# A Mutant Rhodopsin Photoproduct with a Protonated Schiff Base Displays an Active-State Conformation: A Fourier-Transform Infrared Spectroscopy Study†

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ABSTRACT: In the rhodopsin mutant E113A/A117E the position of the protonated Schiff base counterion, $Glu^{113}$, is moved by one helix turn from position 113 to 117. The photoreaction of this mutant pigment was studied by Fourier-transform infrared (FTIR) difference spectroscopy. At acidic pH, formation of a 474-nm absorbing photoproduct previously characterized biochemically as a species that activates transducin caused infrared absorption changes typical of metarhodopsin II (MII) formation in native rhodopsin. Specific spectral alterations revealed a localized perturbation near the protonated Schiff base in the dark state. In addition, an infrared band assigned to the C=O stretching vibration of $Glu^{113}$ in MII of rhodopsin was abolished in the mutant. Absorption changes caused by $Asp^{83}$ and $Glu^{122}$ C=O stretching vibrations characteristic of rhodopsin MII formation were not affected. At alkaline pH, mutant E113A/A117E formed predominantly a 382-nm absorbing photoproduct. It displayed infrared-difference absorption bands significantly different from those of native MII over a large spectral range. These results support the conclusion that the 474-nm photoproduct of mutant E113A/A117E, despite a protonated Schiff base linkage, displays a predominantly MII-like conformation capable of catalyzing guanine-nucleotide exchange by transducin.

Photoreception in vertebrates employs a signal transduction pathway typical of G protein[1] -coupled receptor systems. In contrast to ligand-activated hormone receptors, the seven transmembrane helix receptor rhodopsin in the disc membranes of rod outer segments (ROS) of the retina is activated by light. The experimental advantage of a physical activation mechanism, as well as the ease by which large amounts of bovine rhodopsin or site-directed mutants of rhodopsin can be prepared, has rendered this system an intensely studied model for G protein-coupled signal transduction. Of particular interest is the elucidation of intramolecular changes which ultimately lead from an inactive receptor conformation in dark rhodopsin to the light-induced active conformation capable of catalyzing nucleotide exchange in transducin.

Visible light is absorbed by 11-*cis*-retinal which is covalently linked to $Lys^{296}$ of the apoprotein opsin via a protonated Schiff base (Oseroff & Callender, 1974). The positive charge of the Schiff base is stabilized by the negatively charged $Glu^{113}$ side chain in the third transmembrane helix (Nathans, 1990; Sakmar et al., 1989; Zhukovsky & Oprian, 1989). The chromophore isomerizes to the *all-trans* geometry (Shoenlein et al., 1991), thereby triggering a cascade of protein conformational changes which are thermally activated and characterized by absorption changes in the UV−visible range. A key event is the deprotonation of the Schiff base during formation of the metarhodopsin II (MII) photoproduct, which is accompanied by a shift of the absorption maximum ($\lambda_{max}$) of the *all-trans*-retinylidene Schiff base to 380 nm (Doukas et al., 1978). The MII state activates transducin (Kibelbek et al., 1991), and chemical modification of the active site lysine has demonstrated that in native rhodopsin Schiff base deprotonation is a prerequisite for signal transduction (Longstaff et al., 1986).

Previous studies of a detergent-solubilized rhodopsin mutant in which $Glu^{113}$ was replaced by Gln (E113Q) showed that an unprotonated Schiff base of 11-*cis*-retinal in the dark state of the recombinant pigment was able to form a MII-like photoproduct which activated transducin (Fahmy & Sakmar, 1993). A net deprotonation of the Schiff base was not required for photoactivation when a neutral amino acid occupied the position of the Schiff base counterion. To reconcile these results with the requirement of Schiff base deprotonation in native rhodopsin, we suggested that the deprotonation of the Schiff base may serve to neutralize $Glu^{113}$. Therefore, net deprotonation of the Schiff base may no longer be essential for the formation of a MII-like conformation in recombinant pigments already carrying a neutral side chain at position 113. The possible importance of neutralization of $Glu^{113}$ for rhodopsin activation is supported by the constitutive activity of mutant opsins in which $Glu^{113}$ was replaced by neutral amino acids (Cohen et al., 1992; Robinson et al., 1992). The hypothesis that Schiff base deprotonation is not essential *per se* but provides a light-sensitive mechanism to neutralize $Glu^{113}$ can be tested in two ways. First, the proposed protonation of $Glu^{113}$ in MII can be probed by FTIR-difference spectroscopy as an infrared absorption increase in the frequency range of C=O stretching vibrations of protonated carboxylic acid groups

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[1] Abbreviations: Con-A, conconavalin-A; FTIR, Fourier-transform infrared; G protein, guanine nucleotide-binding regulatory protein; MI, metarhodopsin I; MII, metarhodopsin II; R*, photoactivated rhodopsin; ROS, rod outer segment.

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between 1700 and 1800 $cm^{-1}$. Second, rhodopsin mutants might be found which form photoproducts containing a protonated Schiff base, yet activate transducin due to a neutral amino acid side chain introduced at position 113.

The mutant E113A/A117E in which the retinylidene Schiff base counterion was moved from position 113 to 117 bound 11-*cis*-retinal and displayed a $\lambda_{max}$ value of 491 nm (Zvyaga et al., 1993, 1994). Illumination produced a pH-sensitive mixture of 382- and 474-nm absorbing forms, which contained an unprotonated and a protonated Schiff base, respectively. Transducin activation rates correlated with the amount of 474-nm photoproduct, indicating that the mutant pigment constituted the hypothesized active receptor state with a protonated Schiff base (Zvyaga et al., 1994). Another mutant (G90D) was suggested possibly to activate transducin under conditions where the photoproduct carried a protonated Schiff base (Rao et al., 1994).

Here, we have used FTIR-difference spectroscopy to monitor chromophore isomerization as well as changes of the protonation state and the hydrogen-bond strength of internal carboxylic acid groups during formation of the 382-nm and 474-nm photoproducts of mutant E113A/A117E. The infrared bands provide criteria to assess MII-like conformations by comparison with the rhodopsin to MII difference spectrum of bovine rhodopsin. The spectra obtained from the 474-nm photoproduct of mutant E113A/A117E are consistent with an active (MII-like) receptor conformation containing a protonated Schiff base and confirm the conclusions of biochemical studies (Zvyaga et al., 1994). Furthermore, these results support the finding of a net transfer of the Schiff base proton to $Glu^{113}$ in native rhodopsin (Jäger et al., 1994).

## MATERIALS AND METHODS

*Materials.* Sources of reagents and materials have been reported (Chan et al., 1992; Fahmy et al., 1993; Zvyaga et al., 1993, 1994).

*Preparation of Rhodopsin and Rhodopsin Mutants.* Conconavalin-A (con-A) purified rod outer segment (ROS) rhodopsin was prepared in 0.02% dodecyl maltoside as described (König et al., 1989). The mutant E113A/A117E gene was prepared as described (Zvyaga et al., 1994). The mutant pigment was purified for FTIR studies according to procedures described (Fahmy et al., 1993).

*UV−Visible Absorption Spectroscopy.* The photoreaction of the mutant pigment was monitored in hydrated films prepared in the same way as for infrared spectroscopy. Recombinant pigment in 0.02% (w/v) dodecyl maltoside (5−10 μL, ~300 pmol) and either 50 nmol of sodium phosphate buffer (pH 4.8) or 100 nmol of imidazole buffer (pH 8.5) were dried by a gentle stream of $N_2$ on a quartz window, rehydrated and cooled to 0 °C on the sample stage of a λ-19 Perkin-Elmer spectrophotometer. Illumination (1 min) was carried out through fiber optics with a 150-W slide projector equipped with a 495-nm long-pass filter. Light-induced difference spectra were obtained by subtracting the absorption spectrum obtained before illumination from that after illumination. To estimate pH of the film sample, a wet indicator strip was pressed onto the hydrated pigment film after the experiment.

*FTIR-Difference Spectroscopy.* Infrared-difference spectra were obtained from ~1.2 nmol of recombinant material in $H_2O$ or $D_2O$ as described (Fahmy et al., 1993). Buffers were identical with those used in the UV−visible experiments. Calcium fluoride windows were used to support the hydrated films. Measurements were performed on a home-built FTIR spectrometer equipped with a IFS-88 interferometer (Bruker) and a MCT detector (EG & Judson). Photoactivation was elicited as in UV−visible experiments. FTIR-difference spectra with a spectral resolution of 2 $cm^{-1}$ were calculated from 128 scans before and after illumination as described (Ganter et al., 1990). For a reference spectrum con-A purified ROS rhodopsin in 0.02% dodecyl maltoside was used under conditions identical to the recombinant material.

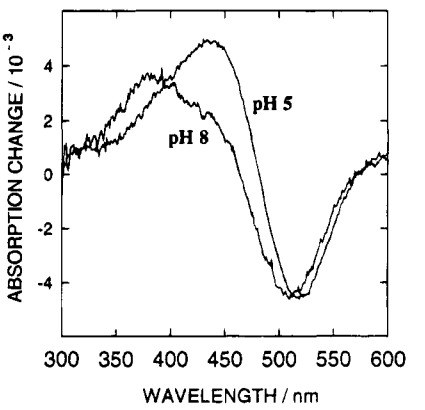


FIGURE 1: UV−visible absorption photobleaching-difference spectra of hydrated films of rhodopsin mutant E113A/A117E. Experimental conditions are described in Materials and Methods.

## RESULTS

*Biochemical Characterization of Mutant Pigment.* The preparation and biochemical characterization of mutant pigment E113A/A117E has been reported (Zvyaga et al., 1993, 1994). As a control for FTIR experiments, UV−visible absorption photobleaching-difference spectra of the mutant were recorded of hydrated films on quartz windows prepared identically to FTIR samples. When dried and rehydrated in the presence of phosphate buffer (final pH ~ 5), illumination of the film at 0 °C produced the 474-nm photoproduct (Figure 1). This species decayed thermally to the 382-nm photoproduct with a half time of ~50 min. The 382-nm form was predominantly formed at alkaline pH when the film was prepared in the presence of 100 nmol imidazole (final pH of ~8) (Figure 1). Comparison of the traces in Figure 1 shows that the 474-nm photoproduct could be obtained quantitatively, whereas the 382-nm form was only partly formed as shown by the shoulder at ~460 nm in the absorption difference spectrum obtained at pH 8. These conditions allowed the FTIR-difference spectra to be recorded of the formation of the 474-nm photoproduct at acidic pH and of a photoproduct mixture dominated by the 382-nm form at alkaline pH. Since the scan speed was fast, 128 interferograms could be averaged within 1 min after illumination. During this time only a negligible amount of the 474-nm form decayed to the 382-nm form.

*FTIR-Difference Spectra of Mutant E113A/A117E and ROS Rhodopsin.* It has been shown by previous resonance Raman (Lin et al., 1992) and FTIR studies (Fahmy et al., 1993) on detergent-solubilized recombinant rhodopsin that no significant perturbations are introduced into the spectra by the COS-cell expression and purification procedures. Furthermore, the spectra obtained from detergent-solubilized recombinant rhodopsin are nearly identical to those of

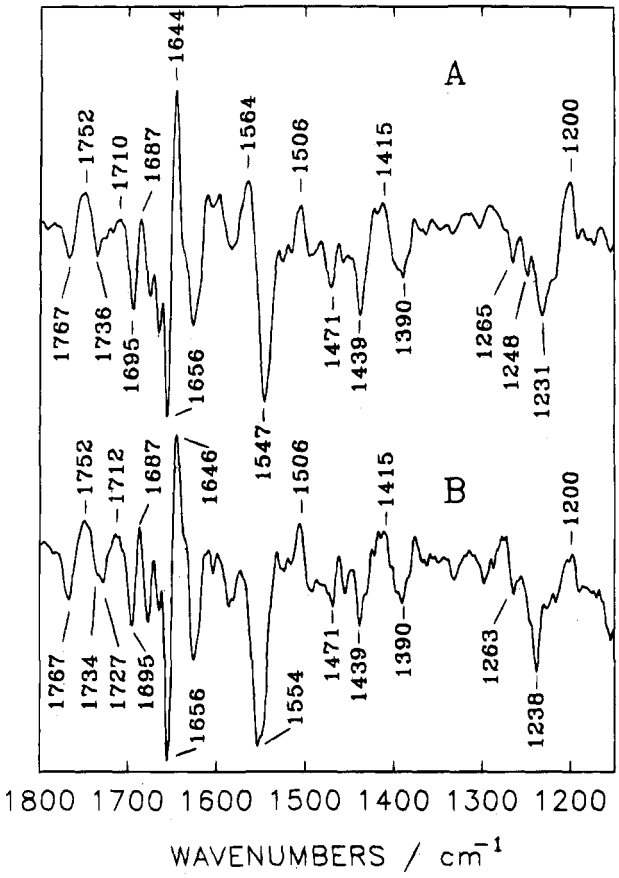


FIGURE 2: Infrared difference spectra of the photoreaction of mutant E113A/A117E and ROS rhodopsin at acidic pH. Spectra were recorded at 0 °C at pH ~5. Illumination was carried out through a 495 nm long-pass filter. One hundred twenty-eight interferograms were averaged before and after photoconversion for each spectrum. Bands of photoproducts point upwards, those of the dark states downwards. (A) Infrared absorption changes during formation of the 474-nm mutant photoproduct. (B) Infrared absorption changes during MII formation in ROS rhodopsin.

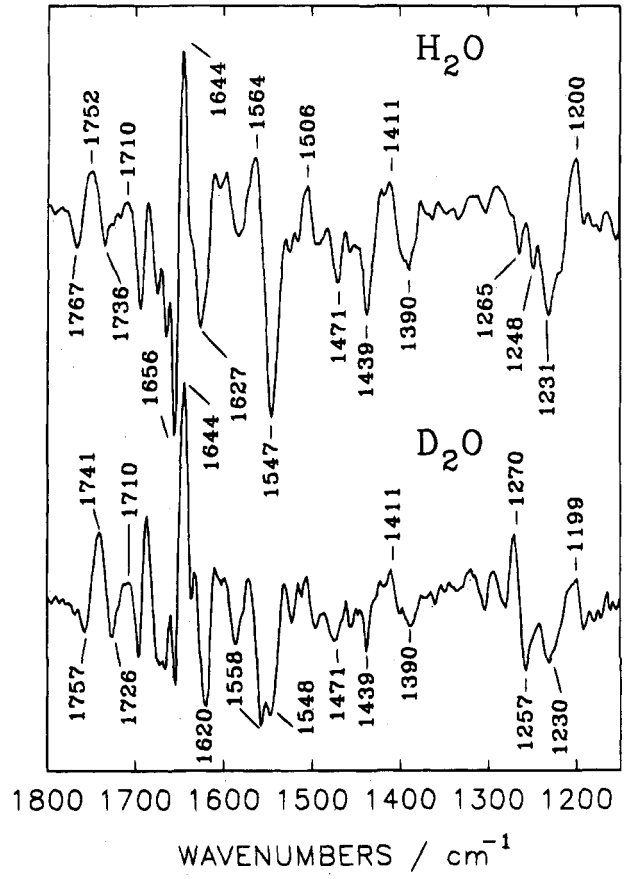


FIGURE 3: Effect of H/D isotope exchange on infrared absorption changes during formation of the 474-nm mutant photoproduct. Spectra in the presence of $H_2O$ and $D_2O$ are presented. Convention and experimental conditions are as given in the legend to Figure 2.

recombinant rhodopsin reconstituted into vesicles (Rath et al., 1993). Therefore, we have used spectra of detergent-solubilized ROS rhodopsin as a reference to assess mutant E113A/A117E.

*FTIR-Difference Spectra of the Mutant 474-nm Photoproduct.* Figure 2A shows the infrared absorbance changes of the mutant pigment obtained at pH 5 in $H_2O$ when the 474-nm species was formed. Infrared bands of the photoproduct point upward and those of the dark state point downward. Figure 2B shows the corresponding spectrum of ROS rhodopsin. The C=C stretching vibration of the 11-*cis*-retinal Schiff base in the dark state of the mutant pigment is expected between 1530 and 1560 cm$^{-1}$, but cannot be assigned unequivocally due to the overlapping amide II absorption changes. Based on the inverse relationship between visible absorption maximum and C=C stretching frequency of retinal polyenes (Doukas et al., 1978; Rimai et al., 1973) a C=C stretch close to 1550 cm$^{-1}$ is expected for the 490-nm absorbing dark state of the mutant. Therefore, the strong negative band at 1547 cm$^{-1}$ most likely contains the C=C stretching vibration of the mutant pigment.

The spectral range between 1200 and 1300 cm$^{-1}$ ("fingerprint region") comprises absorption bands of the C–C stretching vibrations coupled to C–H bending motions of the retinal Schiff base. The position of these bands is sensitive to the retinal geometry. The major negative absorption band at 1238 cm$^{-1}$ in ROS rhodopsin is a highly delocalized mode caused by the $C_{12}$–$C_{13}$ and the $C_{14}$–$C_{15}$ stretching vibrations coupling to H in plane bending modes at $C_{14}$, $C_{15}$, and the Schiff base nitrogen of the 11-*cis*-retinal Schiff base (Palings et al., 1987; Ganter et al., 1988). Comparison with the position and relative intensity of this vibration in the mutant difference spectrum argues for a mutational effect on the coupling of these modes in the 11-*cis*-retinal chromophore of the mutant, thereby shifting the main absorption band by 7 cm$^{-1}$ to 1231 cm$^{-1}$. This has also been observed in the difference spectrum of the single amino acid replacement mutant E113A (Jäger et al., 1994). The band shift is most likely caused by a steric perturbation near $C_{12}$–$C_{13}$ caused by the alanine introduced at position 113 (Lin et al., 1993) and by a different interaction of the Schiff base with the repositioned counterion, $Glu^{117}$.

Interestingly, when measured in $D_2O$ the fingerprint bands of the mutant spectrum (Figure 3) look almost identical to those of ROS rhodopsin (not shown). Such a behavior supports the notion that the spectral changes introduced by the mutation are indicative of different coupling of the involved modes to the NH bending vibration. The coupling is abolished when the NH bend is replaced by an ND bending mode of much lower frequency than the CH bending motions. Consequently, the mutational perturbation near the Schiff base is no longer reflected in the fingerprint region. Therefore, the spectral alterations caused by the mutation do not indicate significant changes of the chromophore geometry, but rather subtle perturbations near the Schiff base.

The down-shift of the 1238-$cm^{-1}$ band to 1231 $cm^{-1}$ uncovers a negative absorption at 1248 $cm^{-1}$ in the mutant pigment, which is usually discernible only as a shoulder in the 1238-$cm^{-1}$ band. A distinct band at 1263 $cm^{-1}$ not assigned to a particular infrared active mode but close in frequency to the Raman active $C_{11}H + C_{12}H$ in-plane rock (Palings et al. 1987) of 11-*cis*-retinal in rhodopsin is reproduced in the mutant spectrum. Similarly, a distinct absorption of the mutant photoproduct is observed at 1200 $cm^{-1}$ which in ROS rhodopsin is most likely caused by the $C_{14}-C_{15}$ stretching vibration of the *all-trans*-retinal chromophore (Palings et al., 1987). In summary, the similarity of the prominent vibrational bands of the chromophore to those of ROS rhodopsin in $H_2O$ and in $D_2O$ indicates that an 11-*cis* to *all-trans* photoisomerization occurs in the mutant pigment.

In spite of the different Schiff base protonation states in the 474-nm photoproduct of E113A/A117E and MII of rhodopsin similar chromophore-protein interactions can be inferred from the very well reproduced bands at 1506, 1471, 1439, 1415, and 1390 $cm^{-1}$. These absorption changes are caused by yet unidentified amino acid side chains which are sensitive to the light-induced retinal isomerization. Some alterations in the mutant spectrum, however, indicate minor conformational perturbations. A small positive band at 1460 $cm^{-1}$ in rhodopsin is reduced in the spectrum of the mutant.

The absorption changes in the amide II region, which comprise the coupled C−N stretching and N−H bending vibrations of the polypeptide backbone, agree with a slightly different conformational change in the mutant as compared with rhodopsin. A prominent photoproduct band at 1564 $cm^{-1}$ is observed in the mutant spectrum which is absent from the control spectrum of rhodopsin. The sensitivity of this band to $H_2O/D_2O$ exchange (Figure 3) supports the assignment to an amide II vibration rather than to the C=C stretch of the mutant photoproduct. The amide I absorption changes between 1620 and 1700 $cm^{-1}$, which are caused by the polypeptide C=O stretching vibrations, are very well reproduced in the mutant spectrum. This is not only the case for the prominent bands at 1656, 1644, and 1627 $cm^{-1}$ but also for subtle absorption changes between 1660 and 1700 $cm^{-1}$. Only a small negative band at 1727 $cm^{-1}$ usually observed in MII difference spectra is lacking in the mutant spectrum. This band was found earlier to vanish in a $Glu^{122}$ single replacement mutant as well (Fahmy et al., 1993).

The large majority of conformation-sensitive bands in the 474-nm mutant photoproduct is characteristic of a MII-like conformation. The conformational similarity between MII of rhodopsin and the 474-nm photoproduct is further supported by absorption changes of membrane-embedded carboxyl groups. In a previous FTIR study on detergent-solubilized COS-cell rhodopsin, we assigned infrared absorption changes to weakly hydrogen-bonded protonated carboxylic acid side chains in the hydrophobic receptor domain. $Asp^{83}$ (Fahmy et al., 1993; Rath et al., 1993) and $Glu^{122}$ (Fahmy et al., 1993) were found to be protonated in both dark rhodopsin and in MII. Changes in the hydrogen bond strength of both residues during photoactivation give rise to difference bands at 1767 (negative)/1750 $cm^{-1}$ (positive) and 1734 (negative)/1745 $cm^{-1}$ (positive), respectively. The 474-nm photoproduct of mutant E113A/A117E exhibits absorption changes at identical positions as shown in Figure 2A. Since these bands are characteristic of the MII conformation (Klinger & Braiman, 1992), the 11-*cis* to *all-trans* isomerization in the mutant pigment causes conformational changes in the membrane-embedded domain that affect the hydrogen-bond environments of $Asp^{83}$ and $Glu^{122}$ in the same way as in rhodopsin even though Schiff base deprotonation does not occur.

The intensity of the positive band at 1712 $cm^{-1}$ assigned to the C=O stretching mode of a protonated carboxylic acid group newly formed in MII is significantly reduced in the 474-nm photoproduct. This vibration has been assigned to protonation of the counterion, $Glu^{113}$, in MII of rhodopsin (Fahmy et al., 1993; Jäger et al., 1994). Since the counterion is replaced by Ala in mutant E113A/A117E, a lack of this band was expected and it was crucial to investigate the residual absorbance of the 474-nm photoproduct at 1710 $cm^{-1}$. Replacement of $H_2O$ by $D_2O$ shifts the C=O stretching frequency of a protonated carboxyl group to lower wavenumbers. This provides a criterion for the assignment of the carbonyl absorption to a protonated carboxylic acid group. The spectrum in Figure 3 shows the effect of $D_2O$ on the absorption changes. Clearly, the difference bands of $Asp^{83}$ and $Glu^{122}$ are down-shifted by ~10 $cm^{-1}$ as previously observed, whereas the residual absorbance at 1710 $cm^{-1}$ is not affected by the isotope exchange. Therefore, the $Glu^{113}$ to Ala replacement specifically abolished a $D_2O$-sensitive band at 1712 $cm^{-1}$ assigned to protonated $Glu^{113}$ in MII.

*FTIR-Difference Spectrum of the Mutant 382-nm Photoproduct.* Figure 4 compares the difference spectra of mutant E113A/A117E and rhodopsin obtained at alkaline pH where the 382-nm form of the mutant photoproduct dominated. The 382-nm species did coexist with some amount of the 474-nm species (compare Figure 1 for a visible control spectrum). The absorption changes in the fingerprint region are identical to those of the 474-nm form with the exception of a small additional difference band at 1276/1272 $cm^{-1}$ and a gain in intensity of the band at 1205 $cm^{-1}$. However, large spectral deviations from the spectrum of the 474-nm species (Figure 2A) and from the spectrum of MII at alkaline pH (Figure 4B) occur over a broad spectral range. This is most pronounced in the intense additional absorption at 1521 $cm^{-1}$ in the dark state of the mutant and by the lack of the negative band at 1390 $cm^{-1}$ usually observed in the dark state of rhodopsin. Whereas the absorption of the MII state is weak at 1411 $cm^{-1}$, a relatively strong band occurs in the 382-nm photoproduct at this position. Conversely, no absorption increase is observed at 1508 $cm^{-1}$ in the mutant spectrum where the MII spectrum displays a distinct band. The general difference between mutant and ROS rhodopsin in the 1390 to 1510 $cm^{-1}$ range is the significant pH sensitivity of the difference bands of the mutant spectra. In contrast, absorption changes of MII formation in rhodopsin at alkaline pH (Figure 4B) are almost identical to those observed at acidic pH (Figure 2B).

The absorption changes in the amide II region are also considerably different from those observed upon formation of the 474-nm species or of MII at alkaline pH. Instead of the intense negative band at 1558 $cm^{-1}$, a major difference band is observed at 1552/1568 $cm^{-1}$. Although an increase in pH affects the amide I/II absorption changes in rhodopsin, the effects are again more pronounced in the mutant. Whereas the increase in pH mainly abolishes the intense negative amide I absorption at 1656 $cm^{-1}$ in rhodopsin (Figure 4B versus Figure 2B), the relative intensity of the

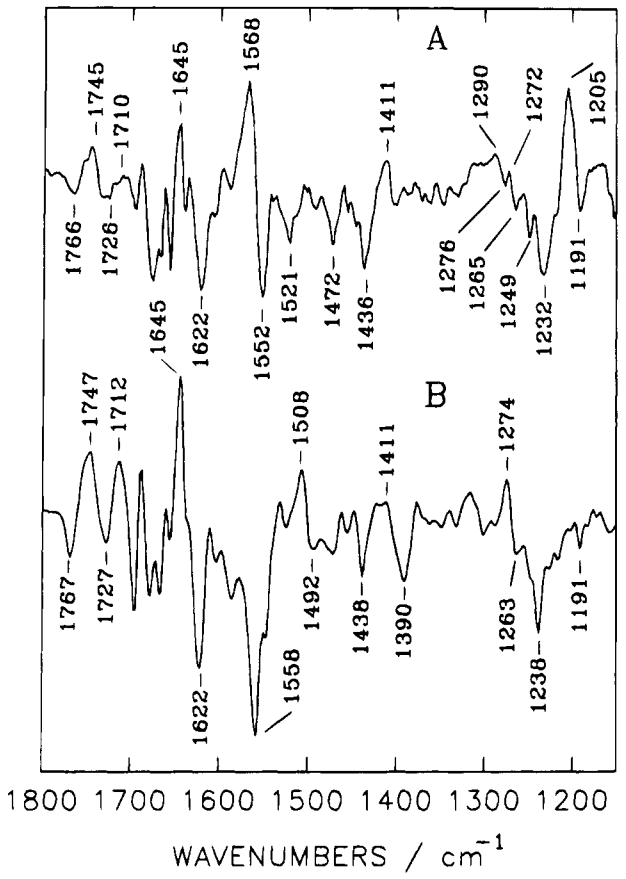


FIGURE 4: Infrared-difference spectra of the photoreaction of mutant E113A/A117E and ROS rhodopsin at alkaline pH. Convention and experimental conditions are as in the legend to Figure 2, except that spectra were recorded at pH ~8. (A) Infrared absorption changes during formation of predominantly the 382-nm mutant photoproduct. An unknown amount of the 474-nm-absorbing photoproduct also contributes to infrared absorption changes under these conditions. (B) Infrared absorption changes during MII formation of ROS rhodopsin at alkaline pH. Due to the presence of dodecyl maltoside detergent, metarhodopsin I (MI) formation usually observed at alkaline pH does not occur.

1690 cm$^{-1}$ difference band is drastically reduced and a new negative band at 1640 cm$^{-1}$ is observed in the mutant difference spectrum. This indicates that at alkaline pH the polypeptide backbone of mutant E113A/A117E undergoes conformational changes which differ to a larger extent from those in rhodopsin than is the case for the 474-nm photoproduct at pH 5. In particular, an absorption difference typical of the rhodopsin to MII conversion at 1695 cm$^{-1}$ (negative)/1687 cm$^{-1}$ (positive) is reduced to about one-half of the usual intensity, where the intensities of the negative fingerprint bands are taken as a relative measure.

The frequencies of the absorption changes of carbonyl stretching vibrations of protonated carboxyl groups look identical to those observed during formation of the 474-nm species. However, normalizing again to the size of the fingerprint absorption changes, the magnitude of the C=O stretch absorption differences for the 382-nm photoproduct formation is significantly smaller than for the 474-nm form. Taking also into account that a certain amount of the 474-nm species is still formed at alkaline pH, it seems that the 382-nm form contributes little if at all to the absorption differences observed between 1700 and 1800 cm$^{-1}$.

In summary, the difference spectrum of the mutant 382-nm form differs to a much larger extent from the MII control spectrum than does the spectrum of the 474-nm photoproduct. In particular, typical MII bands are missing from the 382-nm photoproduct and the existence of additional negative bands suggests that at alkaline pH the dark state of the mutant is already significantly perturbed and thereby unable to form a MII-like conformation after photoisomerization of the chromophore. Does Glu$^{117}$, which successfully stabilizes the Schiff base proton in the dark state, also replace the proton acceptor function of Glu$^{113}$ in the photoproduct at alkaline pH when Schiff base deprotonation is allowed to occur? If so, a new photoproduct band absent from the spectrum of the 474-nm species would be expected. As pointed out, all bands in this range appear smaller than in the spectrum in Figure 2A arguing against a C=O stretching vibration assignable to the protonation of Glu$^{117}$ in the 382-nm photoproduct. In principle such a band may be superimposed with the positive lobes of the C=O stretching difference bands assigned to Asp$^{83}$ and Glu$^{122}$ between 1750 and 1740 cm$^{-1}$. To check this possibility, we have prepared the triple-replacement mutant E113A/A117E/D83N. The infrared absorption changes in the 1700−1800-cm$^{-1}$ range show that the removal of Asp$^{83}$ isolates the difference band of protonated Glu$^{122}$ (data not shown) as previously reported for the single mutation D83N (Fahmy et al., 1993). No positive band attributable to protonation of Glu$^{117}$ was observed. Therefore, it is likely that the Schiff base proton is released to the aqueous phase. This may also explain the pH sensitivity of the Schiff base protonation state in the photoproduct of mutant E113A/A117E. This pH sensitivity is not observed in rhodopsin where a net proton transfer from the Schiff base to the internal proton acceptor Glu$^{113}$ occurs (Jäger et al., 1994).

## DISCUSSION

FTIR-difference spectra were recorded from the double-replacement mutant E113A/A117E in order to assess conformational similarities between the mutant photoproducts and the MII state of rhodopsin. Besides minor deviations in the amide II region, absorption changes typical of the rhodopsin to MII transition were observed for the formation of the 474-nm absorbing mutant photoproduct obtained at acidic pH. The vibrational modes agree with an 11-*cis* to *all-trans* isomerization. In addition, changes in the hydrogen-bond strength of Asp$^{83}$ and Glu$^{122}$, which are specific for MII formation, were identical to those of rhodopsin (Fahmy et al., 1993; Rath et al., 1993).

At alkaline pH, 11-*cis* to *all-trans* photoisomerization caused spectral changes in the amide I and amide II regions that deviated more severely from those observed in rhodopsin, indicating larger protein conformational perturbations. The characteristic absorption changes of the C=O stretching vibrations of Asp$^{83}$ and Glu$^{122}$ were also reduced in the 382-nm form. These results support the notion that the 474-nm photoproduct, rather than the 382-nm form, possesses conformational features similar to light-activated rhodopsin and is mainly responsible for light-dependent transducin activation by the mutant (Zvyaga et al., 1994).

Regarding the intramolecular mechanism of photoactivation in rhodopsin, the data imply that after photoisomerization, the deprotonation of the Schiff base is not required to form an active receptor conformation. In order to reconcile this result with the reported requirement for Schiff base deprotonation for R* formation in native rhodopsin (Longstaff

et al., 1986), we propose that this deprotonation provides a mechanism to couple a light-induced sterical event (i.e., photoisomerization of retinal) to an electrostatic alteration in the chromophore binding site (i.e., neutralization of the counterion $Glu^{113}$). In fact, mutant opsins with neutral amino acids at position 113 activate transducin in the absence of chromophore (Cohen et al., 1992; Robinson et al., 1992). However, the protonation state of the $Lys^{296}$ side chain could not be monitored in those experiments, rendering neutralization of $Lys^{296}$ and $Glu^{113}$ equally possible to explain receptor activation. The present results suggest that in native rhodopsin the neutralization of $Glu^{113}$ after photoisomerization, in addition to the role of Schiff base neutralization (Longstaff et al., 1986), is important for formation of a fully active state, R*. A more detailed discussion of the possible role of $Glu^{113}$ will be presented elsewhere (Fahmy et al., 1994).

The neutralization of $Glu^{113}$ is achieved by mutation in pigment E113A/A117E irrespective of the Schiff base protonation state so that photoisomerization alone can trigger the formation of R*, as was the case for mutant E113Q (Fahmy & Sakmar, 1993). The results show that if a suitable environment can be established to stabilize the Schiff base proton after photoisomerization by a newly introduced counterion, then an active receptor conformation can be obtained with a protonated Schiff base, as in the 474-nm photoproduct of mutant E113A/A117E. On the other hand, the lack of transducin activation by the mutant E113A/A117E in the dark shows that neutralization of $Glu^{113}$ alone, although important for light-dependent receptor activation, is not sufficient to induce the active receptor conformation.

The proposed model of $Glu^{113}$ neutralization via Schiff base deprotonation implies a net proton transfer from the Schiff base to $Glu^{113}$ in rhodopsin. Evidence for this mechanism is provided by the assignment of the C=O stretching vibration (1712 $cm^{-1}$ in rhodopsin) of a protonated carboxyl group in MII to $Glu^{113}$ based on the FTIR-difference spectra of single amino acid replacement mutants (Jäger et al., 1994). The assignment is consistent with the spectra of the double-replacement mutant as shown by the lack of this band in the spectrum of the 474-nm photoproduct and loss of $D_2O$ sensitivity of the small residual band at this position.

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