Identification of Glutamic Acid 113 as the Schiff Base Proton Acceptor in the Metarhodopsin II Photointermediate of Rhodopsin†

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ABSTRACT: In order to investigate the molecular mechanism of rhodopsin photoactivation, site-directed mutants of bovine rhodopsin were studied by Fourier-transform infrared (FTIR) difference spectroscopy. Rhodopsin mutants E113D and E113A were prepared in which the retinylidene Schiff base counterion, Glu^{113}, was replaced by Asp and Ala, respectively. FTIR difference spectra were recorded and compared with spectra of recombinant native rhodopsin. Both mutant pigments formed photoproducts at 0 °C with vibrational absorption bands typical of the metarhodopsin II (MII) state of rhodopsin. The FTIR difference spectrum of E113D was nearly identical to that of rhodopsin. A positive band at 1712 cm^{-1} caused by the protonation of an internal carboxylic acid in rhodopsin was shifted slightly to 1709 cm^{-1} in mutant E113D. E113A was studied at acidic pH in the presence of chloride as an inorganic counterion to the protonated Schiff base. The 1712-cm^{-1} (1709-cm^{-1}) band was absent in the FTIR difference spectrum of mutant E113A. Therefore, we have assigned the 1712-cm^{-1} absorbance band to the C=O stretching vibration of protonated Glu^{113} in MII of rhodopsin. These results show that the Schiff base counterion of rhodopsin, the carboxylate side chain of Glu^{113}, becomes protonated during MII formation.

Rhodopsin is the photoreceptor which initiates the visual transduction cascade in the outer segments of the retinal rod cell (Stryer, 1991). It is a member of the superfamily of seven-transmembrane helix, G protein[1]-coupled receptors. Photoisomerization of the 11-*cis*-retinal chromophore (Shoenlein et al., 1991), which is covalently bound via a protonated Schiff base (PSB) linkage (Oseroff et al., 1974) to Lys^{296} (Hargrave, 1983; Ovchinnikov, 1982), is followed by a thermal relaxation process leading to the active receptor state, R*. In the dark, the positive charge of the PSB is stabilized by a negatively charged carboxylate at position Glu^{113} (Nathans, 1990; Sakmar et al., 1989; Zhukovsky & Oprian, 1989). R* is spectrally characterized as metarhodopsin II (MII) and contains an all-trans chromophore bound via an unprotonated *all-trans*-retinylidene Schiff base (SB) linkage (Doukas et al., 1978). Since formation of MII is accompanied by proton uptake (Arnis & Hofmann, 1993; Wong & Ostroy, 1973) rather than by proton release, it has been suggested that the Schiff base proton is transferred to an internal amino acid side chain, which acts as a proton acceptor, during MII formation (Arnis & Hofmann, 1993).

In a previous FTIR study of mutant rhodopsin pigments, we showed that among three putative membrane-embedded carboxylic acid side chains, Asp^{83} and Glu^{122} were protonated in dark rhodopsin. We suggested that the PSB counterion, Glu^{113}, was likely to serve the role of a proton acceptor during Schiff base deprotonation (Fahmy et al., 1993). Here, we have investigated infrared absorption changes during the MII-like photoproduct formation in the single amino acid replacement mutants E113D and E113A. The spectral changes were correlated with the mutations to prove the assignment of a C=O stretching vibrational band at 1712 cm^{-1} in the FTIR difference spectrum of rhodopsin. This band corresponds to the carboxylic acid group of Glu^{113}, which becomes protonated in MII.

EXPERIMENTAL PROCEDURES

Preparation of Mutants. Genes for the single amino acid replacement opsin mutants E113D and E113A were prepared by restriction fragment replacement in a synthetic opsin gene as described (Ferretti et al., 1986; Franke et al., 1988; Sakmar et al., 1989). After transient expression in monkey kidney cells (Oprian et al., 1987), 11-*cis*-retinal was added in the dark. Pigments were purified in dodecyl maltoside buffer by an immunoaffinity adsorption procedure (Franke et al., 1992; Oprian et al., 1987). Elution of the pigment from the affinity resin was carried out with an octadecapeptide in the presence of 0.02% dodecyl maltoside and 0.5 mM sodium phosphate, pH 6.5. The peptide was subsequently removed and the samples were concentrated as described (Fahmy et al., 1993).

FTIR Spectroscopy. About 1.25 nmol of each preparation was dried onto an AgCl window under a N_2 stream. The samples were rehydrated, sealed with a Ge window, and cooled to 0 °C. Measurements were performed on a spectrometer equipped with an IFS-88 interferometer (Bruker) and a HgCdTe detector (EG & G-Judson) as previously described (Fahmy et al., 1993). Illumination (1 min) was performed within the spectrometer using a 150 watt slide projector with a long-pass filter (>495 nm). Mutant and native spectra were averaged over 512 and 256 scans, respectively. Difference spectra were calculated as described (Siebert et al., 1983; Ganter et al., 1990). For rhodopsin and mutant E113D, about 500 nmol of sodium phosphate buffer (pH 5.5) was added,

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[1] Abbreviations: FTIR, Fourier-transform infrared; G protein, guanine nucleotide-binding regulatory protein; MII, metarhodopsin II; PSB, protonated Schiff base; R*, light-activated rhodopsin; ROS, rod outer segment; SB, Schiff base.

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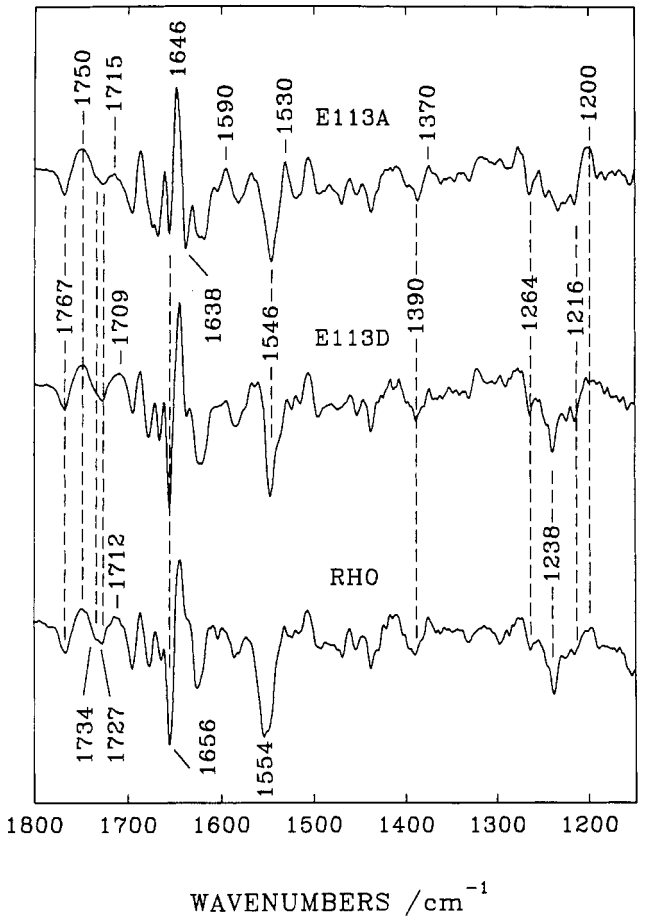

FIGURE 1: Infrared absorption difference spectra of the photoreactions of mutants E113A, E113D, and COS cell rhodopsin. Experiments were carried out at 0 °C on detergent-solubilized pigments. The photoreaction was induced by 1-min illumination through a 495-nm long-pass filter. Spectral resolution is 2 cm^{-1}.

and for mutant E113A, about 200 nmol of sodium phosphate buffer (pH 4.2) and 800 nmol of sodium chloride were added. For deuterium isotope-exchange experiments, 0.5 μL of D_2O was deposited in the sample holder for rehydration of the dried film.

RESULTS

The mutant opsin E113D regenerates with 11-*cis*-retinal to form a pigment with a visible absorption maximum (λ_{max}) of ~505 nm independently of pH. Mutant E113A forms a pigment absorbing at 506 nm at acidic pH if chloride is present as an exogenous counterion to the protonated Schiff base as reported earlier (Sakmar et al., 1989, 1991). In order to investigate structural changes accompanying the photoreaction of rhodopsin and to determine putative protonation changes of the carboxylic acid side chain at position 113, we have recorded FTIR difference spectra of three recombinant pigments. Figure 1 compares the light-induced difference spectra of the two mutant pigments with that of recombinant rhodopsin in the spectral range from 1150 to 1800 cm^{-1}. Photoproduct bands point upward, whereas the dark-state pigment bands point downward. Below about 1150 cm^{-1}, the absorption of dodecyl maltoside causes increased noise. Therefore, the hydrogen-out-of-plane (HOOP) vibrations of the chromophore are not resolved.

In the "fingerprint region" between 1200 and 1300 cm^{-1}, which is characteristic of the retinal chromophore geometry, three negative bands are observed at 1216, 1238, and 1264 cm^{-1} in the spectra of rhodopsin and E113D. The main contribution to absorption changes in this region is caused by the 1238-cm^{-1} band, which has been described as a coupling of C_{12}–C_{13}, C_{14}–C_{15} stretching vibrations and N–H, C_{14}–H, and C_{15}–H bending vibrations (Palings et al., 1987; Ganter et al., 1988a,b). Obviously, an aspartate can structurally and functionally replace the glutamate counterion without affecting the mutual coupling of the different infrared modes. On the basis of the good correspondence between the fingerprint bands in the E113D mutant and rhodopsin spectra, an 11-cis to all-trans photoisomerization of the chromophore can be inferred for the mutant.

In contrast, a larger impact of the Glu^{113} to Ala replacement is observed in the presence of an inorganic chloride counterion. The lack of the 1238-cm^{-1} band of the 11-*cis*-retinal chromophore in the spectrum of mutant E113A suggests that the C–C stretching and C–H bending vibrations couple differently to the N–H bending mode of the SB. Such a mutational effect is not surprising because the replacement of the organic counterion by chloride has been found to weaken the hydrogen bond strength of the PSB in this mutant (Lin et al., 1992). Instead of the 1238-cm^{-1} vibration, a negative band at 1233 cm^{-1} is apparent. This band shift uncovers a negative absorption at 1250 cm^{-1} normally only present as a shoulder in the dominating 1238-cm^{-1} band. Another band coupling to the N–H vibration absorbs at 1390 cm^{-1} in COS cell rhodopsin. This band has been described as a combination of the N–H with the C_{14}–H and C_{15}–H bending vibrations in ROS rhodopsin (Ganter et al., 1988a,b). The frequency of this vibration is again reproduced in the spectrum of mutant E113D. However, it is slightly downshifted to 1387 cm^{-1} in the spectrum of E113A. Besides these alterations, other difference bands at 1264 and 1216 cm^{-1}, which are characteristic of the 11-cis to all-trans isomerization of the retinal chromophore, are reproduced in the mutant E113A spectrum. Therefore, the described spectral alterations in the fingerprint region are indicative of a local perturbation of the Schiff base environment in E113A rather than reflecting gross changes in the retinal geometry.

A specific effect on the Schiff base has in particular been inferred from a shift of the resonance Raman band of the C=N stretching vibration in E113A (1645 cm^{-1}) versus native rhodopsin (1656 cm^{-1}) (Lin et al., 1992). In agreement with the resonance Raman band assignment, the infrared difference spectrum of E113A shows a reduction of the negative band at 1656 cm^{-1} caused by the C=N stretching mode in dark rhodopsin. A concomitant increase of the negative band at 1638 cm^{-1} in the mutant difference spectrum may be due to the expected C=N stretch at 1645 cm^{-1} but is superimposed with the strong absorption increase at 1646 cm^{-1}. In the dark state of mutant E113D, the C=N stretching vibration must be similar to that of rhodopsin since the typical negative band at 1656 cm^{-1} is reproduced.

The C=C stretching vibration of the retinal chromophore is expected to absorb between 1530 and 1560 cm^{-1}. Both mutant pigments exhibit a large negative band at 1546 cm^{-1}, whereas a broader band with a major contribution at 1554 cm^{-1} is observed in dark rhodopsin. A direct assignment of these bands to the respective C=C stretching vibrations cannot be made because of the overlapping amide II absorption changes of the polypeptide backbone. The inverse relation between the C=C stretching frequency and the visible absorption of a retinal polyene (Doukas et al., 1978; Rimai et al., 1973) suggests that the infrared mode is lower in both mutants as compared with rhodopsin since the visible absorp-

tion is red shifted by ~6 nm in both mutant E113A (with chloride counterion) and mutant E113D. Therefore, at least part of the shift to lower frequency of the predominant negative bands in the mutant spectra is most likely due to a downshift of the C=C stretching frequencies in the mutant pigments. However, altered amide II absorption changes of the polypeptide backbone also contribute to the observed differences. This is the case in particular for mutant E113A, which exhibits an absorption increase at 1530 cm^{-1} absent in the MII photoproduct of rhodopsin.

In addition, amide I absorption changes are affected. In the difference spectra of rhodopsin and the mutant E113D, three negative bands are observed between 1656 and 1700 cm^{-1}. The intensities of these bands increase slightly with frequency. This is not observed in the spectrum of mutant E113A where the distinct negative band at 1695 cm^{-1} is present, but the two lower frequency modes overlap in a broad negative band. It should be emphasized, however, that the absorption changes represent less than $^1/_{1000}$th of the total amide absorption and do not indicate global changes in the protein conformation. In particular, the band pattern between 1510 and 1400 cm^{-1}, which mainly comprises absorption changes of as yet unidentified amino acid side chains, is very well reproduced in both mutants. This is evidence that the chromophore–protein interactions in the mutants are typical of the formation of MII-like photoproducts after 11-cis to all-trans photoisomerization.

In spite of some protein conformational perturbations introduced by the Glu^{113} to Ala replacement, and to a minor extent by the Glu^{113} to Asp replacement, absorption changes of the C=O stretching vibration of protonated carboxylic acid side chains in the hydrophobic core of the pigment are reproduced in both mutants. The difference bands at 1767/1750 cm^{-1} and 1734/1745 cm^{-1} are typical of the rhodopsin to MII transition and have recently been assigned to Asp^{83} (Fahmy et al., 1993; Rath et al., 1993) and Glu^{122} (Fahmy et al., 1993), respectively. Bands typical of MII formation were expected because both recombinant pigments have been shown to activate transducin in a light-dependent fashion (Sakmar et al., 1989; Zvyaga et al., 1993).

However, in the control spectrum of rhodopsin, the formation of MII is accompanied by an additional absorption increase at 1712 cm^{-1}. This band does not have a negative counterpart in the C=O stretching frequency range (i.e., between 1700 and 1800 cm^{-1}). Negative absorptions in this region have been previously paired with their respective photoproduct bands by the assignments to frequency shifts of the C=O stretching vibrations of Asp^{83} and Glu^{122} (Fahmy et al., 1993). The only additional negative band observed in this frequency interval is the mode at 1727 cm^{-1}, which is typically observed in the rhodopsin/MII difference spectrum and may represent an unusually high frequency amide I mode rather than the C=O stretching vibration of a protonated carboxyl group because it does not shift upon H/D isotope exchange (Ganter et al., 1988a,b; see also Figure 2). Therefore, the band at 1712 cm^{-1} has to be assigned to the C=O stretching vibration of a carboxyl group that becomes protonated in MII and was unprotonated in dark rhodopsin. The frequency of the C=O vibration of the unprotonated carboxylate in rhodopsin should lie well below the 1700–1800-cm^{-1} region. The 1712-cm^{-1} absorption is affected by Glu^{113} replacements. In mutant E113D, it is shifted to 1709 cm^{-1}, whereas in E113A it is significantly reduced in intensity.

The residual weak positive absorption at 1715 cm^{-1} of the E113A photoproduct was further investigated by recording

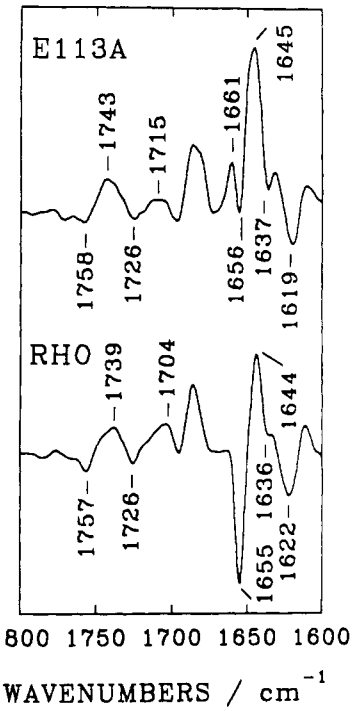

FIGURE 2: Infrared absorption difference spectra of the photoreactions of mutants E113A and COS cell rhodopsin in D_2O. Isotope-sensitive infrared bands in the spectral range of C=O stretching vibrations of protonated carboxyl groups are shown. H_2O/D_2O exchange was carried out as described in Experimental Procedures. Experimental conditions are given in the legend to Figure 1.

difference spectra in D_2O. The C=O stretching vibration of protonated carboxylic acid groups typically shifts by ~10 cm^{-1} to lower frequency upon H/D isotope exchange. Figure 2 shows that in the spectrum of E113A the entire band pattern caused by the C=O stretches of Asp^{83} and Glu^{122} shifts down by 7–10 cm^{-1} to the 1758–1726-cm^{-1} range as is the case with the corresponding bands in the rhodopsin spectrum. As expected, the positive band at 1712 cm^{-1} in MII of RHO is sensitive to the isotope exchange and is shifted to 1704 cm^{-1}. In contrast, the residual absorbance at 1715 cm^{-1} of the MII-like form of mutant E113A in H_2O (Figure 1) persists when measured in D_2O (Figure 2). These results prove the previous tentative assignment of the C=O stretching mode at 1712 cm^{-1} in MII of rhodopsin to the protonated carboxylic acid group in the side chain of Glu^{113}. Therefore, MII formation is accompanied by protonation of the Schiff base counterion, Glu^{113}.

In principle, the symmetric and antisymmetric stretching vibrations of the carboxylate at position 113 in dark rhodopsin also could be assigned on the basis of mutational effects in the 1350–1450-cm^{-1} and 1580–1600-cm^{-1} frequency range, respectively. An absorption change compatible with such an assignment is a weak negative band at 1395 cm^{-1}. This band is part of a broader feature with a negative peak at 1390 cm^{-1} (Figure 1). In mutant E113D, the band is narrower due to a lack of the 1395-cm^{-1} component. Concomitantly, the positive band at 1370 cm^{-1} is reduced, suggesting that the (negative) 1395-cm^{-1} absorption has shifted under the (positive) 1370-cm^{-1} mode in the mutant. In mutant E113A, the 1395-cm^{-1} component again is reduced, but no reduction of the 1370-cm^{-1} band is observed. These spectral changes suggest an assignment of the 1395-cm^{-1} absorption to the symmetric stretching vibration of the Glu^{113} carboxylate in rhodopsin. The corresponding vibration of the substituted aspartate in E113D seems to absorb near 1370 cm^{-1}. It is

completely absent in the spectrum of mutant E113A, consistent with the removal of a carboxylic acid group from this position. The antisymmetric carboxylate stretching frequency of the ionized Glu^{113} side chain in dark rhodopsin is expected to cause a negative band close to 1600 cm^{-1}. Interestingly, a positive band at 1590 cm^{-1} occurs in the difference spectrum of mutant E113A. This band is not observed in the difference spectrum of rhodopsin nor in that of mutant E113D. Thus, it may be caused by the lack of an overlapping negative band, which in native rhodopsin compensates the 1590-cm^{-1} absorption increase. Therefore, the mutational effect on the infrared absorption agrees with a symmetric stretching vibration of Glu^{113} at 1590 cm^{-1}, which is abolished upon replacement of Glu^{113} by Ala. The notion that Glu^{113} contributes to absorption changes near 1600 cm^{-1} is supported by a slight alteration of the band shape around 1600 cm^{-1} in the difference spectrum of E113D, indicating a band shift rather than a complete loss of a negative band.

DISCUSSION

We have used site-directed mutagenesis in combination with FTIR difference spectroscopy to study the infrared absorption changes that occur during the rhodopsin to MII transition. The FTIR difference spectra of both mutants E113D and E113A are consistent with 11-cis to all-trans photoisomerization of their retinal chromophores. Both mutants form photoproducts similar to the transducin-activating MII state of native rhodopsin as deduced from "marker bands" only observed in the MII intermediate. However, both mutations specifically affect the C=O stretching mode at 1712 cm^{-1} of a protonated carboxylic acid group newly formed in MII. The band is shifted to lower wavenumbers in the MII-like photoproduct of mutant E113D and is abolished in the MII-like photoproduct of mutant E113A. Thus, the observed carbonyl vibration at 1712 cm^{-1} in MII is assigned to the protonated carboxyl group of Glu^{113}. Since this group is ionized in the dark state of rhodopsin, the salient result of the present study is the evidence of a protonation of the Schiff base counterion, Glu^{113}, in the MII intermediate of rhodopsin.

An aspartate residue at position 113 can functionally substitute for the glutamate counterion by stabilizing the protonated Schiff base in the dark state and also provides an alternative proton acceptor in the MII-like mutant photoproduct as shown by the occurrence of a positive C=O stretching vibration at 1709 cm^{-1}. Since Glu^{113} affects the chemical environment of the Schiff base, the data suggest that protonation of Glu^{113} and deprotonation of the Schiff base in MII occur as an internal proton transfer. If so, the proton-transfer reaction is not precisely tuned to the distance between the counterion and the Schiff base because an aspartate side chain, which is shorter by one methylene group, can substitute as a proton acceptor. In addition, nuclear magnetic resonance data indicate that, in ROS rhodopsin, the carboxylate of Glu^{113} is closest to C_{12} of the chromophore, rather than to the Schiff base nitrogen (Han et al., 1993). Hence, 11-cis to all-trans isomerization might be expected to change mainly the relative orientation of the Schiff base to the counterion.

We suggest that the proposed net proton transfer may be due predominantly to exposure of the Schiff base to a hydrophobic protein environment in the MII photoproduct, which does not provide stabilizing electrostatic interactions. This is supported by the fact that the Schiff base is not titratable in MII and by the observation that a solvent chloride anion cannot stabilize a Schiff base proton in the MII-like photoproduct of mutant E113A (Sakmar et al., 1991; Zvyaga et al., 1993). Despite a lack of precise knowledge regarding the distance between the imine proton and its counterion, the carboxylate may still be the closest functional group that can accommodate the destabilized proton. Concurrently, the Glu^{113} carboxylate would itself be destabilized due to the lack of a nearby balancing positive charge. The Schiff base environment in MII must be close to Ala^{117} as well, since replacement of this residue by Glu allows the Schiff base to stay protonated even after photoisomerization (Zvyaga et al., 1994). Many of the amino acids adjacent to Ala^{117} are of hydrophobic character.

Interestingly, a water molecule has been also included in several models of the retinal-binding site (Birge, 1988, 1990; Birge & Zhang, 1990; Rafferty & Shichi, 1981; Ganter et al., 1988a,b). The low frequency of the C=O stretching vibration of protonated Asp^{113} in mutant E113D and of Glu^{113} in rhodopsin indicates strong hydrogen bonding of the respective carboxyl groups. This may indicate that, after photoisomerization, the putative water is involved in a hydrogen bond interaction with the protonated counterion rather than with the Schiff base, which is shielded in the proposed hydrophobic area around Ala^{117}. Schiff base deprotonation is necessary for the formation of the active receptor conformation, R^*, which catalyzes nucleotide exchange in transducin (Ganter et al., 1991; Longstaff et al., 1986). The data suggest that Schiff base deprotonation is mechanistically coupled to Glu^{113} neutralization.

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