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The influence of the 13-methyl group of the retinal on the photoreaction of rhodopsin revealed by FTIR difference spectroscopy

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Abstract. The photoreaction of rhodopsin regenerated with 11-*cis*-13-demethyl-retinal was investigated by FTIR difference spectroscopy. The measurements show that the chromophore experiences different twists in the modified bathorhodopsin as compared to normal bathorhodopsin and that the twists are relaxed in the additional intermediate batho-lumirhodopsin. Whereas the missing methyl group influences the lumi-metarhodopsin-I transition, a metarhodopsin-I-meta-rhodopsin-II difference spectrum very similar to that of unmodified rhodopsin is observed. The significance of the steric interaction for regulating the photoreaction is discussed.

Key words: Retinal analogue – Retinal protein interaction

Introduction

The visual pigment rhodopsin consists of the protein opsin and the 11-*cis* retinal chromophore, which is bound to the protein via a protonated Schiff base to lysine 296. The first photo-intermediate which can be trapped at low temperature (80 K), bathorhodopsin, already contains a photoisomerized all-trans retinal chromophore. A later intermediate, metarhodopsin-II, which arises at about 0 °C, was shown to have a deprotonated Schiff base. It is this intermediate which triggers the visual enzymatic cascade by activating the G-protein transducin (Stryer 1986). It was shown that both isomerization (Fukada et al. 1984) and deprotonation (Longstaff et al. 1986) are prerequisites for the activation, demonstrating that the interaction is limited selectively to the metarhodopsin-II intermediate (Emeis et al. 1982). To allow for this specific interaction, protein conformational changes must be initiated by the isomerization of the chromophore. Therefore, it is of special interest to investigate the chromophore-protein interaction. By regenerating rhodopsin with a large variety of retinal analogues, information on the retinal binding pocket was obtained (Derguini and Nakanishi 1986).

The steric hindrance by the protein induces large twists of the retinal chain in bathorhodopsin and causes unusually high intensities of the hydrogen-out-of-plane bending (HOOP) vibrations in both the resonance raman and the infrared difference spectra (Eyring et al. 1980; Bagley et al. 1985). Since, in the bathointermediate of rhodopsin regenerated with 11-*cis*-9-H-retinal, these modes have low intensity, it was concluded that the chromophore is not twisted (Eyring et al. 1980; Ganter et al. 1989). The same was observed for the blue-shifted intermediate of rhodopsin regenerated with 9-*cis*-5,6-di-hydro-retinal, which is stabilized at 80 K (U. M. Ganter et al., submitted).

The importance of the steric interaction between the chromophore and the protein in regulating the photoreaction was demonstrated by using retinal analogues from which bulky groups are removed (11-*cis*-9-H-retinal, 9-*cis*-13-H-retinal) and which are more flexible (9-*cis*-5,6-H_2-retinal). These retinal analogues cause an altered photoreaction (Albeck et al. 1989; Shichida et al. 1981; Ganter et al. 1989). In 9-H-rhodopsin, transducin is activated to only a very limited extent (Ganter et al. 1989). For 5,6-H_2-isorhodopsin, intermediates of the photoreaction decay at lower temperatures than in native rhodopsin and, additionally, some of the molecular changes during the photoreaction occur at an earlier intermediate (U. M. Ganter et al., submitted).

Despite the altered photoreaction, 13-H-rhodopsin is capable, if bleached, of activating the phospho-diesterase (Ebrey et al. 1980) and transducin (R. R. Rando: private communication). An additional intermediate between bathorhodopsin and lumirhodopsin, batho-lumirhodopsin, which can be stabilized at 95 K, exhibits a blue-shifted absorption maximum (475 nm) (Shichida et al. 1981). Here, we report on FTIR investigations of 13-H-rhodopsin. As with 5,6-H_2-isorhodopsin, some molecular

Abbreviations: 9-H-: 9-demethyl; 13-H-: 13-demethyl; 5,6-H_2-5,6-dihydro; HOOP: hydrogen-out-of-plane; FTIR: Fourier transform infrared

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changes occur in 13-H-rhodopsin at a lower temperature than in normal rhodopsin. In addition, removal of the 13-methyl group causes different molecular changes for the lumi-metarhodopsin-I transition.

Materials and methods

All manipulations involving rhodopsin were performed under dim red-light or in darkness. Rod outer segments were isolated as previously reported (Siebert et al. 1983) and stored at 200 K. 11-*cis*-13-H-retinal was synthesized as previously reported (Gärtner et al. 1980). For regeneration, rhodopsin was bleached in a solution of 0.1 *M* phosphate buffer pH 7, 0.01 *M* hydroxylamine and 1 m*M* DTT at 293 K with white light. To remove hydroxylamine, the bleached membranes were washed three times with a solution of 0.01 *M* phosphate buffer pH 7 and 1 m*M* DTT and resuspended in a solution of 1 m*M* phosphate buffer pH 5.5 and 1 m*M* DTT. A three-fold molar excess of 11-*cis*-13-H-retinal dissolved in ethanol was added, and the regeneration mixture was gently stirred for 12 h. The volume of ethanol was less than 1% of the volume of the suspension.

For the infrared investigations, approximately 5 nM of the suspension of rod outer segments were dried under a stream of dry nitrogen onto a AgCl window and rehydrated with 1 µl H_2O or D_2O. The films were sealed with a germanium window. The actual amount of H_2O (D_2O) on the film was less than 1 µl and varied somewhat for the different measurements. The hydration, however, was high enough to allow for a more than 90% conversion to metarhodopsin-II at 273 K, pH 5.5. The sample was mounted in a continuous flow cryostat equipped with CsJ windows. The samples were cooled to the desired temperature. Photoproducts were obtained at 80 K (batho-rhodopsin), 123 K (batho-lumirhodopsin), 180 K (lumi-rhodopsin), 233 K (metarhodopsin-I) and 273 K (meta-rhodopsin-II) by illuminating the pigment with light of wavelengths between 435 and 475 nm.

IR difference spectra were obtained on a Bruker FT-IR spectrophotometer model IFS 113v equipped with a HgCdTe-detector. The measurements were carried out as previously reported (Siebert et al. 1983); spectral resolution is 2 cm^{-1}, zero filling is 2 and no smoothing of the spectra was performed. First, a single-beam spectrum of the unilluminated sample was recorded. The sample was then illuminated within the FTIR instrument and a second single-beam spectrum was recorded. From the two single-beam spectra the rhodopsin – photoproduct difference spectrum was calculated. The convention is such that negative bands are due to the initial state, positive bands to the photoproduct. For each single-beam spectrum 1024 scans were accumulated. They were divided in four blocks of 256 scans each for baseline control as described in Siebert et al. (1983). To increase the signal/noise ratio, up to four difference spectra of different measurements were co-added. To correct for variations in sample concentration and for different photo-conversion efficiencies, the difference spectra shown are scaled to an internal standard, equally visible in all measurements, i.e. the negative band at 1184 cm^{-1}. Since the most reproducible conversion was obtained for the rhodopsin – batho-lumi-rhodopsin difference spectrum, amounting to more than 90% conversion, this spectrum served as an absorbance calibration standard.

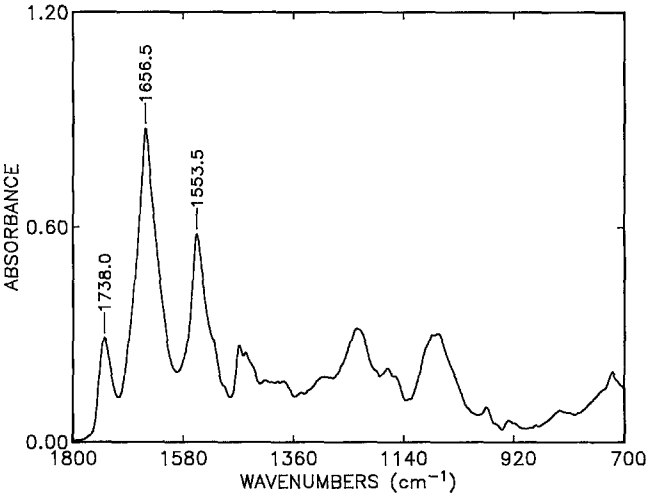

Fig. 1. Absorption spectrum of the rhodopsin sample (rod outer segments) regenerated with 11-*cis*-13-H-retinal, hydrated with 1 µl of H_2O. Ordinate scale: absorbance units. On this scale, no differences can be seen between unilluminated and illuminated samples

Results and discussion

A typical absorption spectrum of the rhodopsin sample is shown in Fig. 1. The large bands at 1656.5 and 1553.5 cm^{-1} are due to the amide-I and amide-II vibrations, respectively. The ester carbonyls of the lipids cause the band at 1738 cm^{-1}. Comparing this absorption spectrum with the difference spectra of Fig. 2, it is obvious that the largest spectral changes induced by the photoreaction amount to only 1% of the amide-I absorption of the sample. Therefore, at this ordinate scale, no differences between the spectra of the unilluminated and illuminated samples can be seen.

Figure 2 shows the FTIR difference spectra for the various photo-intermediates of 13-H-rhodopsin. In 13-H-bathorhodopsin, the HOOP modes have altered positions and strongly reduced intensities as compared to native bathorhodopsin. This is probably partially due to the missing methyl group at position 13 which leads to a decoupling of the HOOP-modes from vibrations of this methyl group. This assumption is supported by results obtained for 20-D_3-bathorhodopsin, where an upshift of the high-frequency mode from 921 to 931 cm^{-1} (Ganter 1989; Palings et al. 1989) and a reduction in i.r.-intensity (Ganter 1989) were observed. But the very low intensity of the other modes suggests that the twist of the retinal is also reduced. A correlation between i.r.-intensity of the HOOP modes and twists has been derived recently (Fahmy et al. 1989). Owing to the steric hindrance of the 13-methyl group with 10-H, the chromophore of rhodopsin is twisted around the 11-12 double bond. This explains the band at 967 cm^{-1} of normal rhodopsin

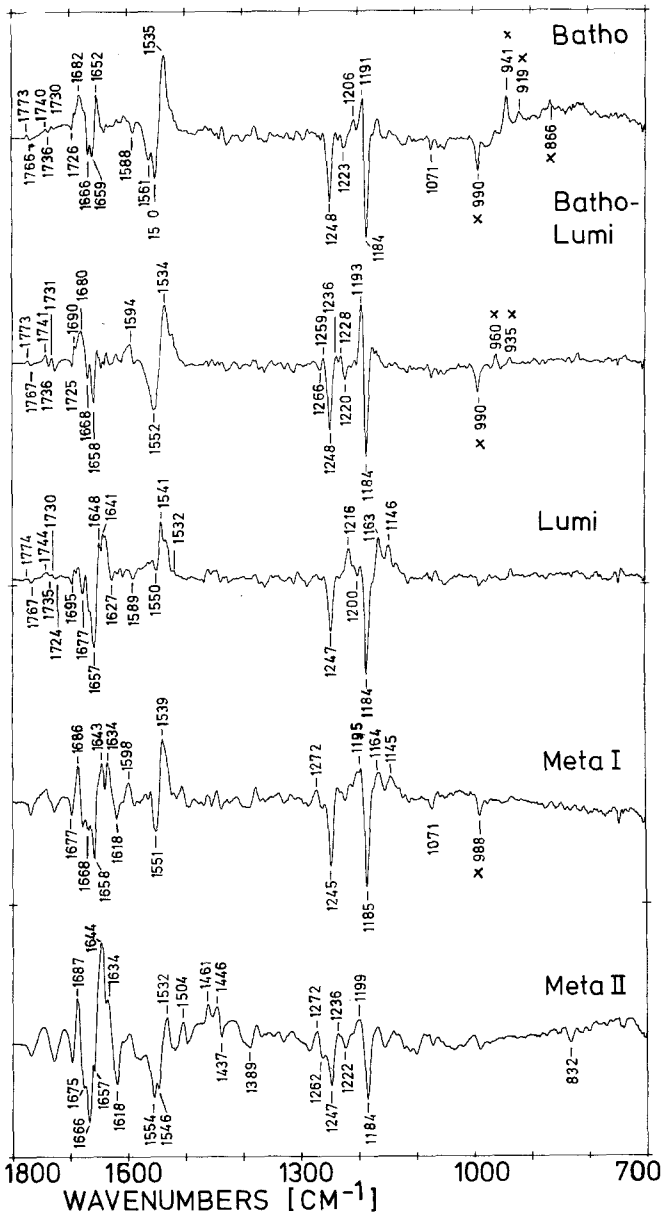

Fig. 2. FTIR difference spectra for the various photointermediates of 13-H-rhodopsin measured in H_2O. Negative bands are due to the initial state, positive bands to the photoproduct. The amplitude of the band at 1184 cm^{-1} in the batho-lumi difference spectrum is approx. −0.008 with respect to the base-line. The traces are from top to bottom: bathorhodopsin measured at 80 K; batholumirhodopsin at 123 K; lumirhodopsin at 180 K; metarhodopsin-I at 233 K; metarhodopsin-II at 273 K. HOOP modes are indicated by crosses. As mentioned in Materials and Methods, the batho, lumi and meta-I difference spectra have been scaled to the same size for the band at 1184 cm^{-1}. In the meta-II difference spectrum, due to the large bands between 1700 and 1600 cm^{-1}, the scaling is reduced, resulting in a size of the 1184 cm^{-1} band of about 65%

(Ganter et al. 1989), which has been assigned to the A_2-combination of the 11,12-HOOP (Palings et al. 1989). Although this A_2-combination is normally i.r. inactive, in the twisted chromophore this mode can derive i.r.-intensity by coupling with skeletal modes. In 13-H-rhodopsin, the steric hindrance is removed, resulting in an i.r. inactive A_2-mode. Therefore, the negative band at 990 cm^{-1} cannot be assigned to this mode. Because it is shifted in D_2O medium to 980 cm^{-1}, it can probably be assigned to the C_{15}-H HOOP mode.

The comparison of the difference spectrum obtained at 123 K (batho-lumirhodopsin) with the batho- and lumirhodopsin difference spectra, clearly shows that a new intermediate is obtained at this temperature as is evident from changes in the carbonyl, amide-I, amide-II and fingerprint bands. This is in contrast to the results of Shichida et al. (1981) who obtained, at 95 and 113 K, only a mixture of rhodopsin, bathorhodopsin and batholumirhodopsin. But because the authors mentioned that batho-lumirhodopsin is photoinsensitive, it seems possible that raising the temperature to 123 K may result in a higher yield. The upshift of the HOOP's and the further reduction of their intensities show that at 123 K the twists are already completely relaxed. The ethylenic modes suggest that the absorption maximum of this intermediate is similar to that of the red-shifted bathorhodopsin in contradiction to the results of Shichida et al. (1981) who claim a blue-shifted intermediate. However, it should be noted that, in contrast to bacteriorhodopsin, in the case of rhodopsin to deduce the absorption maxima from the apparent ethylenic modes in the i.r. difference spectra appears to be questionable (Ganter et al. 1988; Ganter et al. 1989). This is especially evident for unmodified metarhodopsin-I, for which the ethylenic mode would predict a red-shift as compared to rhodopsin; the UV-vis spectra, however, clearly demonstrate the blue-shift. The similarities of the ethylenic modes of 13-H-lumirhodopsin and 13-H-metarhodopsin-I to those of the corresponding intermediates of unmodified rhodopsin (Ganter et al. 1989) indicate that the absorption maxima are also similar.

In the bathorhodopsin and lumirhodopsin difference spectra the carbonyl vibrations above 1700 cm^{-1} (Fig. 3) are very similar to those of unmodified rhodopsin. Since the bands are stronger in the batho-lumirhodopsin difference spectrum, the environmental changes of the carbonyl groups are larger in this intermediate. This indicates that, although the chromophore is relaxed, the protein still remains in a conformation similar to rhodopsin. It, therefore, experiences larger distortions which only relax into the new structure with the formation of lumirhodopsin. A similar behaviour has been recently observed for 9-H-rhodopsin (Ganter et al. 1989).

Based on a negative band at 1737 cm^{-1} and a positive band at 1710 cm^{-1} in the metarhodopsin-I difference spectrum of native rhodopsin, a deprotonation of one carboxyl group with the concomitant protonation of another one have been deduced (Ganter et al. 1989). In the corresponding spectrum of 13-H-rhodopsin, instead of the negative band, a differential band centred at 1740 cm^{-1} is present and the positive band, which is shifted in D_2O, is very weak. Thus, instead of a deprotonation, only an environmental change of a protonated carboxyl group occurs as in lumirhodopsin; the other group becomes protonated, if at all, to a very limited extent. In contrast to unmodified metarhodopsin-I, the high-frequency carboxyl group undergoes a larger change in hydrogen bonding, almost approaching the situation of metarhodopsin-II. As in native rhodopsin, a differential band centred at 1729 cm^{-1} can be attributed to an

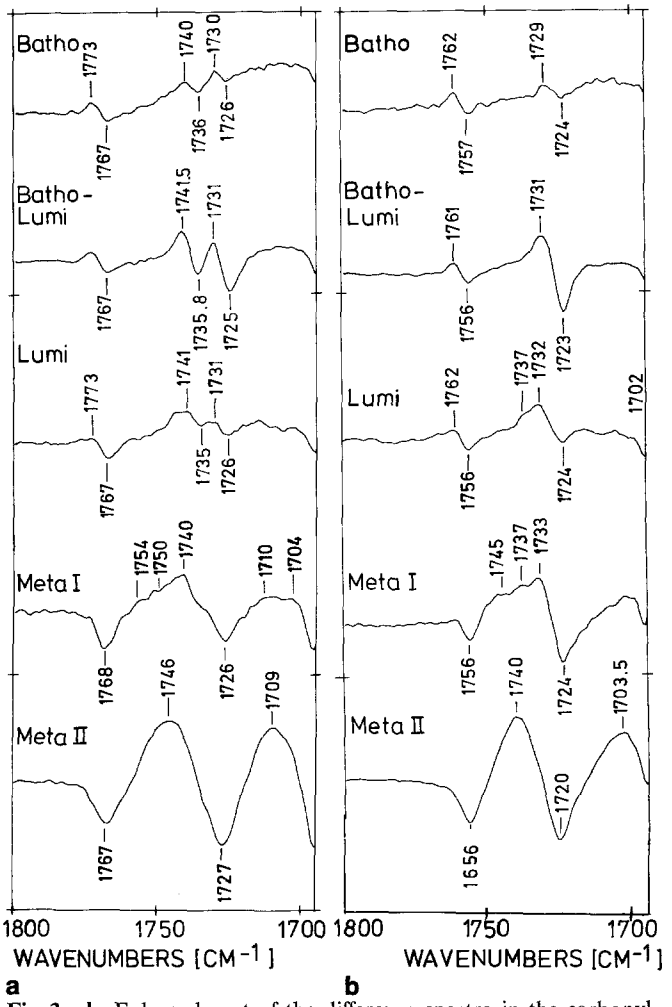

Fig. 3a, b. Enlarged part of the difference spectra in the carbonyl spectral range. **a** H_2O; **b** D_2O. The traces are from top to bottom: bathorhodopsin; batho-lumirhodopsin; lumirhodopsin; meta-rhodopsin-I; metarhodopsin-II. The absorbance of the band at 1741.5 cm^{-1} in the batho-lumi difference spectrum is approx. 0.0005 with respect to the base-line. The spectra are scaled to equal size for the band at 1184 cm^{-1} (Fig. 2)

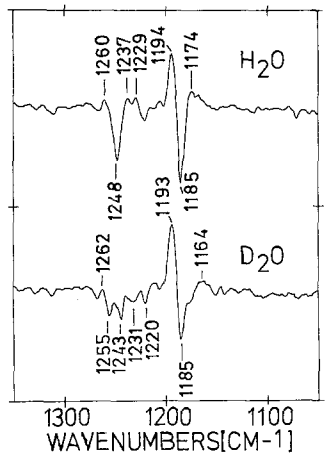

Fig. 4. Enlarged part of the spectral region of the fingerprint bands in the 13-H-rhodopsin – batho-lumirhodopsin difference spectrum. Top trace: measurement in H_2O medium; bottom trace: measurement in D_2O medium. As in Fig. 2, the absorbance of the band at 1185 cm^{-1} with respect to the base-line in the batho-lumi difference spectrum is −0.008

amide-I band of a twisted peptide chain, which is upshifted in bathorhodopsin and lumirhodopsin (Ganter et al. 1989). It was concluded that, in native metarhodopsin-I, the twist relaxes causing a large down-shift of this band, and thereby increasng the intensity of the negative rhodopsin band. Since this increase is also observed for 13-H-rhodopsin, it can be assumed that this downshift of the band and the corresponding peptide relaxation occur also in 13-H-metarhodopsin-I.

The carbonyl vibrations observed in the metarhodopsin-II difference spectra of 13-H-rhodopsin (Fig. 2) are identical to the vibrations previously described for native rhodopsin (Ganter et al. 1989). Also, the patterns of the amide-I and the amide-II bands are in excellent correspondence (Fig. 2). Thus, both metarhodopsin-II intermediates have the same conformation. This is in agreement with the observation that bleached 13-H-rhodopsin is capable of activating the phosphodiesterase (Ebrey et al. 1980).

Some controversy exists about the rhodopsin line at 1238 cm^{-1} which, upon measurement in D_2O, is shifted to 1246 cm^{-1}. This line was assigned to a mode consisting of the 12,13 and 14,15 single bond stretching vibrations and the $C_{15}H$, $C_{14}H$ and NH-bending modes (Ganter et al. 1988). In the resonance Raman spectra published by Palings et al. (1987), no H/D effect was observed for this band and the 1238 cm^{-1} line was assigned to the 12,13-stretching vibration only. Because of the missing methyl group, the 12,13-stretching vibration of 13-H-rhodopsin is downshifted (Mathies et al. 1987) and most likely absorbs at 1184 cm^{-1} (Fig. 2). But, at 1248 cm^{-1}, a strong line is still observed for 13-H-rhodopsin, which splits into three lines at 1255 cm^{-1}, 1243 cm^{-1} and 1231 cm^{-1} upon deuteration of the Schiff base (Fig. 4). Obviously, the modes are decoupled in 13-H-rhodopsin, resulting in a vibration at 1248 cm^{-1} for the bending modes and a second absorption at 1184 cm^{-1} for the stretching mode. By deuteration of the Schiff base, the bending modes become further decoupled.

As in 5,6-H_2- and 9-H-rhodopsin, in which the steric interaction of the chromophore with the protein is also reduced, the relaxation of the cis-trans isomerization and of the induced protein conformational changes proceeds at lower temperature than in native rhodopsin. This shows again the importance of the steric interaction between the protein and the retinal for regulating the photoreaction. In contrast to 9-H-rhodopsin, the low temperature intermediates are mainly changed and the metarhodopsin-II pigment adopts a conformation very similar to native rhodopsin. This is in agreement with the observation that only 9-H-rhodopsin shows a greatly diminished capability for transducin activation (Ganter et al. 1989).

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