Interactions of the β-Ionone Ring with the Protein in the Visual Pigment Rhodopsin Control the Activation Mechanism. An FTIR and Fluorescence Study on Artificial Vertebrate Rhodopsins†

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ABSTRACT: The photoreactions of rhodopsin regenerated with three 9-cis retinal analogs, modified at or in the vicinity of the β-ionone ring (namely 5,6-epoxy, 7,8-diH, diethyl-acyclic) have been investigated by UV–vis and FTIR difference spectroscopy. In parallel, the ability to catalyze the GDP → GTP exchange of G-protein (transducin) has been monitored by time-dependent fluorescence spectroscopy. The first photoproduct obtained with all three pigments at liquid nitrogen temperature is a blue-shifted intermediate (BSI), followed by a lumi-like intermediate at 170 K. For the 5,6-epoxy-ISO and 7,8-diH-ISO pigment we obtain two further intermediates similar to the META-I and META-II states of native RHO. For the diethyl-acyclic-ISO pigment only one further intermediate can be stabilized at 280 K. As compared to META-II the respective photoproduct exhibits striking differences. The latter two pigments have also been investigated in the solubilized lipid-free state (detergent: dodecyl maltoside) at 280 K. For the 5,6-epoxy-ISO pigment, the UV–vis, FTIR, and activation data agree with the formation of a META-II-like photoproduct (81% activation). Less META-II formation is observed for the 7,8-dihydro-ISO pigment in membranes (65% activation), but full formation in detergent (100% activation). Neither the membrane-bound nor the solubilized diethyl-acyclic-ISO pigment forms a META-II-like intermediate (18% and 0% activation, respectively). Therefore, we conclude that the substitution of the β-ionone ring by two ethyl groups abolishes steric interactions with the protein, which are essential for META-II formation. The UV–vis and FTIR spectroscopic data are discussed in connection with the biochemical data and the molecular events are interpreted in terms of chromophore–protein interaction.

Within the large family of G-protein-coupled receptors the visual pigments are the only proteins containing a chromophore (11-cis retinal), thereby coupling a light signal to a neuronal signal. Upon illumination, the 11-cis chromophore, which is covalently bound to a lysine (Lys 296 in rhodopsin) (Ovchinnikov, 1982; Hargrave et al., 1983) via a protonated SB (Oseroff & Callender, 1974), isomerizes to the all-trans geometry (Palings et al., 1987; Bagley et al., 1985). Therefore, conformational changes in the protein are triggered which convert RHO[1] from the inactive to the active state, capable of transducin activation. A sequence of reaction intermediates can be trapped at low temperature, allowing spectroscopic measurements of the transitions occuring after photoisomerization. In RHO, the PSB is stabilized in the hydrophobic interior by the counterion Glu 113 (which is deprotonated in the dark state) and constrains the protein into the inactive form. After light isomerization, the SB is deprotonated. This deprotonation is essential for the active form of RHO, as has been demonstrated by methylation of the SB (Longstaff et al., 1986; Ganter et al., 1991b) or by mutations at position 296 and/or 113 which destabilize the charge equilibrium (Sakmar et al., 1989; Robinson et al., 1992). Resonace Raman and FTIR spectroscopy in combination with isotopically labeled retinals have revealed the geometry of the chromophore in the dark state RHO and in the BATHO and LUMI photointermediates by assignment of the C–C stretching and C–H out-of-plane bending (HOOP) vibrations (Palings et al., 1989; Palings et al., 1987; Bagley et al., 1985; Ganter et al., 1988). The C=N stretching vibrations of the SB in RHO and the photointermediates have also been studied extensively and compared to those of model compounds, thereby providing information on the protonation state of the SB linkage and the hydrogen bonding strength to it (Ganter et al., 1988; Siebert et al., 1983; Palings et al., 1987; Baasov et al., 1987). Besides the electrostatic interaction in the vicinity of the SB, the role of the chromophore structure for the activation process is of interest. To obtain further insight into these questions, RHO was regenerated with modified chromophores (Ottolenghi & Sheves, 1987; Derguini & Nakanishi, 1986; Balogh-Nair & Nakanishi, 1982). Experiments with retinal analogs, modified at or close to the β-ionone ring, emphasized the role of the cyclohexene group (Okada et al., 1991; Shichida et al., 1987; Albeck et al., 1989; Randall et al., 1991). It was shown that for several of these pigments, instead of a BATHO intermediate, a blue-shifted (BSI) photoproduct is stabilized at 80 K, and time-resolved studies showed that this intermediate follows BATHO (Randall et al., 1991; Albeck et al., 1989). We have recently studied by FTIR spectroscopy the photoreaction of the 5,6-diH-pigment and have shown that the chromophore in the BSI intermediate is already in a relaxed conformation

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[1] Abbreviations: FTIR, Fourier transform infrared spectroscopy; RHO, rhodopsin; ISO, iso-rhodopsin; BATHO, batho-rhodopsin; LUMI, lumi-rhodopsin; META-I, meta-rhodopsin-I; META-II, meta-rhodopsin-II; SB, Schiff base; PSB, protonated SB; HOOP, hydrogen out of plane vibrations; GTPγS, guanosine-5′-τ-thio triphosphate; DM, dodecyl maltoside; diH, dihydro.

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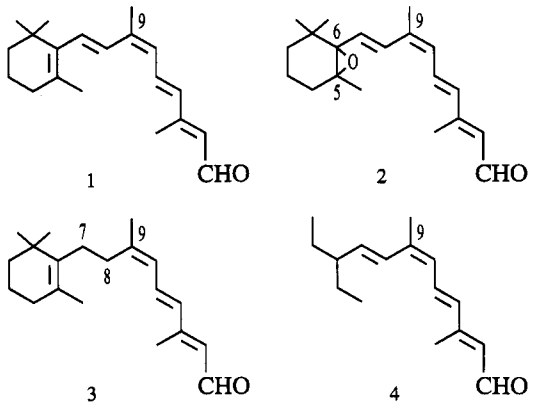


FIGURE 1: Analog retinals: 1, 9-cis; 2, 5,6-epoxy-9-cis; 3, 7,8-diH-9-cis; 4, diethyl-acyclic-9-cis.

(Ganter et al., 1991a). The importance of the methyl group at C_9 in contrast to that at C_{13} for the photoreaction and transducin activation was demonstrated in experiments using 9-desmethyl and 13-desmethyl chromophores (Ganter et al., 1989, 1990). But still, details of the interaction between the chromophore and the protein are not fully understood. Photoreaction-induced FTIR difference spectroscopy is a powerful technique to resolve molecular changes of the retinal, the peptide backbone, and single amino acid side chains (Siebert, 1984, 1993; Braiman & Rothschild, 1988; Rothschild et al., 1983; Fahmy et al., 1991).

In this paper we report investigations on three artificial pigments, in which the 11-cis chromophore is replaced by 9-cis retinal analogs modified at or close to the β-ionone ring (Figure 1). In these chromophores, due to changes in conjugation, the ring part or its replacement is expected to be more flexible with respect to the conjugated chain than in unmodified retinal where the β-ionone ring is included in the conjugated chain. In addition, in the diethyl-acyclic pigment, in which the cyclohexene ring is replaced by two flexible ethyl groups, the steric interaction with the protein is expected to be significantly altered. It will be shown from FTIR difference spectroscopy that all regenerated pigments form a BSI intermediate at low temperature, in agreement with UV–vis data (Randall et al., 1991). The difference spectra of the BSI, LUMI, and following photoproducts are compared to those of unmodified RHO, and they are discussed in terms of chromophore–protein interaction. In addition, the capability of the pigments to activate transducin is related to the conclusions reached from the spectroscopic data.

MATERIALS AND METHODS

Preparation of the Pigments. The chromophores were synthesized as previously described (Albeck et al., 1989; Randall et al., 1991). We prefered the synthesis of 9-cis chromophores, since the synthesis is easier to perform and the retinals are more stable. The use of 9-cis chromophores has no influence on the formation of the later photoproducts. About 100 nmol of membrane-bound RHO was washed by centrifugation in 20 mL of phosphate buffer (1 mM KH_2PO_4, 1 mM DTT, pH 5.5) for 30 min at 4 °C and 46 000 rpm. Subsequent resuspension in hydroxylamine (10 mM NH_3-OHCl) and illumination with a 150-W slide projector for 2 min yielded the retinal free opsin. Hydroxylamine was removed by three washes (as above). All subsequent steps were carried out under dim red light. From the required amounts of retinal analogs (molar ratio retinal/opsin 1:1) dissolved in hexane the solvent was evaporated by a N_2 stream. Subsequently, the chromophores were dissolved in ethanol, and the bleached membranes were added (ethanol approximately 1% of final volume). The mixture was stirred overnight at 20 °C. To remove the excess retinal, the regenerated membranes were washed three times with 2% bovine serum albumin (BSA) in phosphate buffer (40 mM KH_2PO_4, pH 7.4). To prepare lipid-free pigments in DM, the membranes were dissolved in the detergent and RHO was purified by Concanavalin-A (Con-A) affinity chromatography as described (Arnis & Hofmann, 1993).

IR Sample Preparation. About 2.5 nmol of RHO in membranes or 2.5 nmol in detergent were dried under a N_2 stream onto a AgCl window and afterwards rehydrated with approximately 0.5 μL of H_2O and immediately sealed with a Ge window.

FTIR Measurements. All spectra were measured as described previously (Siebert & Mäntele, 1983; Ganter et al., 1988). To obtain spectra of the BSI intermediate, the sample was illuminated at 93 K with light between 435 and 470 nm; the LUMI intermediates were obtained at 170 K under the same illumination conditions. For the subsequent photoproducts the spectra are denoted by their recording temperature. A long-pass filter was used for illumination (cut-on at 470 nm).

UV–vis Measurements. All spectra were recorded on a Perkin-Elmer Lambda 17 spectrophotometer connected to a PC. The samples were measured at room temperature and at pH 5.5. The photoreaction was evoked by illumination with white light of a 150-W slide projector. For the 7,8-diH pigment, spectra after 15 s and after 2 min are shown (curves B_1 and B_2, respectively, Figure 2, middle), and for the 5,6-epoxy and diethyl-acyclic the illumination period was 2 min (curve B, Figure 2, top and bottom, respectively).

Activation Measurements. G-protein was isolated from thoroughly washed ROS by elution with GTP (Heck & Hofmann, 1993). Traces of opsin were removed by a Con-A column (S. Jäger, to be published). A spectrofluorometer (Spex Fluorolog-2) was used to measure the fluorescence enhancement at 340 nm. The excitation wavelength was 300 nm. Samples containing G-protein (0.4 μM) and pigments (80 nM) were prepared at 23 °C in two different buffers: 10 mM Hepes pH 7.5, 130 mM NaCl, 5 mM $MgCl_2$, for membrane suspensions and 10 mM MES pH 6, 130 mM NaCl, 5 mM $MgCl_2$, 0.4 mM DM, in case of solubilized pigments. G-protein activation was induced by adding GTPγS to a final concentration of 20 μM. Under these conditions, the META-I/II equilibrium is not influenced by G-protein and the activation data are a measure of actual META-II formation. All pigments were illuminated for 10 s under white light prior to nucleotide addition. Note that the membrane samples were sonicated (Branson Sonifier S 125, 80-W, 10 s) to obtain a uniform size distribution. All measurements could be fitted by the function $A(t) = A_{max}(1 - \exp(-kt))$, where A is the amplitude and k the rate constant in s^{-1}. For native RHO the value obtained for k was set to 100% activity.

G-protein concentration was determined by the method of Breadford (1976) using bovine serum albumin as standard, whereas the pigment concentration was calculated on the basis of the absorbance at 450–500 nm using ϵ = 40 000 M^{-1} cm^{-1} as an upper limit. For the Con-A purified probes it is possible to calculate ϵ out of the determined protein/retinal absorbance ratios (11-cis: A_{280nm}/A_{500nm} = 1.8; 7,8-diH: A_{280nm}/A_{435nm} = 1.55; diethyl-acyclic A_{280nm}/A_{460nm} = 2.2).

RESULTS

UV–vis Spectra. Figure 2 shows the absorption spectra and the bleaching reaction of the regenerated pigments at

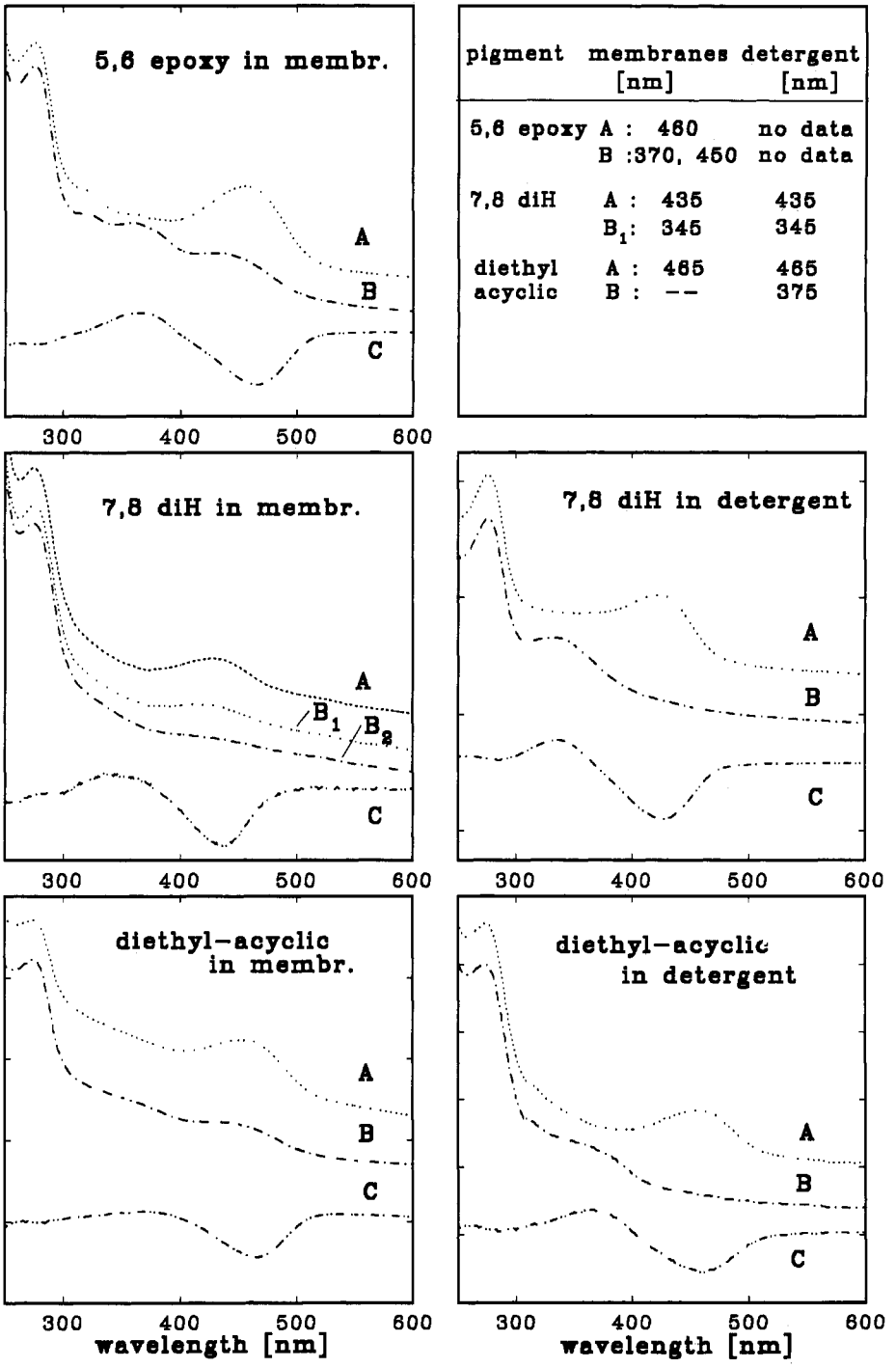
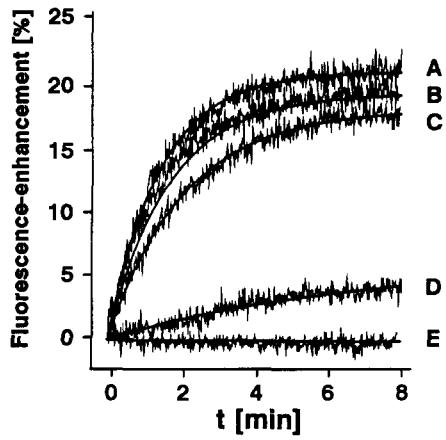


FIGURE 2: UV–vis spectra of the nonilluminated (A) and illuminated (B) samples and the difference spectra (C: B – A). For better illustration the difference spectra of the 7,8-diH and diethyl-acyclic, both in membranes, are multiplied by a factor of 5 and 1.5, respectively. For the 7,8-diH two B-spectra are shown: B_1, 15 s; B_2, 120 s. The table in the upper right corner shows the absorption maxima of the artificial pigments.

room temperature. A discussion about opsin shift and effects regulating the wavelength is presented in a review by Derguini and Nakanishi (1986). Traces A and B represent the spectra of the nonilluminated and illuminated samples, and trace C shows the subtraction.

The absorption maximum of 5,6-epoxy-ISO is blue-shifted (460 nm) compared to RHO. Illumination produces a mixture of species absorbing at 450 and 370 nm. The product absorbing in the UV probably represents META-II and the other species META-I or META-III. These spectra are similar to those of the related pigment 5,6-diH-ISO (Ganter et al., 1991a). We estimate that approximately 83% META-II is formed. 7,8-diH-ISO in membranes exhibits a different behavior. Its absorption maximum is at 435 nm, and it shows some META-II formation (absorption at 345 nm) in the case of a short illumination period (B_1: 15 s). Here, spectrum C = B_1 – A is weighted by a factor of 5 for better illustration. For light exposure longer than 2 min, the 435-nm absorption disappears completely together with the photoproduct absorption located at 345 nm. Since for the longer exposure times there is no evidence of a photoproduct band, it is plausible that illumination causes, in addition, also a gradual decomposition of the chromophores. In the case of 7,8-diH-ISO in membranes, a rough estimate shows that about 60% META-II is formed. For diethyl-acyclic-ISO, exhibiting an absorption maximum at 465 nm, no META-II formation is observed. For this pigment illumination (2 min or longer) causes only a reduction of the absorption band with a concomitant small and broad



Exponential fit

	membranes T=23°C, pH 7.5		detergent[1] T=23°C, pH 6	
	k [1/s]	≙ [%]	k [1/s]	≙ [%]
A	0.013	100	0.0049	100
B	0.0105	81		
C	0.0085	65	0.0042	86[2]
D	0.0024	18	0	0
E	0	0	0	0

FIGURE 3: G-protein activation by different pigments: A, native RHO; B, 5,6-epoxy-ISO; C, 7,8-diH-ISO; D, diethyl-acyclic-ISO; E, G-protein allone (control). Time courses and exponential fits are presented for the *membrane bound* pigments. The table shows the rate constants in 1/s and % and includes also the data for the *detergent*[1] samples. Superscript 2 indicates that number is calculated for ϵ = 40 000. It has to be corrected by a factor of 1.2 to account for the higher ϵ of 7,8-diH-ISO.

absorbance increase between 300 and 400 nm (Figure 2, bottom). One possible explanation involves a slightly blue-shifted intermediate with lower extinction coefficient, which is formed following light absorption. Illumination of samples solubilized in DM (<30 s) produces pronounced absorptions at 345 and 375 nm for the 7,8-diH and diethyl-acyclic pigments, respectively.

G-Protein Activation. When transducin binds GTPγS enhancement of the intrinsic fluorescence is observed (Higashijima et al., 1987; Hofmann, 1993). Thus, the fluorescence is a direct monitor of the activation of transducin. It is expected that pigments that are capable of transducin activation are those for which a deprotonated SB with an absorption maximum in the UV is formed after illumination (Longstaff et al., 1986; Robinson et al., 1992). In Figure 3, we compare for RHO and the artificial pigments the time-course of the fluorescence increase, following the addition of GTPγS. The slopes of the linear part represents the capability of the photoproducts to activate transducin. They are related to that of unnmodified RHO.

The ability of activating transducin is only slightly reduced for 5,6-epoxy-ISO (81% with respect to the unmodified pigment) and is about the same for 5,6-diH-ISO (75%) (not shown). For 7,8-diH-ISO in membranes, activation is reduced to 65%. A similar activation was observed for 7,8-diH-ISO reconstituted into lipid vesicles (Calhoon & Rando, 1985). Almost no activation (18%) is observed for diethyl-acyclic-ISO. The remaining activation of 18% can be attributed to the residual activity of opsin in membranes (data not shown). The UV–vis spectra show for 7,8-diH- and diethyl-acyclic-ISO that the detergent DM favors a UV-absorbing photoproduct. Therefore, both pigments were also assayed in this detergent. Under these conditions 86% activation is obtained for 7,8-diH-ISO, using solubilized unmodified RHO as reference, but still no activation for diethyl-acyclic-ISO (0%). The 86% activity of 7,8-diH-ISO has to be corrected for by a factor of about 1.2 (see Materials and Methods) to account for the higher extinction coefficient of this pigment. Therefore, this pigment is fully active. The results are summarized in the table of Figure 3.

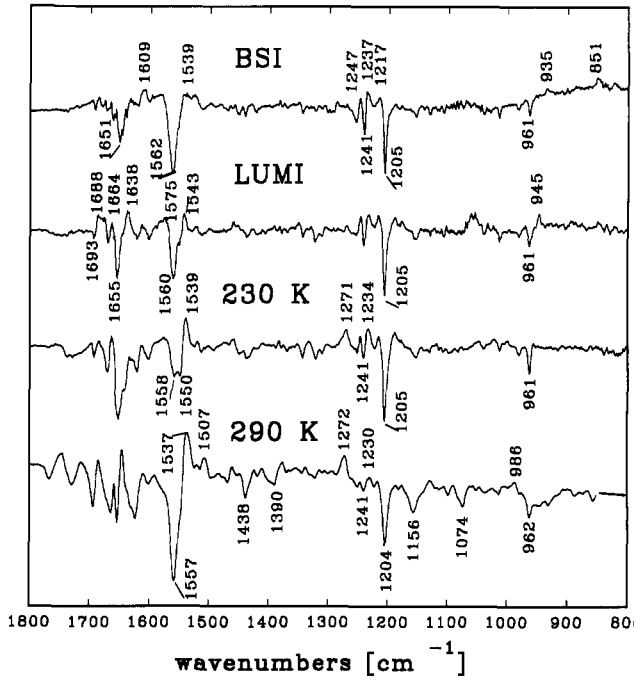


FIGURE 4: FTIR difference spectra of the 5,6-epoxy-ISO, BSI, LUMI, 230 K, and 290 K, recorded at 93, 170, 230, and 290 K, respectively. For the spectrum measured at 290 K the pH was adjusted to 5.5 (phosphate buffer). Photoproduct bands point upwards, ISO bands point downwards.

FTIR Difference Spectra. (1) 5,6-Epoxy-ISO (Presented in Figures 4 and 7B). BSI. The spectrum measured at 93 K is characterized by mainly negative bands. There exists a correlation between the UV–vis absorption maximum of protonated retinylindene Schiff bases and the IR ethylenic stretching vibration: a red-shift of the absorption maximum causes a downshift of the ethylenic mode (Heyde et al., 1971; Sulkes et al., 1978; Callender & Honig, 1977). The negative band at 1562 cm^{-1} can be assigned to the mode of 5,6-epoxy-ISO. In the unmodified pigment, the modes of RHO and BATHO have comparable intensity and are located at 1559 and 1535 cm^{-1}, respectively (Ganter et al., 1989). No corresponding downshifted band can be seen in the 5,6-epoxy-

ISO spectrum. Therefore, it is clear that for this pigment no BATHO can be stabilized at 93 K. The UV–vis data indicate the formation of the BSI intermediate (Randall et al., 1991), which would be expected to have an upshifted ethylenic mode. However, similar to the 5,6-diH-BSI intermediate (Ganter et al., 1991a), no such mode can be deduced for 5,6-epoxy-BSI. Twists in the chromophore are indicated by the intensity of the hydrogen out of plane (HOOP) vibrations (Fahmy et al., 1989). But only low HOOP intensities at 935 and 851 cm^{-1} for BSI and at 961 cm^{-1} for ISO are present. As for 5,6-diH-BSI, we can conclude that the chromophore is already in a relaxed conformation. The negative band at 961 cm^{-1} is similar to the band of unmodified ISO (Siebert et al., 1983). Therefore, the ring modification does not influence the twists of the chromophore near C_{11}–C_{12}. The negative bands in the fingerprint region are characteristic of the ISO state (e.g., they show up in the ISO → LUMI difference spectrum of unmodified RHO, not shown), and the same bands are observed in the BSI difference spectrum of 5,6-diH-ISO (Ganter et al., 1991a). Many small bands appear in the amide-I area, which are caused by distortions of the peptide backbone, but they are difficult to interprete at a molecular level.

LUMI. In the following LUMI photoproduct only the fingerprint region is nearly conserved. There is a HOOP mode at 945 cm^{-1}, which belongs to coupled modes trans to a double bond. Its low intensity shows that the chromophore is essentially planar. In the amide-I region more and larger bands show up and indicate larger changes of the peptide backbone.

230 K. The next spectrum was recorded at 230 K to stabilize META-I. The intensity of the HOOP mode is further reduced, partially due to the overlap with the negative band. The amide-I region is similar to the META-I spectrum of unmodified rhodospin (Figure 7A). Similarily, in the spectral range of the C=O stretch of protonated carboxyl groups good agreement is obtained. However, the intensity of the band at 1700 cm^{-1} is greatly reduced. Because of the different positions of the ethylenic modes, the band pattern between 1530 and 1550 cm^{-1} deviates from that observed for unmodified META-I.

290 K. Comparison of Figure 7A with Figure 7B shows that a META-II- like intermediate is formed at 290 K. An almost conserved band pattern is observed in the range of carbonyl stretches, which monitor the protonation state of carboxylic acids. Within the amide-I region, however, two bands characterizing the unmodified META-II spectrum (1670 and 1633 cm^{-1}) are reduced to shoulders in the difference spectrum of the artificial pigment.

(2) 7,8-diH-ISO (Presented in Figures 5 and 7C). BSI. Following the arguments presented for the 5,6-epoxy pigment, it can be concluded that no BATHO but the BSI intermediate is formed. Within the limits imposed by the noise, no clear positive HOOP mode can be seen. In the fingerprint region the strongest contribution is the 1203 cm^{-1} band. The small shift from 1205 to 1203 cm^{-1} observed for the 7,8-diH-ISO can be expected because the C_7–C_8 single bond disrupts the C=C conjugation resulting in a less delocalized π-electronic system. Again, the small bands from 1695 to 1600 cm^{-1} indicate small distortions of the peptide backbone.

LUMI. No clear HOOP bands can be detected. As compared to the BSI spectrum, the fingerprint region exhibits essentially the same band structure. Stronger bands appear in the amide-I region, but the band pattern differs somewhat from that observed for the 5,6-epoxy pigment. Interestingly,

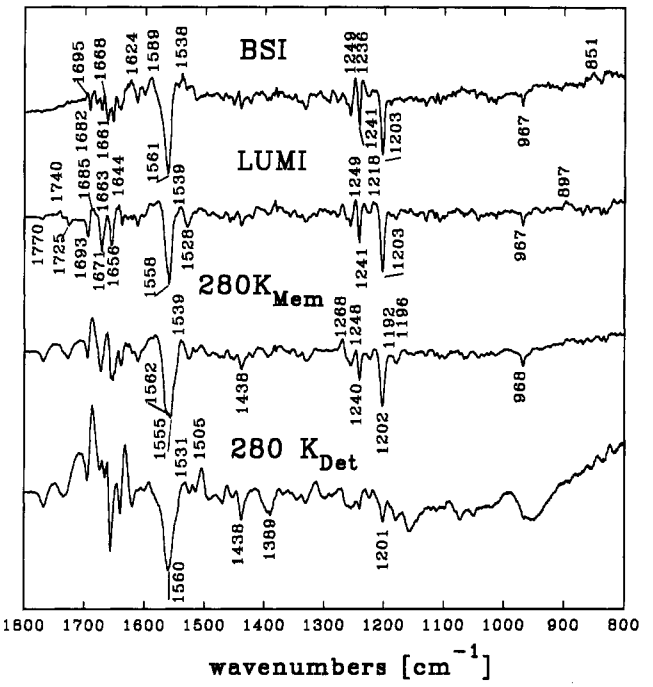


FIGURE 5: FTIR difference spectra of the 7,8-diH-ISO, BSI, LUMI, 280-K_{Mem}, and 280-K_{Det}, recorded at 93, 170, and 280 K, respectively. The suffix describes whether the pigment is membrane embedded or detergent solubilized.

only with this modified pigment we observe the two small carboxyl difference bands characteristic of the spectrum of unmodified LUMI (Ganter et al., 1988). It is a remarkable result that no META-I like intermediate could be stabilized. According to our FTIR data, at temperatures typical of META-I (230 K up to 260 K), the 7,8-diH-ISO pigment was still trapped in the LUMI conformation. By raising the temperature to 280 K a further intermediate could be stabilized. This final photoproduct is denoted by 280-K_{Mem}, to distinguish it from the 280-K_{Det} spectrum, which was measured from a sample solubilized in DM.

280-K_{Mem}. Probably due to contributions from the amide-II mode, the ethylenic band broadens and contains two contributions at 1562 and 1555 cm^{-1}. While the amide-I region of this spectrum is still reminiscent of the LUMI spectrum, the carboxyl region already exhibits META-II patterns.

280-K_{Det}. In contrast, the 280-K_{Det} spectrum displays all essential characteristics of META-II (Figure 7C) both for the carboxyl and amide-I regions. The reduced intensity of the fingerprint bands can be attributed to an orientational effect: in the hydrated films, the membranes–and thus also the chromophores–are preferentially oriented parallel to the AgCl window, whereas random orientation is obtained for solubilized pigments. Therefore, in the hydrated films the chromophore bands are favored. Because of high detergent concentration the base line around 1000 cm^{-1} is distorted.

(3) Diethyl-acyclic-ISO (Presented in Figures 6 and 7D). BSI. Similar to the 5,6-epoxy pigment, the HOOP mode shows up at 933 cm^{-1} with low intensity. Also, the spectral changes in the fingerprint region resemble the BSI spectra of this pigment. The broad negative ethylenic band at 1558 cm^{-1} with a shoulder at 1551 cm^{-1} now has a blue-shifted counterpart at 1574 cm^{-1}, caused by the photoproduct. Similar to the other BSI spectra, many small bands in the amide-I area are observed.

LUMI. The LUMI spectrum has a HOOP band at 947 cm^{-1} and the fingerprint structure is nearly conserved. For the ethylenic bands, a conspicuous decrease of the intensities

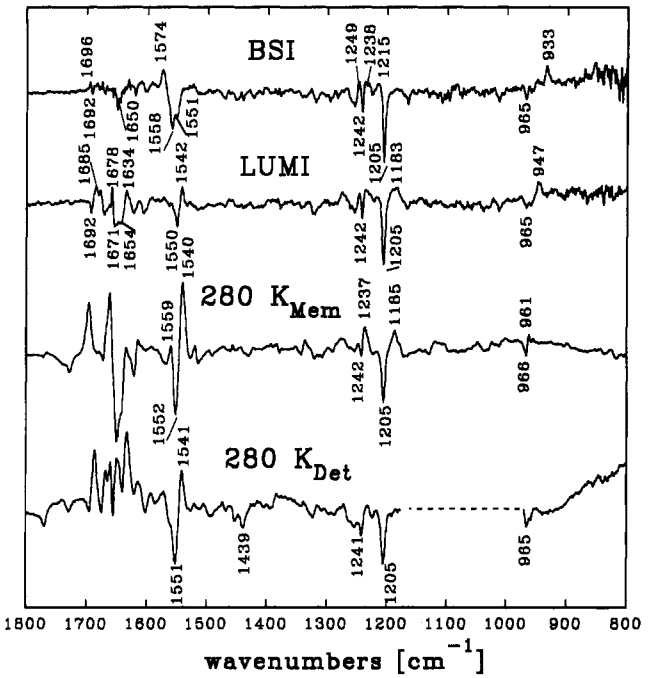


FIGURE 6: FTIR difference spectra of the diethyl-acyclic-ISO, BSI, LUMI, 280-K_{Mem}, and 280-K_{Det}, recorded at 93, 170, and 280 K, respectively. The two latter spectra are from membrane-bound and detergent-solubilized pigments, respectivly. Because of base-line distortions between 970 and 1200 cm^{-1}, the data of the 280-K_{Det} spectrum are replaced by a dashed line.

together with a redshifted band of the photoproduct (1550 cm^{-1} → 1542 cm^{-1}) is observed. This indicates that the positive and negative bands strongly overlap. As compared to the BSI spectrum, the amide-I bands have gained intensity.

280-K_{Mem}. The spectrum denoted as 280-K_{Mem} is obtained in the temperature range from 250 to 280 K. Between the corresponding intermediate and LUMI no further photoproduct could be stabilized. Due to their overlap, the HOOP modes at 961 cm^{-1} (positive) and 966 cm^{-1} (negative) have low intensity. In contrast to the LUMI intermediate spectrum sharp photoproduct bands at 1237 and 1185 cm^{-1} are now revealed. The ethylenic bands (1552/1540 cm^{-1}), the amide-I bands below 1680 cm^{-1}, and the carbonyl bands of carboxyl groups above 1720 cm^{-1} are similar to those of unmodified META-I. However, the strong and narrow band at 1697 cm^{-1} represents a unique feature. As in the case of the 5,6-epoxy pigment, the strong and broad band with maximum at 1700 cm^{-1} is missing. From this spectrum, it can be inferred that no META-II is formed, even at 280 K and pH 5.5.

280-K_{Det}. In order to favor META-II, we measured the corresponding spectrum of this pigment solubilized in DM. Under these conditions, some band positions in the amide-I region characteristic of META-II are observed. The relative intensities, however, differ remarkably. In the carbonyl region, two negative bands at 1769 cm^{-1} and at 1729 cm^{-1} appear, whereas only one photoproduct band is observed at 1741 cm^{-1}. The 1551 and 1541 cm^{-1} ethylenic bands are similar to the 280-K_{Mem} spectrum. The results clearly demonstrate that also in DM no META-II photoproduct is obtained.

DISCUSSION

The aim of this work is to elucidiate the chromophore geometry role in the activation mechanism of RHO. For this, artificial RHO pigments were constructed from three retinal analogs. The steric interactions in the neighborhood of the β-ionone ring are especially addressed. The FTIR difference

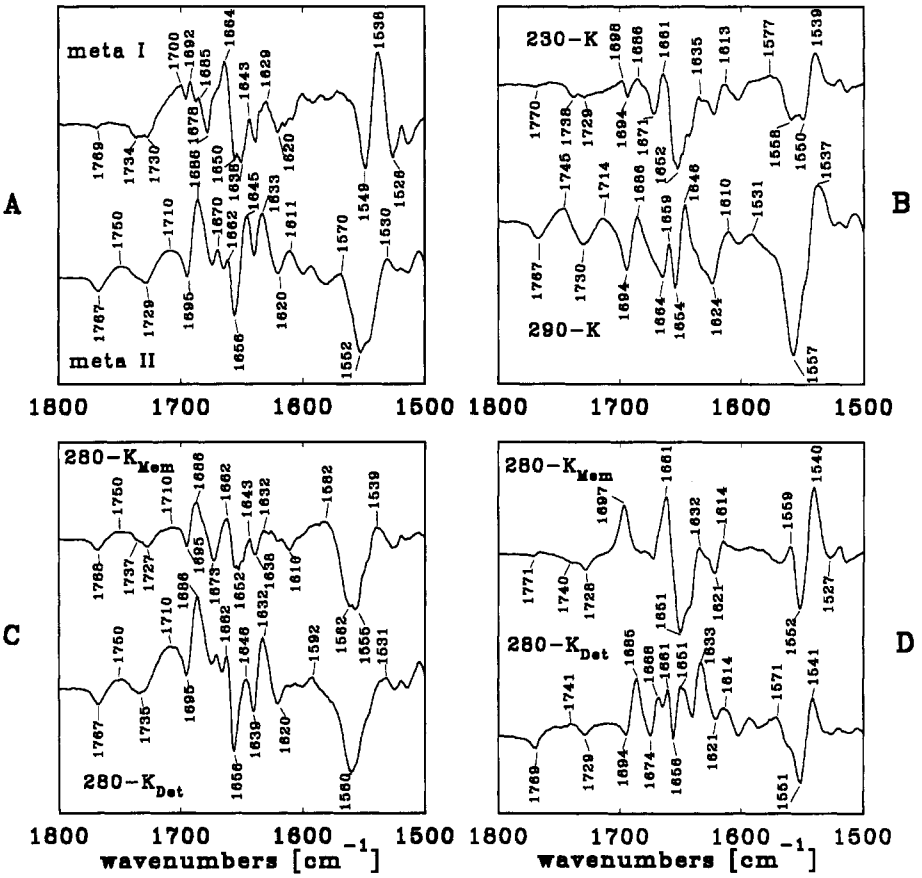


FIGURE 7: FTIR difference spectra of the later photoproducts of unmodified RHO (A), 5,6-epoxy-ISO (B), 7,8-diH-ISO (C), and diethyl-acyclic-ISO (D) in the wavenumber region between 1800 and 1500 cm^{-1} (the lower two traces in Figures 3, 4, 5).

spectra serve as a monitor for the molecular changes, whereas the fluorescence data provide direct information on the capability of the room-temperature photoproduct to activate transducin.

(a) Low-Temperature Photoproducts

As has been stressed in the Results, no BATHO intermediate can be stabilized at low temperatures for all three artificial pigments, but a BSI intermediate is obtained at 93 K. Similar phenomena were observed for the 5,6-diH-ISO pigment (Ganter et al., 1991a). Since in the fingerprint region no significant changes take place from BSI to LUMI, the geometry of the chromophore must be very similar. All three analog pigments exhibit low HOOP intensities, both in BSI and LUMI. This indicates that already in BSI the chromophore relaxes to a nontwisted geometry. The band positions at 933 cm^{-1} (diethyl-acyclic) and 935 cm^{-1} (5,6-epoxy), which are higher than that observed for BATHO (921 cm^{-1}), but lower than for a coupled HOOP trans a double bond, show that the special interaction with the protein near C_{12} observed for BATHO (Palings et al., 1989) is still present although with reduced strength. Similar to unmodified LUMI (Ganter et al., 1988), in the LUMI spectra of the artificial pigments the HOOP mode is further upshifted, indicating that this interaction is no longer acting on the chromophore.

The protein absorptions from 1700 to 1600 cm^{-1} define the two low-temperature photoproducts, BSI and LUMI, of each pigment in a very characteristic manner, but no assignments are presently available. In the LUMI spectrum of 7,8-diH-ISO the typical pattern for the carboxyl region known for native LUMI, indicating that two carboxyl groups change their environment (Ganter et al., 1988), appear. For the other two pigments no distinct bands caused by carboxyl groups can be detected. From C_9 to the Schiff base, the chromophore geometry in LUMI appears to be very similar to that of native LUMI. With respect to amide-I bands, the largest deviations are observed for the acyclic pigment.

(b) 230 and 290 K Photoproducts

In the following discussion we concentrate on the protein absorptions (1800–1600 cm^{-1}). We take into account our recent results on the assignment of carbonyl absorptions to specific carboxyl groups which have been obtained by FTIR measurements of recombinant RHO (Fahmy et al., 1993).

Unmodified META-II (Figure 7A). Two carboxyl groups change their environment. They appear as difference bands (Asp 83: 1767 → 1750 cm^{-1}, Glu 122: 1734 → 1745 cm^{-1}). The positive band at 1710 cm^{-1} has been interpreted as being caused by Glu 113, which is protonated in META-II. The amide-I and amide-II bands reflect conformational changes of the peptide backbone, and their sensitivity to the structure of the cytosolic loops has been demonstrated (Ganter et al., 1992). Due to the overlap with the ethylenic modes, the amide-II band is difficult to evaluate for the three pigments. However, the similarity in the amide-I region of the spectra taken at room temperature and of the META-II spectrum of native RHO can serve as evidence, that the corresponding photoproducts are capable of G-protein activation.

5,6-Epoxy-ISO (Figure 7B). In the 230 K spectrum the negative carboxyl bands and the shape of the amide-I region show good agreement with the spectrum of unmodified META-I. As has already been observed for the 5,6-diH pigment (Ganter et al., 1991a), the broad positive band at 1700 cm^{-1} of native META-I caused by a carboxyl group is missing. So far, we are unable to assign this band to a specific group. We have tentatively ascribed it to the protonation of the counterion Glu 113 and the negative band at 1734 cm^{-1} to the formation of a new counterion (Ganter et al., 1989). The latter band, however, could be assigned to Glu 122. If the interpretation derived for unmodified RHO is correct, the change in the counterion in META-I would be inhibited for these pigments. Since the spectrum measured at 290 K exhibits the typical carboxyl band pattern of unmodified META-II, Glu 113 is now protonated. Two photoproduct bands (1670 and 1633 cm^{-1}) in the amide-I region only appear as shoulders. This indicates that the intermediate differs from META-II. As indicated by the UV–vis spectra, it may contain contributions from META-I or META-III. It has been described that bands characteristic of META-II decay in the transition to META-III (Rothschild et al., 1987; Klinger & Braiman, 1992). Therefore, it appears more plausible that already some META-III is formed under the measuring conditions. In this connection it should be mentioned that the temperature has to be raised to 290 K to obtain a spectrum exhibiting features similar to META-II. At lower temperatures, the bands caused by META-I dominate. The higher temperature could explain the presence of META-III. Indications for a faster decay to META-III have also been obtained for the 5,6-diH pigment (Ganter et al., 1991a). The reduced G-protein activation is in agreement with the presence of META-III (or META-I). In conclusion, illumination of this pigment leads to a META-II-like photoproduct. The META-II/III and META-I/II equilibria, however, are altered, leading to a reduced amount of META-II formation. Both changes in equilibria result in a decrease of G-protein activation. The rough estimate of META-II formation of 83% (UV–vis) agrees with the activation data.

7,8-DiH-ISO (Figure 7C). As already mentioned in the Results no META-I-like photoproduct could be stabilized. In the 280-K_{Mem} spectrum, the region of protonated carboxyl groups reveals bands characteristic of META-II, although with low intensity. However, in the amide-I range only small changes occur as compared to LUMI. Thus, it appears that an intermediate is formed with low yield which has some similarities to META-II, the remaining part being in a state similar to LUMI, suggesting the formation of a LUMI–META-II equilibrium. This interpretation is in agreement with the activation data (65%). The UV–vis data (Figure 2, middle) provide evidence of formation of a deprotonated Schiff base (absorption at 345 nm) in the case of a short illumination period (15 s). As described, the spectra are best interpreted by a photoproduct which first forms a META-II like species and, upon prolonged illumination, undergoes degradation of the chromophore. Illumination conditions for FTIR were chosen as to avoid degradation of the chromophore. From this we deduce that the bands in the carboxyl range are a measure of the active state. The rough estimate of META-II formation of 60% (UV–vis) is in agreement with the activation data.

In order to promote META-II formation we prepared a lipid-free sample of the 7,8-diH pigment in DM which is known to favor this conformation. Since in this preparation regenerated pigment can be separated from bleached RHO, the extinction coefficient can be determined (see Materials and Methods). The UV–vis spectra clearly show a photoproduct absorbing at 340 nm in agreement with the formation of a deprotonated SB, and the FTIR spectrum (Figure 7C, 280-K_{Det}) resembles that of native META-II. In line with these results, full activation is obtained if the fluorescence data are corrected for the higher extinction coefficient. As

shown recently, the detergent rises the enthalpy of META-I (and propably also of RHO and ISO), thus favoring formation of META-II (Arnis & Hofmann, 1993). Under these conditions, even a flexible chromophore such as 7,8-diH-retinal can act as a steric trigger to initiate the conformational changes of META-II. In membranes, however, the more rigid environment partially impedes the trigger function of this chromophore.

Diethyl-acyclic-ISO (Figure 7D). This chromophore has two ethyl groups attached to the conjugated chain instead of the cyclohexene ring. Therefore, an altered steric interaction with the protein can be anticipated. Between 1800 and 1500 cm^{-1} the 280-K_{Mem} spectrum resembles the META-I spectra of RHO and of the 5,6-epoxy pigment. However, with respect to RHO, the broad band around 1700 cm^{-1} caused by a protonated carboxyl group is missing, and with respect to both pigments an additional large band at 1697 cm^{-1} not observed in other spectra can be seen. Thus, in agreement with the UV–vis spectra it can be concluded that very little, if any, META-II is formed from this pigment. The greatly reduced activation (18%) is in congruence with this result.

As in the case of the 7,8-diH pigment, we studied the influence of the detergent DM. The FTIR spectrum (Figure 7D, 280-K_{Det}) is still very different from that observed for a transition to META-II (in contrast to the 7,8-diH pigment). In agreement with this result, no transducin activation is obtained. However, the UV–vis spectra show a clear UV-absorbing photoproduct as it would have been expected for a META-II-like species. In order to resolve this discrepancy we tested by acid denaturation whether the chromophore is still bound to the protein via a Schiff base. The results showed that the band around 375 nm still remained at this position at pH 1.7 (not shown). This is strong evidence that in this species the chromophore is already detached from the protein. Thus, the observed lack of activation is not in conflict with the UV–vis data. It is interesting to note that among the LUMI spectra of the three artificial pigments the largest deviations from the native spectrum are observed for the pigment with the acyclic chromophore. Thus, the replacement of the cyclohexene ring by two ethyl groups impedes the action of the steric trigger even in the case of the solubilized pigment. Similar results have recently been obtained for the 9-desmethyl chromophore (Ganter et al., 1989).

CONCLUSIONS

In agreement with our previous results on the 5,6-diH pigment, the present study shows that increased flexibility of the chromophore at the β-ionone ring enables the stabilization of the BSI intermediate at low temperature. However, no conclusion can be drawn from the formation of BSI with respect to transducin activation. Similar to the removal of the 9-methyl group, the replacement of the cyclohexene ring by two ethyl groups inhibits signal transduction. Therefore, both the ring and the 9-methyl group are important for the steric trigger action. For some chromophores (e.g., 7,8-diH) the reduced efficiency of the steric trigger can be compensated by replacing the membrane environment by the detergent DM, which, by destabilizing META-I favors the formation of META-II. The FTIR spectra have been shown to represent an essential tool for the interpretation of the effects of chromophore modification at a molecular level.

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