformational changes may be significantly altering the protein environment near the Schiff base linkage and thus opening up this site for the eventual hydrolysis of the chromophore.

ACKNOWLEDGMENTS

We thank Drs. Feix and Kusumi at the National Biomedical ESR Center at the Medical College of Wisconsin for helpful discussions and advice. We are grateful to Dr. Walter Waddell, Goodyear, Inc., for the 13-desmethylretinals and Hoffmann-La Roche for the 11-*cis*-retinal.

REFERENCES

Abrahamson, E. W., & Fager, R. S. (1973) *Curr. Top. Bioeng. 5*, 125–200.

Azuma, M., Azuma, K., & Kito, Y. (1973) *Biochim. Biophys. Acta 295*, 520–527.

Balogh-Nair, V., & Nakanishi, K. (1984) in *New Comprehensive Biochemistry* (Tamm, C., Ed.) Vol. 3, pp 283–334, Elsevier Biomedical, Amsterdam.

Baroin, A., Thomas, D. D., Osborne, B., & Devaux, P. F. (1977) *Biochem. Biophys. Res. Commun. 78*, 442–447.

Bennett, N., Michel-Villaz, M., & Kuhn, H. (1982) *Eur. J. Biochem. 127*, 97–103.

Bownds, D. (1967) *Nature (London) 216*, 1178–1181.

Cone, R. (1972) *Nature (London), New Biol. 236*, 39–43.

Crouch, R. (1986) *Photochem. Photobiol. 44*, 803–807.

Crouch, R., & Or, Y. S. (1983) *FEBS Lett. 158*, 139–142.

de Grip, W. J., Gillespie, J., & Rothschild, K. J. (1985) *Biochim. Biophys. Acta 809*, 97–106.

Delmelle, M., Butler, K. W., & Smith, I. C. P. (1980) *Biochemistry 19*, 698–704.

Ebrey, T., Govindjee, R., Honig, B., Pollock, E., Chan, W., Crouch, R., Yudd, A., & Nakanishi, K. (1975) *Biochemistry 14*, 3933–3941.

Ebrey, T., Tsuda, M., Sassenrath, G., West, J. L., & Waddell, W. H. (1980) *FEBS Lett. 116*, 217–219.

Emeis, D., Kuhn, H., Reichert, J., & Hofmann, K. P. (1982) *FEBS Lett. 143*, 29–34.

Fukada, Y., Yoshizawa, T., Ito, M., & Tsukida, K. (1982) *Biochim. Biophys. Acta 708*, 112–117.

Hargrave, P. A. (1982) *Prog. Retinal Res. 1*, 1–51.

Hurley, J. B. (1987) *Annu. Rev. Physiol. 49*, 793–812.

Knowles, P. F., Marsh, D., & Rattle, H. W. E. (1976) ESR Applied to Biomolecules in *Magnetic Resonance of Biomolecules*, pp 237–295, Wiley, New York.

Kropf, A., Whittenberger, B. P., Goff, S. P., & Waggoner, A. S. (1973) *Exp. Eye Res. 17*, 591–606.

Kuhn, H., Mommertz, O., & Hargrave, P. A. (1982) *Biochim. Biophys. Acta 679*, 95–100.

Kusumi, A., & Hyde, J. S. (1982) *Biochemistry 21*, 5978-5983.
Kusumi, A., Ohnishi, S., Ito, T., & Yoshizawa, T. (1978) *Biochim. Biophys. Acta 507*, 539-543.
Kusumi, A., Sakaki, T., Yoshizawa, T., & Ohnishi, S. (1980) *J. Biochem. (Tokyo) 88*, 1103-1111.
Mao, B., Ebrey, T. G., & Crouch, R. (1980) *Biophys. J. 29*, 247-256.
Marsh, D. (1980) *Biochemistry 19*, 1632-1637.
Matthews, R. G., Hubbard, R., Brown, P. K., & Wald, G. (1963) *J. Gen. Physiol. 47*, 215-240.
Papermaster, D. S., & Dreyer, W. J. (1974) *Biochemistry 13*, 2438-2444.
Popp, C. A., & Hyde, J. S. (1981) *J. Magn. Reson. 43*, 249-258.
Ratner, V. L., Bagirov, I. G., & Fesenko, E. E. (1981) *Vision Res. 21*, 251-523.
Renk, G., Grover, T., Crouch, R., Mao, B., & Ebrey, T. G. (1981) *Photochem. Photobiol. 33*, 489-494.
Renk, G. E., Or, Y. S., & Crouch, R. K. (1987) *J. Am. Chem. Soc. 109*, 6163-6168.
Renk, G., Crouch, R. K., & Feix, J. B. (1988) *Biophys. J. 53*, 361-365.
Rousselet, A., Faure, G., Boulain, J., & Menez, A. (1984); *Eur. J. Biochem. 140*, 31-37.
Seigneuret, M., Zachowski, A., Hermann, A., & Devaux, P. F. (1984) *Biochemistry 23*, 4271-4275.
Thomas, D. D., Dalton, L. R., & Hyde, J. S. (1976) *J. Chem. Phys. 65*, 3006-3024.
Wald, G. (1967) *Les Prix Nobel en 1967*, pp 260-280, Nobel Foundation, Stockholm.
Yamamoto, K., & Shichi, H. (1983) *Biophys. Struct. Mech. 9*, 259-267.

ESR and Fluorescence Studies on the Adenine Binding Site of Lectins Using a Spin-Labeled Analogue†

Mary Maliarik, Nike R. Plessas, and Irwin J. Goldstein*
Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109
Giovanni Musci‡ and Lawrence J. Berliner*
Department of Chemistry, The Ohio State University, Columbus, Ohio 43210
Received July 25, 1988

ABSTRACT: The techniques of electron spin resonance (ESR) and fluorescence spectroscopy have been used to study the interaction of a spin-labeled analogue of adenine, N^6-(2,2,6,6-tetramethyl-1-oxypiperidin-4-yl)adenine (I), with several plant lectins. While most adenine derivatives enhanced lectin-induced fluorescence of 1,8-anilinonaphthalenesulfonic acid by binding to a separate, adenine-specific site [Roberts, D. D., & Goldstein, I. J. (1982) *J. Biol. Chem. 257*, 11274-11277], the spin label I caused a *decrease* in this fluorescence with certain lectins. ESR showed the ligand to interact strongly with lectins from lima bean (*Phaseolus lunatus*), *Dolichos biflorus*, and *Phaseolus vulgaris* (PHA); however, no binding was observed with *Griffonia simplicifolia* isolectins A_4 and B_4, soybean agglutinin, or *Amphicarpaea bracteata* lectins. The spin label was highly immobilized by each of these proteins ($2T_{\parallel}$ = 68 G). Apparent affinities of the spin label for the lectins decreased in the order lima bean lectin > PHA erythroagglutinin > PHA leukoagglutinin > *D. biflorus*. Spin-labeled adenine appeared to bind specifically to the adenine binding site of *D. biflorus* and PHA leukoagglutinin, as demonstrated by total abolition of the ESR spectrum of bound spin label by adenine. PHA erythroagglutinin and lima bean lectin bound the analogue with apparent dissociation constants of 5×10^{-5} and 3.2×10^{-5} M, respectively. Several lines of evidence indicate that this derivative binds to at least two sites on lima bean lectin: (1) [^{14}C]adenine was not completely displaced from the lectin by the spin-labeled analogue on equilibrium dialysis; (2) addition of adenine caused a shift in the bound ESR spectrum of the spin-labeled adenine; and (3) the bound ESR spectrum was abolished only by a combination of benzyladenine and 2-toluidinenaphthalene-6-sulfonic acid. Both of these ligands bind at the adenine binding site; however, at higher concentrations, the ligands also bind to the ANS sites [Robets, D. D., & Goldstein, I. J. (1983) *J. Biol. Chem. 258*, 13820-13824]. The distance between the carbohydrate and adenine binding sites on lima bean lectin from ESR studies of spin-labeled adenine I and a *N*-acetyl-D-galactosamine derivative must be greater than 12 Å since no indication of nitroxide-nitroxide interactions was observed.

The carbohydrate binding specificity of plant lectins has been extensively studied and is well described [for reviews, see Goldstein and Hayes (1978), Lis and Sharon (1981), and Goldstein and Poretz (1986)]. Additionally, it has been observed that several lectins also contain binding sites for hydrophobic ligands. For example, Con A[1] is known to bind TNS (Yang & Edelman, 1974) and the phytohormone indoleacetic acid (Edelman & Wang, 1978). Binding of such ligands has since been found to be a common feature of several legume and nonlegume lectins (Roberts & Goldstein, 1983a).

† This research was supported in part by Grants HD 17270 (L.J.B.) and GM 29470 and CA 20424 (I.J.G.) from the U.S. Public Health Service.
‡ Present address: Universita Degli Studi di Roma, Instituto di Chimica Biologica, Piazzale Aldo Moro 5, Rome, Italy.

[1] Abbreviations: Con A, concanavalin A; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonic acid; ANS, 1,8-anilinonaphthalenesulfonic acid; LBL, lima bean lectin; PBS, phosphate buffered saline; ESR, electron spin resonance; PHA-E and PHA-L, *Phaseolus vulgaris* isolectins erythroagglutinin and leukoagglutinin, respectively; GS I-A_4 and GS I-B_4, *Griffonia simplicifolia* isolectins A_4 and B_4, respectively; SBA, soybean agglutinin.

0006-2960/89/0428-0912$01.50/0