Fourier transform infrared studies of active-site-methylated rhodopsin

Implications for chromophore-protein interaction, transducin activation, and the reaction pathway

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ABSTRACT Fourier transform infrared studies of active-site-methylated rhodopsin (ASMR) show that, as compared to unmodified rhodopsin, the photoreaction is almost unchanged up to the formation of lumirhodopsin. Especially, the deviations are much smaller than those observed for the corresponding intermediates of 13-desmethyl-rhodopsin. In metarhodopsin-I, larger alterations are present with respect to the three internal carboxyl groups. Similar deviations have been observed in meta-I of 13-desmethyl-rhodopsin. This indicates that, in agreement with our previous investigations, these carboxyl groups are located in close proximity to the chromophore. Because this latter pigment is capable, when bleached, of activating transducin, our data provide support for the earlier conclusion that deprotonation of the Schiff base is a prerequisite for transducin activation. The positions of the C = C and C–C stretching modes of the retinal suggest that the redshift observed in ASMR and its photoproducts can be explained by an increased distance of the Schiff base from the counterion(s). It is further shown that the photoreaction does not stop at metarhodopsin-I, but that this intermediate directly decays to a metarhodopsin-III-like species.

INTRODUCTION

Rhodopsin, the visual pigment of vertebrate rods, consists of the protein opsin and the chromophore 11-*cis* retinal (1) which is bound to lysine-296 (2) via a protonated Schiff base linkage (3). Upon absorption of light, the chromophore is converted to the all-*trans* isomer by the time the initial photoproduct bathorhodopsin forms (4–6). Bathorhodopsin decays through a series of thermal intermediates to metarhodopsin-II (meta-II) in which the Schiff base is deprotonated. It is this intermediate which has been shown to interact with transducin (7) and which is thought to trigger the activation of the enzymatic amplification cascade (8).

Longstaff and Rando (9) used reductive methylation to prepare a rhodopsin pigment, in which the Schiff base nitrogen is methylated instead of protonated. The absorption maximum of this active-site-methylated rhodopsin (ASMR) is redshifted to 520 nm. Godvindjee et al. (10) showed that after the absorption of light ASMR decays to a metarhodopsin-I-like photoproduct which has an absorption maximum redshifted by 15 nm as compared to normal metarhodopsin-I (meta-I). Because the Schiff base nitrogen is methylated, the meta-I/meta-II transition, which involves the deprotonation of the Schiff base, is blocked. Longstaff et al. (11) showed that ASMR, when illuminated, is not able to activate transducin and hence the first step of the enzymatic cascade is inhibited. They therefore concluded that deprotonation of the Schiff base is a prerequisite for the activation of transducin.

The flash photolysis investigations strongly suggest that the photoreaction of ASMR is not altered before the formation of meta-I. However, definitive proof based on better defined molecular characteristics is desirable here. Methylation of the Schiff base imposes an additional steric hindrance which may influence the photoreaction. Indeed, in recent investigations we have shown that removal of the 9-methyl group of the retinal drastically alters the photoreaction of the modified rhodopsin pigment (12) and that methylation of bacteriorhodopsin's Schiff base inhibits the photoreaction at 80 °K, but allows the photoreaction to proceed up to the formation of the L550 intermediate at room temperature (13). Because Fourier transform infrared (FTIR) difference spectroscopy monitors molecular changes of the chromophore as well as of the protein, it is especially suitable for comparing the photoreaction of ASMR with that of unmodified rhodopsin to establish the photochemical history of ASMR. Observed alterations in the spectral features due to amino acid residues can, in addition, be used to identify groups interacting with the chromophore. In this article we present FTIR difference spectra between ASMR and its photoproducts, and discuss the observed alterations with respect to the native pigment. A molecular mechanism for the redshift of the absorption maximum of ASMR is proposed. Finally, the further temporal evolution of meta-I of ASMR is determined.

MATERIALS AND METHODS

ASMR was prepared as previously reported (9). The detergent solubilized ASMR was reconstituted into a mixture of phosphatidyl-

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serine/phosphatidylcholine lipids (ratio 1:10). In this mixture > 80% of the protein denatured. The FTIR measurements were performed as described (6), with the modification that 1,000 interferograms were accumulated for each single-beam spectrum. The photoreaction was evoked by illuminating ASMR with a slide projector equipped with a 435 cut-off filter and a 470 nm short-wave-pass (SWP)-filter. Bathorhodopsin was obtained at 80 °K, lumirhodopsin at 160 °K, and meta-I at 240 °K. The difference spectrum obtained at 283 °K was used to deduce possible molecular changes after the formation of meta-I. Here, data accumulation was started 2 min after illumination, and the time needed for the accumulation was 4 min. Because the amount of active protein is <20%, the signal-to-noise-ratio in the FTIR-difference spectra of ASMR is worse than in those of the native pigment. To improve the signal-to-noise-ratio, at least three independent measurements for each photoproduct were performed, and the correspondent spectra were added. In addition, because of the intense carbonyl absorption of the lipids used for reconstitution, perturbations of the baseline occur in the region between 1,770 and 1,700 cm^{-1}.

RESULTS AND DISCUSSION

In Fig. 1, the difference spectra between ASMR and its low-temperature photoproducts are shown. Comparison of the difference spectra reveals that, with respect to chromophore bands, the spectra of ASMR resemble those of native rhodopsin measured in D_2O, which are shown in Fig. 2 (for the difference spectra of the native pigment measured in H_2O see reference 12). The similarity, especially with respect to the C = C and C–C stretching modes, indicates that the strong coupling of the N-H bending mode with the different stretching modes of the retinal chain, present in unmodified rhodopsin, is removed by either hydrogen/deuterium (H/D) exchange or by methylation of the Schiff base nitrogen.

The close similarities of the spectra of native rhodopsin and ASMR also demonstrate that, despite the additional methyl group, no major perturbations of the pigment occur. The large 11-HOOP (hydrogen-out-of-plane bending vibration) mode observed at 927 cm^{-1} shows, that in ASMR-bathorhodopsin the chromophore is twisted in a way that is similar but not identical to the way it is twisted in native bathorhodopsin (14). The higher position (927 vs. 920 cm^{-1}) can be explained either by a reduced twist or an altered interaction of the C_{11} = C_{12} region with the postulated second negative charge (14, 15). The new negative band of ASMR at 875 cm^{-1} can tentatively be assigned to the 14-HOOP. If this is correct, it indicates a slightly larger twist in the C_{14}–C_{15} region than in native rhodopsin. By analogy to the 967 cm^{-1} line of native rhodopsin, the negative band of ASMR at 964 cm^{-1} can be assigned to the coupled 11,12-HOOP mode. Because of symmetry, this mode should be infrared inactive. Its strong intensity in the infrared difference spectra can only be explained by a twist of the chromophore caused by the steric hindrance

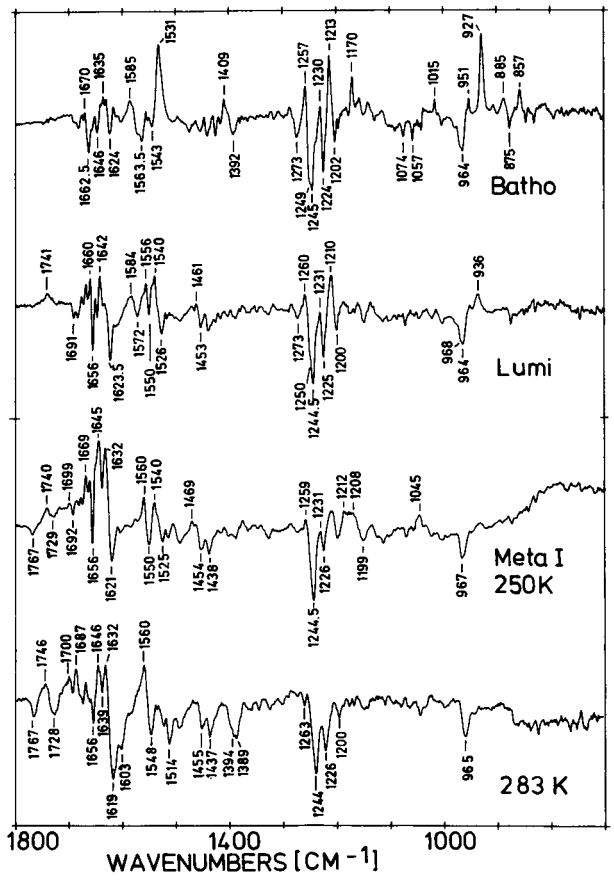

FIGURE 1 The difference spectra between active-site methylated rhodopsin and its photoproducts. *From the top:* bathorhodopsin, lumirhodopsin, metarhodopsin-I, photoproduct obtained at 283 °K.

of the 13-methyl group with 10 H (16). Thus, this shows that the chromophore is twisted around the 11,12 double bond in a way that is similar to the way it is twisted in native rhodopsin. The different position of the HOOP mode of ASM-lumirhodopsin (936 cm^{-1} vs. doublet at 946 and 940 cm^{-1}) demonstrates that its geometry is slightly different from the native one. The same applies to meta-I, for which the HOOP mode at 936 cm^{-1} still has a very low position (950 cm^{-1} in normal meta-I).

The redshift of the electronic absorption maximum from 500 to 520 nm is explained by a larger delocalization of the positive charge of the nitrogen atom along the retinal chain. This delocalization can also be observed in the infrared spectra. Importantly, the downshift of the ethylene mode of bathorhodopsin to 1,531 cm^{-1} (1,536 cm^{-1} in native bathorhodopsin) indicates a smaller force constant for the ethylenic stretching vibration. As with the photoproducts of native rhodopsin (6), the ethylenic bands of the other ASMR photoproducts cannot be assigned unequivocally and, therefore, no shift of these

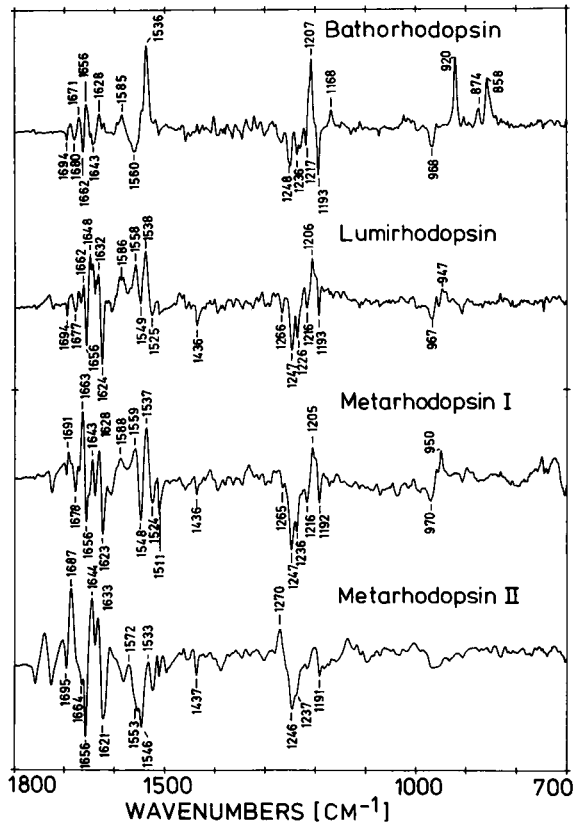

FIGURE 2 The difference spectra of native rhodopsin and its photoproducts in D_2O. *From the top:* bathorhodopsin, lumirhodopsin, metarhodopsin-I, metarhodopsin-II.

bands and no shifts of the corresponding visible absorption maxima can be deduced. It was reported that ASM-meta-I exhibits a redshift similar to ASMR (10). For the C–C single-bond stretching modes of the retinal, a general upshift of the bands is observed. In ASM-bathorhodopsin (native bathorhodopsin), the 10,11 stretching vibration appears at 1,170 cm^{-1} (1,166 cm^{-1}) and the 14,15 mode at 1,213 cm^{-1} (1,207 cm^{-1}). In ASMR (native rhodopsin), the 14,15 mode is observed at 1,202 cm^{-1} (1,192 cm^{-1}) and the 12,13 mode at 1,224 cm^{-1} (1,215 cm^{-1}). Additionally, the lines of ASM-lumirhodopsin (native lumirhodopsin) at 1,212 and 1,208 cm^{-1} (1,198, 1,205 cm^{-1}) also show an upshift. For the assignment of the infrared bands see references 5 and 6. The bands of the unmodified pigment are not influenced by D_2O, i.e., they are not coupled to the N-H bending mode. Therefore, the observed upshifts of the C–C vibrations demonstrate that the charge of the Schiff base nitrogen is more delocalized in ASMR, ASM-bathorhodopsin and ASM-lumirhodopsin than in the native pigment and its intermediates. A similar feature was previously observed for protonated retinal Schiff base model compounds (17, 18). It was shown that by increasing the distance of the protonated Schiff base from its counterion, by changing the counterion from chloride to perchlorate, the C–C stretching vibrations were upshifted. Thus, it appears that the methyl group in ASMR increases the distance from the counterion, causing a redshift of the visible absorption maxima of ASMR, ASM-bathorhodopsin and ASM-lumirhodopsin. (Unfortunately, there is no clear positive band in the fingerprint region of the ASM-meta-I difference spectrum.) The 1,238 cm^{-1} line of native rhodopsin is shifted up to 1,245 cm^{-1} with a shoulder at 1,249 cm^{-1} in ASMR, but this band is also shifted up by deuteration of the Schiff base (6). Therefore, this shift must at least partially be explained by a decoupling of the mode from the N-H bending vibration.

The amide-I bands are slightly altered as compared to those in the bathorhodopsin difference spectrum of unmodified rhodopsin. This indicates that the protein is influenced similarly but not identically during these two transitions. The new negative band at 1,624 cm^{-1} probably represents the C = N stretching vibration of ASMR. Its position is almost identical to that of the deuterated Schiff base of unmodified rhodopsin. This supports the decoupling scheme proposed above. Measurements of the *N*-methyl-*N*-butyl amine retinal Schiff base model compound in methylenechloride showed a C = N stretching vibration at 1,630 cm^{-1}, close to the 1,628 cm^{-1} line observed for the deuterated Schiff base model (13). It is noteworthy that, in contrast to bacteriorhodopsin, for which no transition to K-intermediate could be evoked at 80 °K (13), a normal transition to ASM – bathorhodopsin can be observed at this temperature. This supports the view that the Schiff base is not moved during this transition (in contrast to the transition from bacteriorhodopsin to K-intermediate). This fact could also be deduced from the identical positions of the C = N stretches in rhodopsin and bathorhodopsin and from their similar deuterium isotope shifts (4, 19). In the rhodopsin-lumirhodopsin difference spectra, the C = N stretch of ASMR can be assigned to the band at 1,624 cm^{-1}, and that of lumirhodopsin to the shoulder around 1,634 cm^{-1}. Again, this position is similar to the C = N stretch of the deuterated Schiff base of lumirhodopsin. In the ASM-meta-I, the C = N stretching vibration differs somewhat (1,628 cm^{-1} in native meta-I, 1,632 cm^{-1} in ASM-meta-I). The fact that in all difference spectra of ASMR a new negative band is observed around 1,622 cm^{-1} supports the assignment to the C = N stretching mode of ASMR.

As mentioned above, baseline distortions occur in the region of the carbonyl absorption in the difference spectra of ASMR. Therefore, the carbonyl bands of the rhodopsin-bathorhodopsin transition cannot accurately

be evaluated. The positive band at 1,741 cm^{-1} in the rhodopsin-lumirhodopsin difference spectrum is most probably caused by such a baseline distortion. The remaining small features, however, can, with some caution, be correlated with bands of the normal rhodopsin-lumirhodopsin transition. Large changes of the carboxyl groups occur during the rhodopsin-meta-I transition of ASMR. In Fig. 3 are shown the spectral regions of the carboxyl groups (measured in H_2O and D_2O medium in order to assign the carbonyl vibrations). If one corrects for the baseline, the following conclusion can be drawn: for the unmodified pigment (12), a band of the protonated carboxyl group around 1,767 cm^{-1} is shifted to lower frequencies, but instead of a small shift to 1,764 cm^{-1}, as in native rhodopsin, a much larger shift to ~1,753 cm^{-1} is observed. This is half of the shift to 1,745 cm^{-1} observed in native meta-II (12). The carboxyl group which absorbs at 1,735 cm^{-1} in ASMR and in native rhodopsin does not become deprotonated as in normal meta-I. Instead, it remains protonated, and the corresponding band is shifted to ~1,740 cm^{-1}, a position similar to that observed in lumirhodopsin. As a consequence of this, the protonation of another carboxyl group, which was indicated by the positive band at 1,710 cm^{-1} for native meta-I (12), does not occur in these cases. Because the amide-I bands are strikingly similar in the meta-I difference spectra of ASMR and unmodified rhodopsin, this altered behavior of the carboxyl groups cannot be caused by a different protein conformation. Instead, it is possible that small perturbations of the electrostatic interaction of the Schiff base with its environment, caused by the methylation of the Schiff base, results in this different behavior. For example, the distance to the counterion is enlarged and no hydrogen bond can be formed with the Schiff base nitrogen. As described above for the Schiff base stretching vibrations, a small deviation is observed for meta-I.

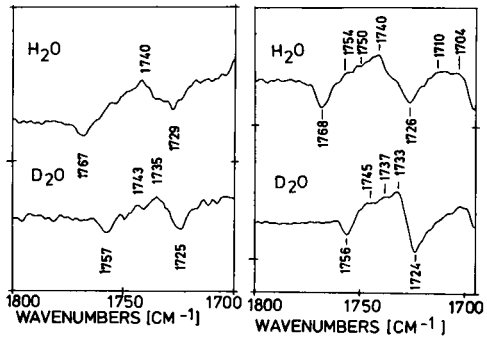

FIGURE 3 The comparison of the rhodopsin-metarhodopsin-I difference spectra of ASMR (*left*) and 13-desmethyl-rhodopsin (*right*) in the carbonyl spectral range; upper traces H_2O, lower traces D_2O.

The difference spectrum obtained at 283 °K shows, with respect to carboxyl groups and amide-I bands, striking similarities to the rhodopsin-meta-III difference spectrum (20, 21). There is a great reduction in the intensity of the bands caused by protonated carboxyl groups and of the amide-I bands at 1,664 cm^{-1} (negative) and 1,644 cm^{-1} (positive) as compared to the corresponding bands in the meta-II difference spectrum. It was previously reported that with the formation of meta-III, part of the protein changes occurring in meta-II are already reversed (20). Therefore, it appears that at 283 °K the photoproduct of ASMR does not stay in the meta-I-like conformation, but decays directly to a meta-III-like species, i.e., without deprotonation of the Schiff base. Some indication for this could already be obtained by the ultraviolet-visible spectra (11): in the spectrum taken 5 min after illumination at 277 °K, a small blueshift of the absorption maximum as compared to the meta-I spectrum can be discerned. A similar decay-time prevails in our infrared experiments. It is generally assumed that meta-III arises from meta-II (22, 23). Because the decay time of meta-I of ASMR is comparable to the formation time of meta-III in the native system, a direct pathway from meta-I is likely to be important in unmodified rhodopsin as well.

Despite the larger size of the additional group, methylation of the Schiff base does not greatly influence the photoreaction up to the formation of lumirhodopsin. The higher positions of the fingerprint modes and the lower positions of the ethylenic modes can be explained by the redshifted absorption maximum and they are not due to geometric alterations. If this and the decoupling of the C = N and C–C stretches from the N-H bending vibration are taken into account, the difference spectra deviate appreciably less from those of the native pigment than those of 13-desmethyl rhodopsin (24). With the formation of meta-I, larger differences become apparent, especially with respect to the carboxyl groups interacting with the Schiff base. However, investigations on 9-desmethyl (12), 13-desmethyl (24) and *cis*-5,6-dihydro-isorhodopsin (Sheves, M., and F. Siebert, unpublished results) have shown that the molecular changes of the carboxyl groups are greatly influenced by the alteration of the steric interactions of the chromophore with the protein. In particular, the meta-I difference spectrum of 13-desmethyl-rhodopsin is very similar to that of ASMR (Fig. 3). Therefore, the influence of methylation of the Schiff base on the three internal carboxyl groups supports our previous conclusion that they must be located in close proximity to the chromophore. The observation that the fingerprint bands of ASMR and its photoproducts are upshifted and the corresponding

ethylenic modes downshifted, in agreement with the redshifted absorption maxima, indicates that the absorption maximum in rhodopsin is partially controlled by the distance of the Schiff base to the counterion.

Interestingly, removal of the 13-methyl group alters the photoreaction and causes larger spectral changes (24). Moreover, 13-desmethyl-rhodopsin is known to activate transducin (Rando, R., R., unpublished results) and, via transducin, the phosphodiesterase (25). Finally, when illuminated, it forms a room temperature intermediate almost identical to normal meta-II (24). Therefore, our investigation supports the conclusions, derived by Longstaff et al. (11), that deprotonation of the Schiff base is a prerequisite for transducin activation.

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