Photoactivation of Rhodopsin Involves Alterations in Cysteine Side Chains: Detection of an S-H Band in the Meta I→Meta II FTIR Difference Spectrum

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ABSTRACT FTIR difference spectroscopy has been used to study the role of cysteine residues in the photoactivation of rhodopsin. A positive band near 2550 cm^{-1} with a low frequency shoulder is detected during rhodopsin photobleaching, which is assigned on the basis of its frequency and isotope shift to the S-H stretching mode of one or more cysteine residues. Time-resolved studies at low temperature show that the intensity of this band correlates with the formation and decay kinetics of the Meta II intermediate. Modification of rhodopsin with the reagent NEM, which selectively reacts with the SH groups of Cys-140 and Cys-316 on the cytoplasmic surface of rhodopsin, has no effect on the appearance of this band. Four other cysteine residues are also unlikely to contribute to this band because they are either thio-palmitylated (Cys-322 and Cys-323) or form a disulfide bond (Cys-110 and Cys-187). On this basis, it is likely that at least one of the four remaining cysteine residues in rhodopsin is structurally active during rhodopsin photoactivation. The possibility is also considered that this band arises from a transient cleavage of the disulfide bond between cysteine residues 110 and 187.

INTRODUCTION

Rhodopsin serves as the primary photoreceptor in scotopic vision (for recent reviews see, e.g., Birge, 1990; Hargrave and McDowell, 1992a, b; Skulachev, 1993). It is a member of the growing family of G-protein-linked receptors, such as the adrenergic and cholinergic receptors, which share the 7-helix transmembrane domain motif as well as several conserved residues (Hargrave and MacDowell, 1992b; Khorana, 1992; Oprian, 1992). However, in contrast to these other receptors that are activated by the binding of a soluble ligand, rhodopsin contains an internal "chromophore," 11-*cis* retinal, which is covalently linked through a Schiff base to a lysine residue of the apoprotein opsin. Activation of rhodopsin occurs via photoisomerization of retinal to an all-*trans* configuration (Green et al., 1977; Suzuki and Callender, 1981).

A key goal in rhodopsin research is to determine how the initial retinal photoisomerization results in binding and activation of the G-protein, transducin (Vuong et al., 1984). Several distinct rhodopsin intermediates have been identified that are involved in this process. After the initial rapid photoreaction (Schoenlein et al., 1991; Yan et al., 1991), there occurs a series of slower thermal transitions (Batho→Lumi→Meta I→Meta II), each intermediate characterized by a different visible absorption band (Yoshizawa and Wald, 1963; Wald, 1968). Only the Meta II intermediate is known to bind and activate the G-protein (Vuong et al., 1984; Kibelbek et al., 1991). Meta II decays upon hydrolysis of the retinal Schiff base to a long-lived intermediate, Meta III, or to the apoprotein opsin and all-*trans* retinal (Matthews et al., 1963; Ostroy, 1977; Van Breugel et al., 1979).

Important progress has been made in using site-directed mutagenesis to identify amino acid residues that are involved in rhodopsin photoactivation (Karnik and Khorana, 1990; Nathans, 1990; Nakayama and Khorana, 1991; Cohen et al., 1992; Khorana, 1992; DeGrip et al., 1993). However, these studies do not provide information about the possible changes in local environment, orientation, and ionization state of these residues during the photoactivation process. For this purpose, we have been developing methods based on Fourier transform infrared (FTIR) difference spectroscopy (for a recent review see Rothschild, 1992), which were first applied to the related system bacteriorhodopsin (Rothschild et al., 1981; Bagley et al., 1982; Siebert and Maentele, 1983). In the case of rhodopsin, information has been obtained about conformational changes of the retinylidene chromophore, protonation/hydrogen bonding changes of Asp and Glu residues and structural changes of the peptide backbone and membrane lipid matrix (Rothschild et al., 1983; Siebert et al., 1983; Bagley et al., 1985; DeGrip et al., 1985; Rothschild and DeGrip, 1986; Ganter et al., 1989; Klinger and Braiman, 1992). Recently, the combination of FTIR difference spectroscopy with site-directed mutagenesis has facilitated the first assignment of bands arising from individual amino acid residues (Fahmy et al., 1993; Rath et al., 1993).

Hubbell and co-workers have recently used the selective spin-labeling of cysteines to probe conformational changes in rhodopsin (Farahbakhsh et al., 1992, 1993). In this paper, we have utilized FTIR difference spectroscopy, which does not require the chemical modification of rhodopsin, to investigate whether cysteine residues are structurally active,

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Abbreviations used: FTIR, Fourier transform infrared; Rho, rhodopsin; Meta I, metarhodopsin I; Meta II, metarhodopsin II; NEM, *N*-ethyl-maleimide; DTNB, Di(thio nitro-4-benzoic acid); D_2O, ^2H_2O.

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e.g., undergo a change in their environment during the rhodopsin photocascade. In contrast to earlier FTIR studies that focused on the spectral region below 1800 cm^{-1}, we have examined time-resolved absorption changes in the region between 2500 and 2600 cm^{-1}, where bands normally arise from the S-H stretch mode of cysteine residues. We show that a small band near 2550 cm^{-1} appears with kinetics that correlates with the formation and decay of the Meta II intermediate. Although the assignment of this band to specific cysteine residues has not yet been possible, we are able to eliminate Cys-140 and Cys-316, located on the cytoplasmic region of the protein by using the sulfhydryl label *N*-ethylmaleimide (NEM). Because the band we observe has only positive intensity in the Rho→Meta II difference spectrum, the possibility also exists that it arises from the transient cleavage of the disulfide bond between the highly conserved residues Cys-110 and Cys-187 (Karnik and Khorana, 1990). Earlier studies on other G-protein-linked receptors have led to the suggestion that cleavage of intramolecular disulfide bridges is a key event in receptor activation (Malbon et al., 1987).

MATERIALS AND METHODS

Production, purification, and NEM labeling of bovine rhodopsin

Bovine rod outer segments (ROS) were prepared as previously described (DeGrip et al., 1980). The A280/A500 ratio of the resulting washed photoreceptor membrane was 2.0 ± 0.1.

Analysis of free thiol groups using DTNB (Research Organics Inc., Cleveland, OH) and modification of cytoplasmic thiol groups by NEM (Merck, Darmstadt, Germany) was performed as described earlier (DeGrip et al., 1975; Regan et al., 1978; DeGrip and Daemen, 1982). The number of cytoplasmic thiol groups (1.8 ± 0.3) was estimated by reaction with DTNB in membrane suspension (buffer A: 20 mM MOPS, 130 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, pH 7.2). The total number of thiol groups (5.8 ± 0.2) was estimated by reaction in SDS-solution (1% (w/v) SDS in buffer A. This reaction was complete in 2 h at room temperature. The analysis was done in an Argon atmosphere to prevent oxidation of thiol groups (DeGrip et al., 1973). The results fully agree with earlier reports (DeGrip et al., 1973, 1975; Chen and Hubbell, 1978; Regan et al., 1978).

Modification of cytoplasmic located thiol groups was accomplished with NEM (20-fold molar excess; 0.2 M phosphate buffer, pH 7.0; reaction time: 30 min at 20°C). Under these conditions, 2.2 ± 0.1 thiol groups were modified in agreement with earlier results (DeGrip et al., 1975; Regan et al., 1978; DeGrip and Daemen, 1982), which correspond to cysteine residues 140 and 316 (Griffith et al., 1979; Farahbakhsh et al., 1992). Similar results were obtained when modification was performed either in membrane suspension or in 20 mM nonylglucose solution, followed by reconstitution through stepwise dilution (DeGrip et al., 1983).

FTIR difference spectroscopy

Fourier transform infrared difference spectra of rhodopsin were recorded using methods similar to those previously reported (Rothschild et al., 1987). Samples were prepared by isopotential spin drying (Clark et al., 1980; DeGrip et al., 1985) of an aqueous suspension of photoreceptor membranes in H_2O, containing approximately 3 nmol of rhodopsin, onto an AgCl window and then rehydrating the film before insertion into a sealed transmittance cell as previously described (Rath et al., 1993). The H_2O or D_2O content of the sample was monitored by measuring the intensity ratio of the 3400 cm^{-1} O-H (or 2600 cm^{-1} O-D) band due to the stretching mode of water to the methyl and methylene C-H stretch bands of the protein and lipid in the 2800–3000 cm^{-1} region. For the deuteration of rhodopsin, the film was first dried for more than 12 h in a dry-air box to remove residual H_2O. This film was then sealed in an IR cell, using a second AgCl window. For the purpose of humidification, small drops (3 μl) of D_2O were placed on this window outside of the IR beam path. The cell was left at room temperature for more than 12 h before inserting into the spectrometer. The Rho→Meta II difference spectra were recorded at temperatures ranging from −15 to +10°C to control the decay rate of the Meta I and Meta II intermediates. The sample was illuminated for 3 min using a 500 nm long band-pass filter, and spectra were recorded at 2 or 8 cm^{-1} resolution for 10 min intervals for several hours before and after illumination (3000 scans for each spectrum at 8 cm^{-1} resolution) on a Biorad FTS-60A spectrometer. Difference spectra were computed by subtracting the spectrum before illumination from each of the spectra after illumination. Data analysis was performed using LabCalc (Galactic Industries, Nashua, NH).

RESULTS

Identification of the S-H stretch band of cysteine

Fig. 1 shows the Rho→Meta II difference spectra for rhodopsin in ROS photoreceptor membranes. Several bands in the region below 1800 cm^{-1} have been previously assigned to specific protein vibrations including the pair of negative/positive bands at 1767/1749 cm^{-1} (C=O stretch mode of the Asp-83 COOH group (Rath et al., 1993)) and at 1686, 1655, and 1643 cm^{-1} (amide I carbonyl (C=O) stretch mode). Bands have also been assigned on the basis of comparison with results from resonance Raman spectroscopy to specific vibrations of the retinylidene chromophore such as at 1548 cm^{-1} (C=C ethylenic stretch), 1238 cm^{-1} (C12-C13 stretch mode) and 969 cm^{-1} (hydrogen-out-of-plane mode) (Oseroff and Callender, 1974; Mathies et al., 1976; Eyring et al., 1982; Palings et al., 1987).

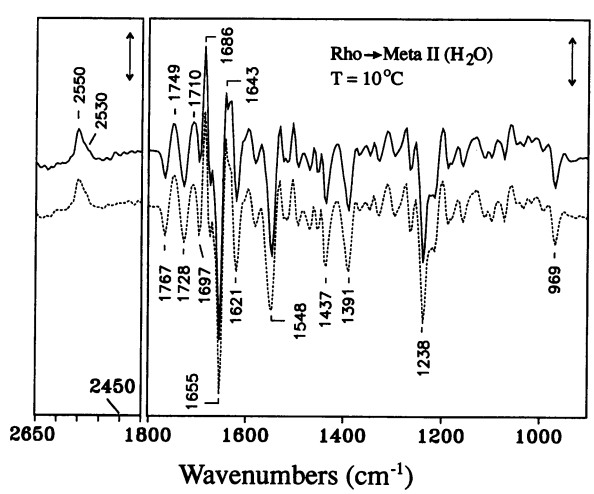

FIGURE 1 Rho→Meta II FTIR difference spectrum of normal rhodopsin (——) and NEM labeled rhodopsin (. . . . . .) in H_2O at 10°C. Each spectrum is the average of 3000 scans (10 min) recorded at 8-cm^{-1} resolution. (*Right* panel) The scale-bar shown is 2×10^{-3} o.d. units for the solid curve. (*Left* panel) The scale-bar shown is 0.3×10^{-3} o.d. units for the solid curve.

In contrast, bands appearing in the region above 1800 cm^{-1} involve mainly the X-H stretch mode (X=C,N,O,S).[1] As shown in Fig. 1 (*left* panel), a small but highly reproducible positive band is found at 2550 cm^{-1} that is less than 25% of the negative intensity of the 1767 cm^{-1} band (note the Y-scale has been expanded approximately 7 times for the left panel relative to the right panel). A small shoulder also appears near 2530 cm^{-1}, which is more distinct in the 2 cm^{-1} resolution difference spectra (data not shown).

The 2525–2580 cm^{-1} region is highly characteristic of the S-H stretch mode of cysteine and has been studied extensively in model compounds and proteins by both infrared and Raman spectroscopy (Alben et al., 1974; Bare et al., 1975; Moh et al., 1987; Li and Thomas, 1991; Li et al., 1992). Significantly, no other protein or chromophore vibrational modes contribute to this region, thus making its appearance in the FTIR difference spectrum a distinctive marker for cysteine residues.

A further indication that the band at 2550 cm^{-1} arises from one or more SH groups is its isotope shift when rhodopsin is exposed to D_2O. In this case, the S-D vibration should downshift to near 1850 cm^{-1} based on normal mode calculations and model compound studies (Alben et al., 1974; Bare et al., 1975; Li et al., 1992). In agreement, a new band is found at 1853 cm^{-1} with a shoulder near 1828 cm^{-1} in the Rho→Meta II difference spectrum of samples hydrated for several hours in D_2O (Fig. 2).[2] As previously reported, many of the bands in the region below 1800 cm^{-1} also have downshifted due to H/D exchange, including the new bands at 1755/1740 cm^{-1} assigned to the C=O stretching mode of the COOD side chain of Asp-83 (Rath et al., 1993). *Thus, we conclude on the basis of the assignment of the 2550 cm^{-1}* band to an S-H group that one or more cysteine residues are perturbed during the Rho→Meta II transition of rhodopsin.

The hydrogen bonding strength of the SH group in cysteines affects the frequency of the S-H stretching vibration. For example, a study of the S-H stretch frequency of model thiol compounds shows that in the absence of hydrogen bonding the frequency is in the 2580–2590 cm^{-1} interval, whereas a downshift of as much as 30–40 cm^{-1} occurs when stronger hydrogen bonds are formed (Bare et al., 1975; Moh et al., 1987; Li and Thomas, 1991). Changes in the cysteine side-chain orientation can also affect the S-H stretch frequency, however much less than hydrogen bonding. For example, the S-H frequency of the P_c and P_h rotamers of model mercaptans differ by approximately 10 cm^{-1} (Durig et al., 1975; Richter and Schiel, 1984; Li and Thomas, 1991). Thus, the band at 2550 cm^{-1} reflects at least one cysteine residue that is strongly hydrogen-bonded in the Meta II intermediate.

[1] For example, a band appears near 3330 cm^{-1} during Meta II formation that has been assigned to the methylene C-H stretch mode of C=C-H groups in unsaturated photoreceptor membrane lipids (DeGrip et al., 1988).

[2] The expected disappearance of the 2550-cm^{-1} band upon H/D exchange is not observable due to the downshift of OD and ND bands into this region of the difference spectrum.

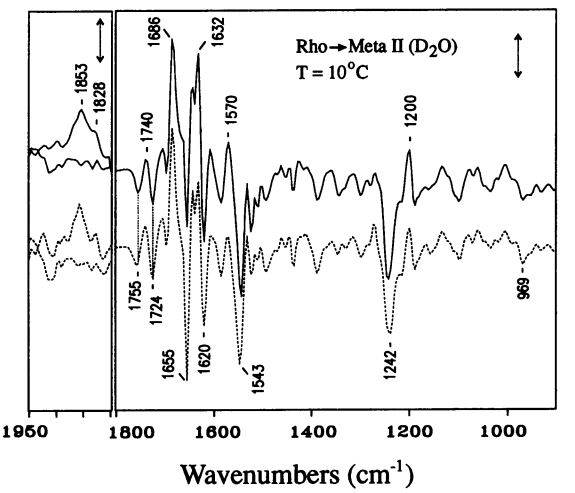

FIGURE 2 Rho→Meta II FTIR difference spectrum of normal rhodopsin (——) and NEM labeled rhodopsin (. . . . . .) in D_2O at 10°C. Each spectrum is the average of 3000 scans (10 min) recorded at 8-cm^{-1} resolution. (*Right* panel) The scale-bar shown is 2×10^{-3} o.d. units for the solid curve. (*Left* panel) The scale-bar shown is 0.17×10^{-3} o.d. units for the solid curve. Lower curves correspond to the same region in H_2O.

The shoulder at lower frequency (both in the H_2O and D_2O spectra) might represent a second cysteine residue with an even stronger hydrogen bonding, although we cannot exclude the possibility that the same cysteine residue also gives rise to the observed shoulder by adopting two different side-chain orientations.

The kinetics of the S-H band intensity parallels Meta II formation and decay

To determine at which stage of rhodopsin bleaching the cysteine bands appear, we obtained time-resolved FTIR difference spectra at low temperature. When rhodopsin is photoactivated at low temperatures (−10 to −15°C) and spectra are recorded at 10-min intervals, the S-H band at 2550 cm^{-1} is observed to increase gradually in intensity (Fig. 3), accompanied by changes in the region below 1800 cm^{-1} that are characteristic of the slow decay of Meta I and formation of Meta II. For example, bands at 1767/1747 cm^{-1} (−/+) and 1686 cm^{-1}, characteristic of Meta II, gradually appear and are correlated with a decay in intensity of the band at 949 cm^{-1} characteristic of Meta I. As shown in the inset, the intensity of the 2550 cm^{-1} S-H band increases at the same rate as that of the 1686 cm^{-1} band characteristic of Meta II, *demonstrating that the appearance of the S-H band is correlated with Meta II formation.*

At higher temperatures (above 0°C), Meta II decay is accompanied by a partial reversal of many of the protein conformational changes that occur during the Rho→Meta II transition (Rothschild et al., 1987; Klinger and Braiman, 1992). This reversal effect can be seen in Fig. 4, which shows the successive FTIR difference spectra recorded during the decay of Meta II at 10°C. Many of the protein bands that

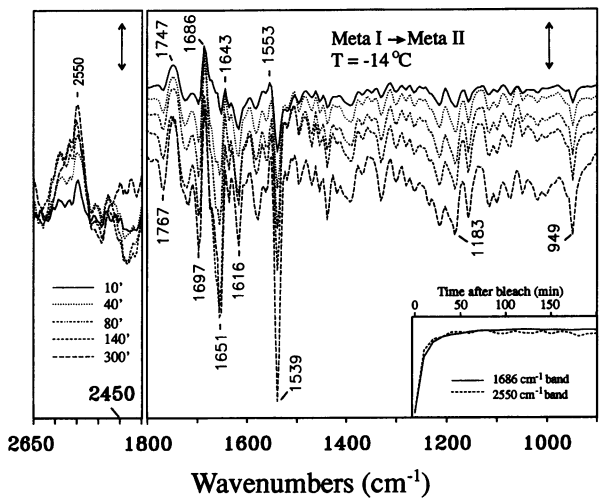

FIGURE 3 Meta I→Meta II FTIR difference spectrum measured at −14°C. Spectra shown are the difference between the first spectrum recorded immediately after the photobleaching and subsequent spectra beginning at the indicated time. Each spectrum is the average of 3000 scans recorded at 8-cm^{-1} resolution. The scale bars shown are 0.5×10^{-3} o.d. (*right* panel) and 0.03×10^{-3} o.d. units (*left* panel). (*Inset*) Kinetic plot of the 1686-cm^{-1} band (*solid* curve), which reflects the formation of Meta II and the 2550-cm^{-1} band (*dashed* curve) assigned to the S-H stretching vibration of difference spectra recorded from a sample at −10°C. *y* axis is the normalized integrated intensity of these bands.

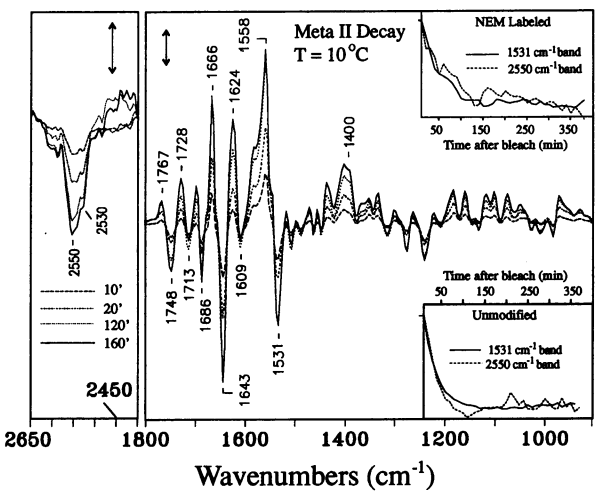

FIGURE 4 Meta II decay FTIR difference spectra recorded at 10°C. Spectra shown are the difference between the first spectrum recorded after photobleaching and subsequent spectra recorded at 10-min intervals. Spectra were recorded at 8-cm^{-1} resolution and are the average of 3000 scans. The scale bar shown is 1.0×10^{-3} o.d. units (*right* panel) and 0.1×10^{-3} o.d. units (*left* panel). Similar difference spectra were obtained for the NEM-labeled rhodopsin (spectra not shown). (*Insets*) Kinetic plot of the 1531-cm^{-1} amide II band (*solid* curve) and the 2550-cm^{-1} S-H band (*dashed* curve) in both unmodified (*bottom right*) and NEM-labeled rhodopsin (*top right*) recorded at 10°C. *y* axis is the normalized integrated intensity of these bands.

appear in the Rho→Meta II difference spectrum (Fig. 1) reverse sign during Meta II decay. However, note that the characteristic chromophore bands at 1548 cm^{-1} (C=C ethylenic stretch), 1238 cm^{-1} (C12-C13 stretch mode) and 969 cm^{-1} (hydrogen out-of-plane mode) are absent because retinal does not reisomerize during the Meta II decay. Significantly, the S-H band also decays in parallel with these protein bands (Fig. 4, *left* panel and *bottom right inset*). Thus, we conclude that the decay of the S-H band is correlated with the decay of the Meta II intermediate.

NEM Labeling of Cys-140 and Cys-316 has no effect on the S-H band

In an attempt to identify the cysteine residue(s) giving rise to the S-H band during Meta II formation, we have used the -SH reagent *N*-ethyl-maleimide (NEM). This reagent reacts selectively with SH groups to form the corresponding *N*-ethyl succinimido-thio ether. In the case of rhodopsin, previous studies have shown that it reacts predominantly with the highly conserved cysteine residues 140 and 316, which are located on the extradiscal (cytoplasmic) side of the photoreceptor membrane (Griffith et al., 1979; Farahbakhsh et al., 1992).

As seen in Figs. 1 and 2, the reaction of these cysteine residues with NEM had no significant effect on the Rho→Meta II difference spectrum for rhodopsin exposed to either H_2O or D_2O. In particular, the S-H band is still present at 2550 cm^{-1} (Fig. 1) or 1853 cm^{-1} in the case of D_2O (Fig. 2), ruling out the possibility that these bands arise from Cys-140 or Cys-316. The kinetics of the decay of the S-H band as well as other protein bands also are not significantly affected by this modification (Fig. 4, *top right inset*) and still correlate with Meta II decay. Also, no significant changes were found due to NEM labeling on the difference spectrum below 1800 cm^{-1} (Figs. 1 and 2). Thus, we conclude that *Cys-140 and Cys-316 undergo no significant changes in hydrogen bonding during rhodopsin photoactivation.*

DISCUSSION

In this paper, we present evidence based on FTIR difference spectroscopy that one or more cysteine residues undergoes a change in its environment during rhodopsin photoactivation. Earlier infrared studies by Alben and co-workers on hemoglobin have established that the S-H stretching vibration can be a sensitive probe of this group's environment in a protein (Alben et al., 1974; Bare et al., 1975; Moh et al., 1987). For example, differences in the frequency and molar extinction have been found for different cysteines in human hemoglobin. The S-H frequency for specific cysteines has also been shown to vary as a function of the binding ligand (i.e., CO, O_2, CN) and is related to alterations in the local environment and bonding interactions of the SH group (Moh et al., 1987).

In the case of rhodopsin, we observe a positive band at 2550 cm^{-1} with no detectable negative component. Based on the correlation of frequency and hydrogen bonding strength of SH groups, one would expect to observe a pair of negative/positive bands if an SH group undergoes a change in hydrogen bonding during the Meta I→Meta II transition. However, the molar extinction coefficient of the S-H stretching

vibration has been found to be highly dependent on SH hydrogen bonding, increasing by a factor of almost 10 for the model compound ethanethiol measured in nonpolar and polar solvents (Alben et al., 1974). Thus, the appearance of a single positive band in the difference spectrum of rhodopsin could be explained if a cysteine SH group undergoes a switch from a weak hydrogen bonding environment (Meta I) to a much stronger hydrogen bonding environment (Meta II).[3]

Although there are 10 cysteine residues in the bovine rhodopsin primary sequence, we are able to exclude two that are highly conserved, Cys-140 and Cys-316, as contributing to the FTIR difference spectrum. In particular, NEM modification of these residues did not alter the 2550 cm^{-1} band. Recently, Farahbakhsh et al. (1993) have shown that a spin-labeled reporter group bound to Cys-140 undergoes a transient change in its environment upon photobleaching, which has the same rise and decay kinetics as Meta II (whereas a spin-label linked to Cys-316 showed no change). However, these findings are not necessarily inconsistent with our results because: (i) The spin-label might artificially induce the observed changes due to a perturbation in the environment of Cys-140[4]; and (ii) the local conformational changes around Cys-140 sensed by the spin-label may not produce changes detected by FTIR. This is possible because FTIR difference spectroscopy will be sensitive only to changes in the microenvironment of the SH group, e.g., hydrogen bonding changes, whereas the spin-label may also probe the environment outside the immediate vicinity of the SH group.

Two other residues, Cys-322 and 323, can also be excluded as contributing to the 2550 cm^{-1} band because they are the palmitylation sites for rhodopsin (Ovchinnikov et al., 1988). Finally, Cys-110 and Cys-187, which are also highly conserved in the rhodopsin family, have been shown to form a disulfide bond (Karnik and Khorana, 1990). Thus, these residues cannot contribute to the 2550-cm^{-1} band unless, as discussed below, the disulfide bond is broken during the lifetime of the Meta II intermediate.

This leaves four cysteine residues as candidates for the assignment of the 2550-cm^{-1} band, three of which are contained inside intramembrane helices D, E, and F (Cys-167, Cys-222, and Cys-264) and one (Cys-185) on the intradiscal loop 2. None of these residues is completely conserved in the rhodopsin family (Hargrave and McDowell, 1992b), making it unlikely that the cysteine(s) we are observing spectroscopically are essential for rhodopsin photoactivation. On the other hand, these cysteines may be sensing structural activity in a nearby region of the protein. For example, simple folding models based on the primary sequence of rhodopsin place Cys-264 in helix-F close to the Schiff base of the retinal formed with Lys-296 on the neighboring helix-G. In this case, Cys-264 might be responding to deprotonation of the Schiff base and possible protonation of the putative Schiff base counterion Glu-113 during Meta II formation (Sakmar et al., 1989; Zhukovsky and Oprian, 1989; Nathans, 1990). In support of such a possible interaction, a homologous region in the F-helix of bacteriorhodopsin has been shown on the basis of FTIR difference spectroscopy and site-directed mutagenesis to be structurally active, most likely due to its proximity to the Schiff base region (Rothschild et al., 1989). An additional candidate is Cys-222, which has been identified as the possible binding site for a hydrophobic spin-label that responds to structural changes upon rhodopsin photobleaching (Farahbakhsh et al., 1992).

An additional possibility is that the appearance of a positive band at 2550 cm^{-1} is due to the transient reduction of a disulfide group that occurs during the Meta I→Meta II transition. In this case, a *positive* band is a natural consequence of the *formation* of two new SH groups.[5] However, confirmation of this possibility would depend on identification of a negative disulfide vibrational bands that are normally located in 485 to 540 cm^{-1} region (Parker, 1983). Reduction of disulfide bonds has also been suggested as a potential mechanism for the activation of other G-protein-coupled receptors (Malbon et al., 1987). However, the simple absence of a disulfide bond does not appear to be sufficient for receptor activation.[6]

A more exact assignment of the 2550 cm^{-1} band to a specific cysteine residue(s) may be possible using site-directed mutagenesis as recently demonstrated for Asp residues (Fahmy et al., 1993; Rath et al., 1993). In this case, individual cysteine residues could be eliminated from the rhodopsin primary sequence, and changes in the S-H vibration could be monitored. Ultimately, such FTIR studies that assigns bands to individual opsin residues should provide an increased understanding of the mechanism of rhodopsin photoactivation. Similar studies should also be possible with ligand-activated receptors such as the nicotinic acetylcholine receptor (Baenziger et al., 1992).

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[3] The absence of a negative band may also be due to a reorientation of an SH group, because in oriented rhodopsin films an S-H bond parallel to the membrane plane will absorb more than one perpendicular to it.

[4] Note that photo-induced changes were not detected when two other spin-labeled probes attached to Cys-140 were used (Farahbakhsh et al., 1992, 1993).

[5] In an earlier study of the role of cysteine residues in rhodopsin using site-directed mutagenesis, it was found that the disulfide bridge formed by Cys-110 and Cys-187 (Karnik and Khorana, 1990) is buried in native rhodopsin, because it does not react with powerful reducing agents such as tri-*n*-butylphosphine unless rhodopsin is in a denatured state. Thus, a transient reduction of this bond might not be easily detected chemically if Cys-110 and Cys-187 were inaccessible to mild thiol reagents such as NEM, DTNB, or iodoacetic acid.

[6] For example, the substitution of Cys-184 in β-adrenergic receptor (homologous to Cys-187 in opsin) with valine does not cause activation of this receptor, although the disulfide bond cannot be formed. Instead, this mutation causes a partial uncoupling from adenylyl cyclase activity due to a loss of the high affinity agonist-receptor-G-protein complex (Liggett et al., 1989).

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