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Fourier Transform Infrared Difference Spectroscopy of Rhodopsin Mutants: Light Activation of Rhodopsin Causes Hydrogen-Bonding Change in Residue Aspartic Acid-83 during Meta II Formation†

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ABSTRACT: Fourier transform infrared (FTIR) difference spectroscopy and site-directed mutagenesis have been used to investigate structural changes which occur during rhodopsin photoactivation at the level of individual amino acid residues. The rhodopsin → bathorhodopsin FTIR difference spectra of the mutants Asp-83 → Asn (D83N) and Glu-134 → Asp (E134D) incorporated into membranes are similar to that of native rhodopsin in the photoreceptor membrane, demonstrating that the retinal chromophores of these mutants undergo a normal 11-*cis* to *all-trans* photoisomerization. Two bands assigned to the C=O stretching mode of Asp and/or Glu carboxylic acid groups are absent in the D83N rhodopsin → metarhodopsin II FTIR difference spectrum. Corresponding changes are not observed in the carboxylate C=O stretching region. The most straightforward explanation is that the carboxylic acid group of Asp-83 remains protonated in rhodopsin and its bleaching intermediates but undergoes an increase in its hydrogen bonding during the metarhodopsin I → metarhodopsin II transition. The mutant E134D produced a normal rhodopsin → bathorhodopsin and rhodopsin → metarhodopsin II difference spectrum, but a fraction of misfolded protein was observed, supporting earlier evidence that Glu-134 plays a role in proper protein insertion and/or folding in the membrane.

Rhodopsin, the primary photoreceptor in scotopic vision, is the predominant component of the photoreceptor membrane of the rod outer segment (ROS). It consists of a seven-helix integral membrane protein and a retinylidene chromophore which is bound through a protonated Schiff base to Lys-296 [for reviews see, e.g., Hargrave (1986) and Birge (1990)]. Upon visible light absorption near 500 nm, the rhodopsin (Rho)[1] chromophore undergoes a rapid 11-*cis* to *all-trans* isomerization (Rho → Batho) (Green et al., 1977; Suzuki & Callender, 1981), followed by a series of slower thermal protein transitions (Batho → Lumi → Meta I → Meta II). In the Meta II stage, rhodopsin binds and activates the G-protein transducin (Vuong et al., 1984). Primary sequence homology between rhodopsin and other G-protein-linked receptors such as adrenergic and cholinergic receptors (Hargrave & MacDowell, 1992; Khorana, 1992; Oprian, 1992) suggests that they all share common features in their signal transduction mechanism.

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[1] Abbreviations: FTIR, Fourier transform infrared; Rho, rhodopsin; Batho, bathorhodopsin; Lumi, lumirhodopsin; Meta I, metarhodopsin I; Meta II, metarhodopsin II.

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In order to understand the working mechanism of rhodopsin, it is necessary to determine at the molecular level the detailed structural changes which occur during each step of photoactivation. One promising approach is Fourier transform infrared (FTIR) difference spectroscopy. It offers a means of probing changes in the orientation, protonation state, and hydrogen bonding of individual chemical groups of both the apoprotein and retinal chromophore [see Rothschild (1992) for a recent review]. This method was first applied to bacteriorhodopsin (bR) (Rothschild et al., 1981; Bagley et al., 1982; Rothschild & Marrero, 1982; Siebert & Mantele, 1983) and rhodopsin (Rothschild et al., 1983; Bagley et al., 1985; DeGrip et al., 1985; Rothschild & DeGrip, 1986; Ganter et al., 1989) and more recently also to halorhodopsin (Rothschild et al., 1988) and sensory rhodopsin I (Bousché et al., 1991b).

In the case of bacteriorhodopsin, the combination of FTIR and site-directed mutagenesis has been particularly useful, enabling bands to be assigned to individual amino acid residues. This approach has led to an increasingly detailed model of proton transport by bR (Braiman et al., 1988a,b, 1992; Gerwert et al., 1989; Rothschild et al., 1990a,b; Bousché et al., 1991a; Fahmy et al., 1992; Maeda et al., 1992). However, a similar approach has not yet been possible for rhodopsin due to the difficulty of isolating sufficient quantities of reconstituted mutant protein suitable for FTIR measurements.

In this paper, we report on the use of FTIR difference spectroscopy to study rhodopsin mutants. Wild-type (WT) and mutant rhodopsin were isolated from an insect cell culture system using recombinant baculovirus as a vector (Janssen et al., 1991). Yields of opsin as high as 1 mg/L of culture were obtained. The opsin was regenerated with 11-*cis*-retinal, purified by affinity chromatography, and reconstituted into native ROS lipids. As described below, this procedure resulted in samples which yielded high quality FTIR difference spectra.

We selected for our initial studies the highly conserved residues Asp-83 and Glu-134. FTIR difference spectroscopy at low temperature shows that the mutants D83N[2] and E134D both undergo a normal 11-*cis*- to *all-trans*-retinal isomerization during the Rho → Batho photoactivation. In contrast, two bands in the Meta I → Meta II transition of rhodopsin previously assigned to protonation changes and/or hydrogen-bonding changes of one or more carboxylic acid groups are absent in the D83N mutant. Corresponding changes are not found, however, in the carboxylate C=O stretching region of the D83N difference spectrum. These results show that Asp-83 is protonated in rhodopsin and undergoes an increase in its hydrogen bonding during the Meta I → Meta II transition.

MATERIALS AND METHODS

Production and Purification of Wild-Type and Mutant Bovine Rhodopsin. All manipulations involving rhodopsin were performed in dim red light (Schott-Jena, RG 645). Site-directed mutagenesis, cloning, production, and propagation of recombinant baculovirus were performed as described (Summers & Smith, 1987; Janssen et al., 1988, 1990, 1991; DeCaluwé et al., 1993). The *Spodoptera frugiperda* cell line IPLB-Sf9 was maintained at 27 °C in TNH-FH medium plus 10% fetal calf serum (FCS), 50 g/mL streptomycin, and 50 units/mL penicillin. Large-scale production of recombinant opsin (V-ops) was achieved in suspension culture (100–1000 mL), stirred at 200 rpm with overlay aeration and with addition of Pluronic-F68 (Fluka) to a final concentration of 0.3%. Viral infections (0.1 volume, MOI = 10) were performed in one third of the final volume during 1–2 h and then supplemented with medium without FCS to a final density of 2×10^6 cells/mL. Cells were harvested at 3 days postinfection (dpi), and regeneration of V-ops into V-Rho was accomplished in total cellular membrane preparations as described (DeCaluwé et al., 1993) with a minor adjustment. The suspension was incubated while being rotated with retina lipids (added as a solution in methanol at 100-fold molar excess over recombinant opsin; final methanol concentration <2%) for 0.5 h at room temperature, prior to addition of 11-*cis*-retinal. Following regeneration, the sample was solubilized in 20 mM dodecyl β-1-maltoside and purified over ConA–Sepharose (Pharmacia) as described (DeCaluwé et al., 1993). The purified V-Rho was mixed with a 100-fold molar excess of retina lipids and concentrated on a Filtron Omega 30K filter. The suspension was layered on top of a sucrose step gradient (10%, 20%, and 45%) followed by centrifugation (4 °C; 100000*g*; 24 h). The resulting V-Rho proteoliposomes were collected from the 20%/45% interface, washed, and stored at −80 °C until further use.

FTIR Difference Spectroscopy. Fourier transform infrared difference spectra of the Rho → Batho and Rho → Meta II were recorded using methods similar to those previously reported (Rothschild et al., 1987; DeGrip et al., 1988). Samples were prepared by isopotential spin drying (Clark et al., 1980; DeGrip et al., 1985) 0.3–0.9 nmol of sample on an AgCl window and then rehydrating prior to insertion into a sealed transmittance cell which was mounted in a Helitran cryostat (Air Products, Allentown, PA). The water content of the sample was checked by monitoring the ratio of the 3400-cm^{-1} OH stretching mode of water to the CH stretching bands of the protein and lipid near 2900 cm^{-1}. The Rho → Batho difference spectra were recorded at 82 K at both 2- and 8-cm^{-1} resolution using a Nicolet Analytical Instruments (Madison, WI) 740 spectrometer. The sample was illuminated with light from a narrow-band 500-nm interference filter for approximately 15 min and photoreversed back to a mixture of rhodopsin and isorhodopsin by illumination for the same period of time with a 600-nm narrow-band interference filter. Difference spectra from at least 25 of these cycles were then averaged. The Rho → Meta II difference spectra were recorded at 10 °C, where the decay of Meta II to Meta III occurs over several hours (Rothschild et al., 1987). The sample was illuminated for 3 min using a 500-nm long-pass filter, and spectra were recorded at 8-cm^{-1} resolution for 10-min intervals before and after illumination (3000 scans for each spectrum) on a Bio-Rad FTS-60 spectrometer. Difference spectra were computed by subtracting the spectra before illumination from each of the spectra thereafter.

RESULTS

Structure of Mutants D83N and E134D. The absolute FTIR absorption spectra of bovine rod outer segments (ROS) and the mutants D83N and E134D are shown in Figure 1. The two intense bands at 1657 and 1545 cm^{-1} are due to the amide I and amide II modes of the protein backbone, respectively. The frequency, intensity, and dichroism of these bands are extremely sensitive to protein secondary structure and in the case of rhodopsin reflect a predominantly α-helical structure (Rothschild et al., 1980). The similar appearance of these bands in the spectra of the dehydrated (Figure 1) and hydrated (not shown) D83N mutant and ROS shows that D83N has a structure very similar to that of native rhodopsin. In contrast,

[2] Designations of the mutants make use of the standard one-letter abbreviations for amino acids. Thus D83N signifies the mutant in which the aspartic acid at position 83 has been replaced by asparagine.

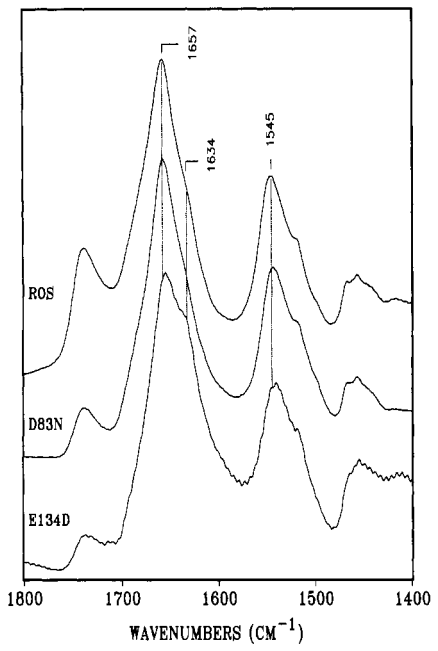

FIGURE 1: Infrared absorption spectra of dehydrated films of ROS and the mutants D83N and E134D obtained at 2-cm^{-1} resolution. Films were prepared on AgCl windows as described under Materials and Methods.

E134D exhibits a prominent shoulder near 1634 cm^{-1} similar to delipidated rhodopsin which is most likely due to increased β-structure and indicates the presence of at least partially misfolded protein (Rothschild et al., 1980).

The Rho → Batho Difference Spectra. The Rho → Batho FTIR difference spectra of reconstituted rhodopsin (WT) and the mutants D83N and E134D are shown in Figure 2. The similarity between these spectra and the corresponding ROS difference spectrum (Figure 2, top) shows that the mutations have relatively little effect on the normal 11-*cis* → *all-trans* isomerization of the retinylidene chromophore. For example, the conformationally sensitive fingerprint region between 1100 and 1300 cm^{-1}, which is due to the C–C stretching modes of the retinal chromophore, is similar in all of the spectra. Only small shifts (2–3 cm^{-1}) are observed in the spectra of the mutant samples, including the 1245-cm^{-1} positive band assigned to the C_{12}–C_{13} stretching mode and the 922-cm^{-1} band assigned to the hydrogen out-of-plane (HOOP) wag of the 11-H. These shifts may be due to a slight change in the conformation of the *all-trans* chromophore of bathorhodopsin. Note that despite the fact that we detect misfolded E134D (see above), a major fraction of this mutant must still be properly folded since it produces a normal Rho → Batho FTIR difference spectrum.

The Rho → Meta II Difference Spectra. Compared to ROS and WT, the D83N Rho → Meta II difference spectra exhibit a dramatic change in the carboxylic acid C=O stretching region with the disappearance of two bands at 1767 cm^{-1} (negative) and 1748 cm^{-1} (positive) (Figures 3 and 4). A similar effect is observed for D83N exposed to D_2O, where the two bands which downshift due to H/D exchange to 1755 and 1740 cm^{-1} in ROS are absent in D83N (Figure 4). These bands have been previously assigned to a protonation and/or hydrogen-bonding change of one or more Asp/Glu residues during the Meta I → Meta II transition (DeGrip et al., 1985) and as discussed below are tentatively assigned to hydrogen-bonding changes in Asp-83. Note that a small residual negative band near 1765 cm^{-1} (1748 cm^{-1} in D_2O) (Figure 4) still appears in D83N. However, it is unlikely that this band

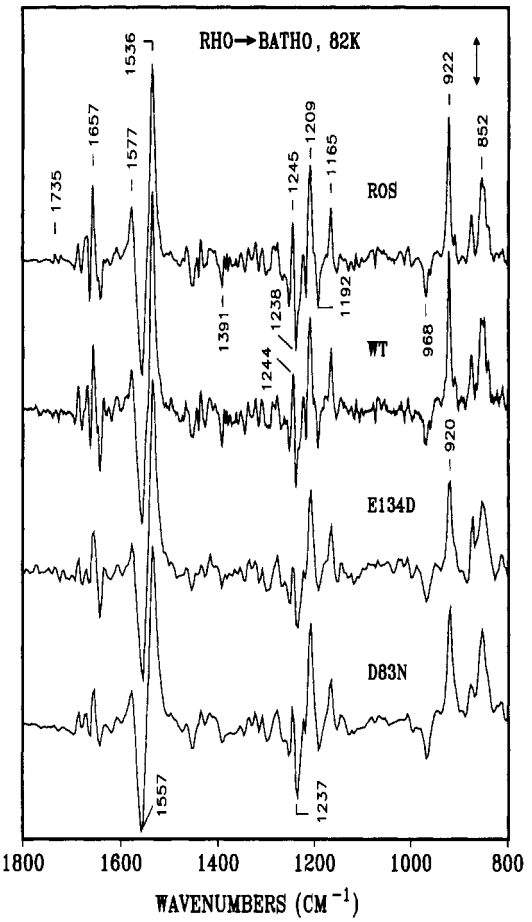

FIGURE 2: Rho → Batho FTIR difference spectra of ROS, wild-type rhodopsin (2-cm^{-1} resolution), and the mutants E134D and D83N (8-cm^{-1} resolution) at 82 K from the photostationary mixtures created by using 500- and 600-nm narrow-band interference filters. The scale bar shown (0.0002 ODU) is for the D83N spectrum, and other spectra were scaled between the 1500- and 1600-cm^{-1} region for comparison.

originates from the same Asp/Glu residues which give rise to the larger band at 1767 cm^{-1} in ROS since a corresponding band at 1755 cm^{-1} is not found for D83N in D_2O.

In contrast to the disappearance of the two bands in the carboxylic acid C=O stretching region, most other regions of the D83N difference spectrum are relatively unaffected by the Asn substitution. For example, the region between 1380 and 1420 cm^{-1}, where bands due to the symmetric C=O stretch of carboxylate groups appear, shows very little change relative to the other three spectra (Figure 3). In contrast to D83N, the E134D exhibits a normal intensity in the 1767/1748-cm^{-1} bands, and no major changes are observed elsewhere in the spectrum.

DISCUSSION

Asp-83 Is Protonated and Undergoes an Increase in Hydrogen Bonding upon Meta II Formation. It has previously been found that bands at 1767 cm^{-1} (negative) and 1748 cm^{-1} (positive) appear in the Rho → Meta II difference spectrum. On the basis of frequency alone, these bands could be assigned to the C=O stretching mode of carboxylic acid (COOH) groups present in both Asp and Glu residues or to the C=O stretching mode of the ester carbonyl of phospholipids. However, since both bands downshift in frequency upon H/D exchange (DeGrip et al., 1985; Ganter et al., 1989), the bands were assigned to carboxylic acid groups which have an exchangeable proton. In addition, these bands cannot be due to the ester carbonyl of phospholipids as they are still present

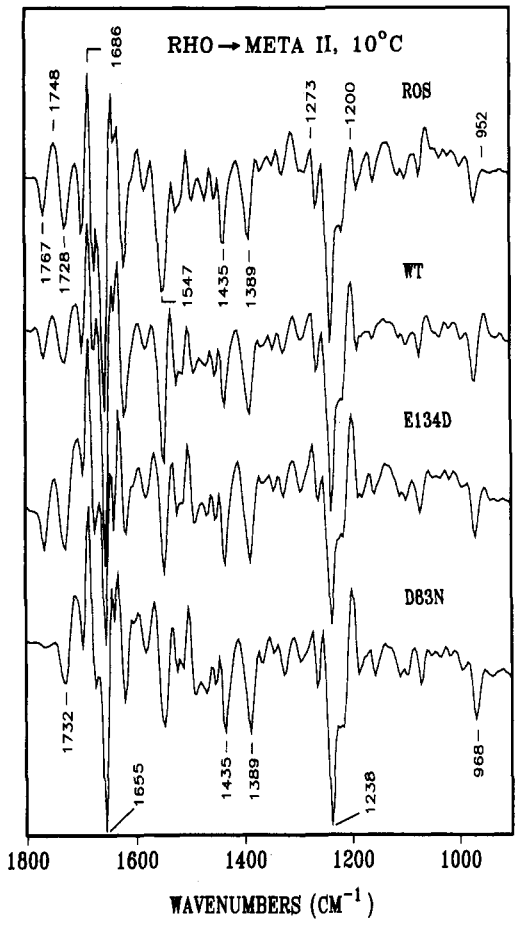

FIGURE 3: Rho → Meta II FTIR difference spectra of ROS, wild-type rhodopsin, and the mutants E134D and D83N (all at 8-cm^{-1} resolution) measured at 10 °C. Each spectrum shown is the average of 3000 scans (about 10 min) after illumination by light with a 500-nm long-pass filter.

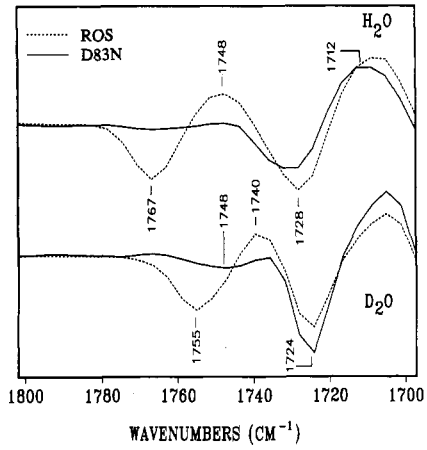

FIGURE 4: Rho → Meta II FTIR difference spectra between the 1700- and 1800-cm^{-1} region of ROS and the mutant D83N in H_2O and D_2O (all at 8-cm^{-1} resolution) measured at 10 °C. All the spectra were scaled using the chromophore band near 1240 cm^{-1} (Figure 3).

in the Rho → Meta II difference spectra of rhodopsin reconstituted into ether-linked phospholipids which lack the ester carbonyl bond (DeGrip et al., 1985).

The disappearance of the 1767/1748-cm^{-1} bands in the D83N Rho → Meta II difference spectrum is a strong indication that either Asp-83 is structurally active in this transition, e.g., it undergoes a change in its hydrogen bonding, or it directly affects the structural activity of a different Asp/Glu residue(s) involved in the Meta II transition. The most straightforward explanation is that these bands arise directly from a downshift in the frequency of the carboxylic acid C=O stretching mode of Asp-83 upon Meta II formation. This could occur, for example, if the hydrogen-bonding strength to the Asp-83 carboxylic acid group increased during Meta I → Meta II transition. A similar, although smaller, frequency shift (1742 to 1748 cm^{-1}) has been detected for the C=O stretching mode of Asp-96 in bacteriorhodopsin during the K to L transition (Braiman et al., 1988a; Gerwert et al., 1989; Bousché et al., 1991a).

We can exclude the possibility that these missing bands are due to a defect in the mutant D83N which blocks Meta II formation. In this case, the difference spectrum would reflect predominantly a Rho → Meta I transition. However, an intense positive band is found at 1686 cm^{-1} which is characteristic of Meta II, whereas the 952-cm^{-1} band characteristic of Meta I is almost absent. Indeed, analysis by UV/vis spectroscopy shows that D83N generates at least as much Meta II as WT under identical conditions (Bovee-Geurts, DeCaluwé and DeGrip, unpublished results).

We also considered the possibility that the two bands at 1767 and 1748 cm^{-1} arise from *two different* Asp/Glu residues which undergo a simultaneous deprotonation/protonation reaction. In this case, we expect both a positive and a negative band to appear in the carboxylic acid C=O stretching region. For example, the positive band at 1748 cm^{-1} could be assigned to the protonation of Glu-113 (the putative Schiff base counterion) and the negative band at 1767 cm^{-1} to deprotonation of Asp-83. However, corresponding bands should also appear in the carboxylate C=O stretching region near 1400 cm^{-1}. As noted above, no changes were observed in the D83N mutant in this region compared to WT, although we still cannot exclude the possibility that carboxylate bands of Asp-83 and Glu-113 effectively cancel out because they have similar intensities and frequencies. However, since the intensity and frequency of these bands are sensitive to a carboxylate group's environment and orientation, we consider complete cancellation unlikely.

The assignment of the 1767/1748-cm^{-1} bands to a simultaneous deprotonation/protonation of Asp-83 and Glu-113 is also unlikely because the D83N mutant exhibits a normal phenotype relative to WT rhodopsin (Zhukovsky & Oprian, 1989; Janssen et al., 1990; Nathans, 1990; Nakayama & Khorana, 1991). UV/vis spectroscopy shows that Meta II formation in D83N is unperturbed. In contrast, if D83N blocked the protonation of Glu-113, we would expect a much larger change in the properties of D83N. For example, substitution of Glu-113 with a neutral residue causes a drop in the Schiff base pK_a from over 8.5 to around 6 (Sakmar et al., 1989; Zhukovsky & Oprian, 1989) which is not observed in D83N.

The assignment of the 1767/1748-cm^{-1} bands to Asp-83 also relates to the question of whether other Asp and/or Glu residues change their protonation state during the Meta I → Meta II transition. This is important since Glu-113, the putative counterion for the retinal Schiff base in rhodopsin (Sakmar et al., 1989; Zhukovsky & Oprian, 1989; Nathans, 1990), could function as the proton acceptor from the Schiff base during Meta II formation in analogy with bacteriorhodopsin, where the Schiff base counterion, Asp-85, functions as the proton acceptor during the L → M transition (Braiman et al., 1988a). In this case, an additional positive band should appear during the Meta I to Meta II transition in the carboxyl stretching region. Bands near1728 cm^{-1} (negative) and 1701 cm^{-1} (positive) appear in the Rho → Meta I difference spectrum and reflect hydrogen-bonding and/or protonation

changes which precede Meta II formation (DeGrip et al., 1985). However, a positive band appears near 1712 cm^{-1} in the Rho → Meta II difference spectrum (Figure 4), possibly indicating a second band associated with Meta II formation as previously noted (DeGrip et al., 1985). In this case, part of the intensity of the positive band near 1712 cm^{-1} might be assignable to Glu-113 protonation. Further studies with other mutants will be necessary to confirm this possibility.

Asp-83 Is Not the Schiff Base Counterion but May Be Located near the Schiff Base. Earlier work led to the suggestion that Asp-83 serves as the counterion for the Schiff base proton in rhodopsin (Kakitani, 1988; Birge, 1990). In this case, replacement of negatively charged Asp-83 by a neutral Asn residue should cause a significant downshift of the C=C stretching frequency similar to that observed in the bacteriorhodopsin mutant D85N, where the Asp-85 counterion is replaced by Asn (Lin et al., 1991; Rath et al., 1993). However, no appreciable shift is observed in the frequency of the C=C stretching vibrations of the D83N chromophore. In addition, D83N exhibits only a minor shift in its absorption maximum compared to WT rhodopsin (Zhukovsky & Oprian, 1989; Janssen et al., 1990; Nathans, 1990; Nakayama & Khorana, 1991). Thus, in agreement with earlier studies we conclude that Asp-83 cannot serve as a counterion for the Schiff base.

Our result is consistent, however, with the possibility that Asp-83 exists in a protonated form and is located close to the Schiff base of retinal. This model would explain why we observe a downshift in the frequency of the C=O stretching mode of Asp-83 upon Meta II formation. In particular, Schiff base deprotonation (Doukas et al., 1978; Smith et al., 1992) would be expected to alter the electrostatic environment of Asp-83. It would also explain why substitution of an Asp by the isomorphous residue, Asn, does not alter significantly the properties of D83N. Both Asp (neutral) and Asn should be capable of forming almost identical hydrogen-bonding interactions. One possibility is that Asp-83 hydrogen bonds directly with the Schiff base and Glu-113. Upon Meta II formation, the loss of a positive charge on the Schiff base would be expected to cause an increase in the Glu-113/Asp-83 interaction. Alternatively, Asp-83 may be involved in structural changes accompanying Meta II formation outside the immediate vicinity of the Schiff base. We also note that since the 1767/1748-cm^{-1} bands downshift due to H/D exchange (DeGrip et al., 1985; Ganter et al., 1989), this means that, similar to the Schiff base proton (Oseroff & Callender, 1974), Asp-83 must be accessible to the external medium.

Mutant E134D Interferes with Rhodopsin Folding. Earlier studies on E134D as well as other mutants involving Glu-134 and Arg-135 suggested that these residues play a role in correct insertion and/or folding of the protein (Janssen et al., 1991). For example, evidence indicates that E134D does not fully glycosylate despite the fact that Glu-134 is on the luminal side of the membrane and the glycosylation sites Asn-2 and Asn-15 are on the cytoplasmic side. FTIR absorption spectroscopy directly confirms that there is a high fraction of misfolded E134D in the reconstituted membranes, while under identical conditions wild type and D83N fold properly. The sensitivity of rhodopsin to the Glu-134 → Asp substitution is surprising since it involves shortening by only one carbon from the Glu side chain. It is possible that this hinders formation of an interaction between Asp-134 and Arg-135 which might be necessary for either recognition by a translocase and proper protein insertion and/or establishment of the proper secondary structure of the protein. Despite the fact that the E134D mutant structure is perturbed, an appreciable fraction must also fold properly in the membrane since normal Rho → Batho and Rho → Meta II difference spectra were obtained. This probably indicates that only the properly folded protein combines with 11-*cis*-retinal to generate a photopigment, as previously suggested (Nathans et al., 1989; Sakmar et al., 1989; Janssen et al., 1991).

CONCLUSIONS

We have demonstrated in this initial study that it is possible to combine FTIR difference spectroscopy and rhodopsin mutagenesis to investigate the structural changes which occur during photoactivation of rhodopsin at the single amino acid level. Our work indicates that the two carboxylic acid C=O stretching bands which appear upon Meta II formation arise directly from an increase in hydrogen bonding of the Asp-83 carboxylic acid group. This could occur, for example, if Asp-83 is protonated and located close to the retinal Schiff base which deprotonates upon Meta II formation. Further tests of these conclusions and additional information about conformational changes which involve other key residues that have been implicated in the rhodopsin G-protein activation mechanism (Karnik & Khorana, 1990; Nakayama & Khorana, 1991; Cohen et al., 1992; Khorana, 1992; DeGrip et al., 1993) should now be possible through further application of this combined approach.

A similar approach should also be possible to investigate other G-protein-coupled receptors such as the muscarinic acetylcholine receptor and the β-adrenergic receptors once they are expressed in sufficient quantities and reconstituted into intact membranes. Although these proteins are not light activated, recent progress has been made in obtaining FTIR difference spectra of such membrane-bound receptors by utilizing attenuated total reflection (ATR) and a flow cell which allows ligands to be introduced directly into the bathing medium (Baenziger et al., 1993).

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