Rhodopsin Activation Affects the Environment of Specific Neighboring Phospholipids: An FTIR Spectroscopic Study

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ABSTRACT Rhodopsin is a member of a superfamily of G-protein-coupled receptors that transduce signals across membranes. We used Fourier-transform infrared (FTIR) difference spectroscopy to study the interaction between rhodopsin and lipid bilayer upon receptor activation. A difference band at 1744 cm^{-1} (+)/1727 cm^{-1} (−) was identified in the FTIR-difference spectrum of rhodopsin mutant D83N/E122Q in which spectral difference bands arising from the carbonyl stretching frequencies of protonated carboxylic acid groups were removed by mutation. As the band was abolished by detergent delipidation, we suggested that it arose from carbonyl groups of phospholipid fatty acid esters. Rhodopsin and the D83N/E122Q mutant were reconstituted into various ^{13}C-labeled 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine vesicles and probed. The 1744-cm^{-1} (+)/1727 cm^{-1} (−) band could be unequivocally assigned to a change in the lipid ester carbonyl stretch upon receptor activation, with roughly equal contribution from both lipid esters. The band intensity scaled with the amount of rhodopsin but not with the amount of lipid, excluding the possibility that it was due to the bulk lipid phase. We also excluded the possibility that the lipid band represents a change in the number of boundary lipids or a general alteration in the boundary lipid environment upon formation of metarhodopsin II. Instead, the data suggest that the lipid band represents the change of a specific lipid-receptor interaction that is coupled to protein conformational changes.

INTRODUCTION

The visual pigment rhodopsin belongs to the superfamily of G-protein-coupled receptors (GPCRs). GPCRs are all intrinsic membrane proteins with seven transmembrane helical segments. Activation of the receptor, by photoisomerization of the 11-*cis*-retinal cofactor in the case of rhodopsin, leads to conformational changes of its cytoplasmic surface. The active receptor can interact with a heterotrimeric G protein to catalyze guanine-nucleotide exchange and initiate signaling (for reviews see Helmreich and Hofmann, 1996; Sakmar, 1998). The formation of the active photoproduct of rhodopsin, metarhodopsin II (MII), is characterized by relative movements of transmembrane helices 3, 5, and 6, as detected by site-directed spin labeling (Farahbakhsh et al., 1993), histidine-zinc chelation (Sheikh et al., 1996), and disulfide cross-linking (Farrens et al., 1996) experiments. These helix movements probably cause the volume increase deduced from the pressure dependence of the metarhodopsin I (MI)/MII equilibrium (Atwood and Gutfreund, 1980; Lamola et al., 1974). Furthermore, the MI/MII equilibrium is influenced by the fluidity of the embedding matrix. A more fluid bilayer (Applebury et al., 1974; Baldwin and Hubbell, 1985; Gibson and Brown, 1993; Mitchell et al., 1992; O'Brien et al., 1977) or fluid detergents (Lamola et al., 1974; Motoyama et al., 1985; Schleicher et al., 1987) favor MII. Thus, it is reasonable to expect that activation of rhodopsin in turn affects its lipid membrane, or detergent, environment.

The physical properties of the membrane may be affected as well by post-translational modification of the receptor. For example, in rhodopsin, Cys^{322} and Cys^{323} near the carboxyl terminus are palmitoylated (Ovchinnikov et al., 1988; Papac et al., 1992). The insertion of the hydrophobic acyl chains into the lipid bilayer forms a putative fourth cytoplasmic loop (Moench et al., 1994). The role of this loop in receptor signaling is not fully understood (for review see Pepperberg et al., 1995), especially considering that the rod G protein, transducin, can be activated in detergent (Ganter et al., 1989; Han et al., 1998) where the fatty acids are probably not anchored. However, it was recently shown that the rhodopsin fourth cytoplasmic loop contains a binding site for the α subunit of transducin and that it also plays a role in regulating the binding of the $\beta\gamma$ subunit (Ernst et al., 2000; Marin et al., 2000). Thus, alterations in lipid-protein interactions may influence this loop, or structural changes of the loop due to its palmitoylation may have an influence on the lipid bilayer.

Many of the signaling events that occur at the membrane surface are dependent on the association of proteins with the phospholipid bilayer, and many of these interactions are facilitated by the post-translational modification of signaling proteins in addition to the GPCRs. These include myristoylation or palmitoylation of G protein α subunits and isoprenylation of G protein γ subunits (transducin is heterogeneously myristoylated and farnesylated). The importance of the two-dimensional arrangement of the signaling complex at the membrane surface has been demonstrated by

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stochastic modeling of visual transduction (Felber et al., 1996; Lamb, 1994). The specific protein-protein interactions occurring at the membrane surface depend on factors such as membrane docking and two-dimensional diffusion of receptors in bilayers.

We recently identified an FTIR difference band at 1744 cm^{-1} (+)/1727 cm^{-1} (−) that was tentatively assigned to lipid ester carbonyl stretching vibrations (Beck et al., 1998b). Here, we present experiments to elucidate the molecular etiology of the lipid band and its relationship to lipid-rhodopsin interactions. We measured the MII spectra of wild-type rhodopsin and of the double mutant D83N/E122Q reconstituted into 1-palmitoyl-2-oleoyl-*sn*-glycero-phosphocholine (POPC) vesicles. Isotope-labeled POPC with ^{13}C at one or both of the two ester functions was also employed. The data show that both ester groups contribute to the lipid band. In addition, the lipid band intensity scales only with the amount of rhodopsin, excluding the possibility that it arises from a bulk lipid effect. The data also exclude the possibility that the lipid band represents a change in the number of boundary lipids or a general alteration in the boundary lipid environment upon formation of MII. Quantitative analysis of the data suggest that the lipid band represents the change of a lipid-receptor interaction linked to protein conformational changes and that only one or a few specific lipid molecules are affected.

MATERIALS AND METHODS

Materials

Site-specific isotopically labeled phospholipids (1-^{13}C-palmitoyl-2-^{13}C-oleoyl-*sn*-glycero-phosphocholine, 1-palmitoyl-^{13}C-2-oleoyl-*sn*-glycero-3-phosphocholine, and 1-^{13}C-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) were prepared by Dr. Walter Shaw at Avanti Polar Lipids (Alabaster, AL). The isotope label content was determined to be 90%, 70%, and 80%, respectively. The 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was from Sigma (St. Louis, MO). Detergents were obtained from Anatrace (Maumee, OH).

Reconstitution of purified rhodopsin and rhodopsin mutant D83N/E122Q into POPC

Disc membranes were isolated from bovine rod outer segments (ROSs) and rhodopsin was solubilized in *n*-octyl-β-D-glucoside (OG) or *n*-dodecyl-β-D-maltoside (DM) and purified using conconavalin-A Sepharose as described previously (Beck et al., 1998b). The rhodopsin double mutant D83N/E122Q was expressed and purified as described (Beck et al., 1998a). All manipulations involving the pigments were carried out under dim red light. Reconstitution of pigments into lipid bilayers was performed, with minor modifications, as described earlier (Beck et al., 1998a). Lyophilized POPC was solubilized in 1.2% (w/v) OG, 1 mM sodium phosphate (pH 6.5) to a final concentration of 5 mg/ml and sonicated in a water bath until the mixture became translucent (~1 min). The appropriate amount of the solubilized POPC was added to pigment (0.4 mg/ml) solubilized in 1.2% OG to achieve the desired lipid to protein ratio. The mixture was again sonicated for 15 s and stored on ice for 1.5 h. The sample (approximately 0.4 ml) was dialyzed against 8 L of 1 mM sodium phosphate (pH 6.5) for 48 h at 7°C using a Pierce microdialyzer system with a 10–20-kDa cutoff membrane. The reconstituted pigment was pelleted at 80,000 × *g* for 16 h at 4°C. The pellet was resuspended in 0.1 mM sodium phosphate buffer to a final concentration of ~1 mg/ml. Because some of the lipid was dialyzed away, the actual lipid:protein molecular ratio was determined experimentally from infrared spectra of the dried samples before rehydration. The size of the C═O band of the lipid ester relative to the amide-I band of the protein is a direct measure of the lipid:protein ratio in the sample. The known value of ~60 lipid molecules/rhodopsin in disc membranes is used as calibration (Kamps et al., 1982), taking a rhodopsin content of ~88% into account. POPC was selected for reconstitution as it is still in the fluid phase at 5°C and guarantees full photo-conversion of rhodopsin to MII. The phase transition of POPC occurs at ~3°C (cited in Pohle et al., 1998).

FTIR spectroscopy

Measurements were performed with a Bruker IFS 28 FTIR spectrophotometer equipped with an MCT detector. A suspension of pigment reconstituted into lipid vesicles (1–1.5 nmol) with a pH of 5.5 (adjusted with 0.5 μmol of sodium phosphate buffer) was dried under a gentle stream of N_2 onto a CaF_2 window. The film was rehydrated with 0.5 μl of H_2O and sealed with a second CaF_2 window. For measurements in ^2H_2O, ^2H_2O (50 μl) was added to the dried film and the sample was again dried and rehydrated with 1 μl of ^2H_2O and equilibrated in the sealed cuvette. The last step was repeated twice. Because the degree of hydration is critical for MII formation, the water content was monitored using the IR absorption band of water at 3300 cm^{-1}. To obtain MII difference spectra, samples at 5°C were illuminated through fiber optics and a long-pass filter (GG435, Schott) using a slide projector. The low pH and high salt concentration of the phosphate buffer enabled full conversion of rhodopsin to MII (Delange et al., 1997). Four sets of 128 scans were collected each before and after the illumination, and the four difference spectra were finally averaged after controlling for distortions. Spectral resolution for all measurements was 2 cm^{-1}.

To investigate the influence of the protein on the C═O band of the lipid, absorbance spectra of samples with different lipid:protein ratios hydrated with ^2H_2O were compared. To obtain absorbance spectra of the samples, their single-beam spectra were ratioed against that of two empty CaF_2 windows. To assure the precise reproducibility of sample preparation, a special Ca_2F window with a circular 8-mm-diameter 4-μm-deep depression that was separated from the rim of the window by a 1-mm-wide channel was employed. The reconstituted pigment was sonicated with a Branson Sonic Power Sonifier and the suspension was dried onto the special Ca_2F window. After rehydration of the film by adding ^2H_2O (~0.5 μl), any excess solvent was displaced into the channel when the second plane window was pressed onto the hydrated film. This technique resulted in samples with highly reproducible solvent content.

RESULTS

ROS rhodopsin and rhodopsin mutant D83N/E122Q were reconstituted into POPC vesicles and studied by FTIR spectroscopy to elucidate the nature of the presumed lipid band at 1744 cm^{-1} (+)/1727 cm^{-1} (−) (Beck et al., 1998b). Synthetic POPC was selected for reconstitution as it guarantees full MII formation even at 5°C and pH 5.5 and also allows the site-specific incorporation of isotopic labels. The isotope shift of the ^{13}C═O stretch was expected to be approximately 40 cm^{-1} (Blume et al., 1988). The comparison of spectra obtained from reconstitutions with either singly or doubly labeled lipid would also clarify whether one or both ester functions contribute to the difference band.

The D83N/E122Q mutant was chosen because, in detergent solution all bands above 1712 cm^{-1} are abolished, and when reconstituted in POPC only the 1744 cm^{-1} (+)/1727 cm^{-1} (−) band is noted above 1712 cm^{-1}.

The results of the reconstitution experiments with rhodopsin mutant D83N/E122Q are shown in Fig. 1. The mutant MII-rhodopsin difference spectra are approximately normalized to the chromophore band at 1238 cm^{-1}. The lack of the hydrogen-out-of-plane (HOOP) mode at 950 cm^{-1} clearly demonstrates almost complete conversion to the MII-like state under the conditions of the study in POPC (Ganter et al., 1989; Jäger et al., 1994). The difference band assigned to the lipids is clearly visible at 1744 cm^{-1} (+)/1727 cm^{-1} (−). It is important to note that the intensity of the lipid band correlates only with the amount of photolyzed pigment. The spectrum of rhodopsin reconstituted into unlabeled POPC at a lipid:protein molecular ratio of approximately 64 was practically indistinguishable from that of rhodopsin reconstituted into egg phosphatidylcholine (Beck et al., 1998b). The band intensity was independent of the lipid:rhodopsin molecular ratio as tested between 39:1 and 108:1 (data not shown). This result provides important constraints concerning the molecular interpretation of the 1744 cm^{-1} (+)/1727 cm^{-1} (−) band as discussed below.

Rhodopsin mutant D83N/E122Q was also reconstituted in POPC that was labeled with ^{13}C at one or both of the C═O groups of the esterified fatty acids at the *sn*1 or *sn*2 positions of the glycerol backbone. Fig. 1 shows that individual labels at either the *sn*1 or *sn*2 positions both reduce the intensity of the difference band compared with the band obtained with unlabeled POPC. Neither individual label completely abolished the band. However, if both C═O groups in the POPC are isotopically labeled, the 1744 cm^{-1} (+)/1727 cm^{-1} (−) difference band disappears. These results confirm that the band is caused by the lipid and clearly show that both ester functions of POPC contribute to the band (Beck et al., 1998b). Surprisingly, in the spectrum obtained with the doubly labeled lipid, a small positive band around 1740 cm^{-1} is still present. As ^2H_2O causes a downshift of this band (data not shown), it arises from a carboxyl group that has been assigned to Glu^{134}, which becomes protonated upon MII formation (Fahmy et al., 2000).

The MII spectrum of rhodopsin in detergent is characterized by bands caused by the C═O stretch of protonated membrane-embedded carboxyl groups (Fig. 2, bottom trace). The difference band at 1767 cm^{-1} (−)/1745 cm^{-1} (+) was assigned to Asp^{83}, and the difference band at 1745 cm^{-1} (+)/1727 cm^{-1} (−) was assigned to Glu^{122}

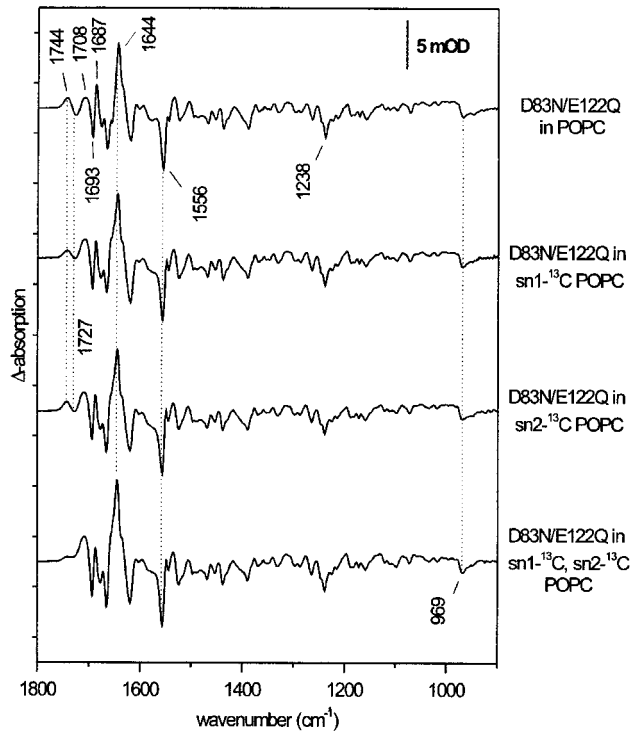

FIGURE 1 FTIR-difference spectra were recorded for mutant rhodopsin D83N/E122Q reconstituted into synthetic phospholipid POPC or isotopically labeled POPC (*sn*1-^{13}C POPC, *sn*2-^{13}C POPC or *sn*1-^{13}C, *sn*2-^{13}C POPC). The vertical bar indicates the approximate absorbance scale of the spectra, which were normalized to the band at 1238 cm^{-1}. Measurements were performed as described in Materials and Methods at 5°C with 2-cm^{-1} spectral resolution.

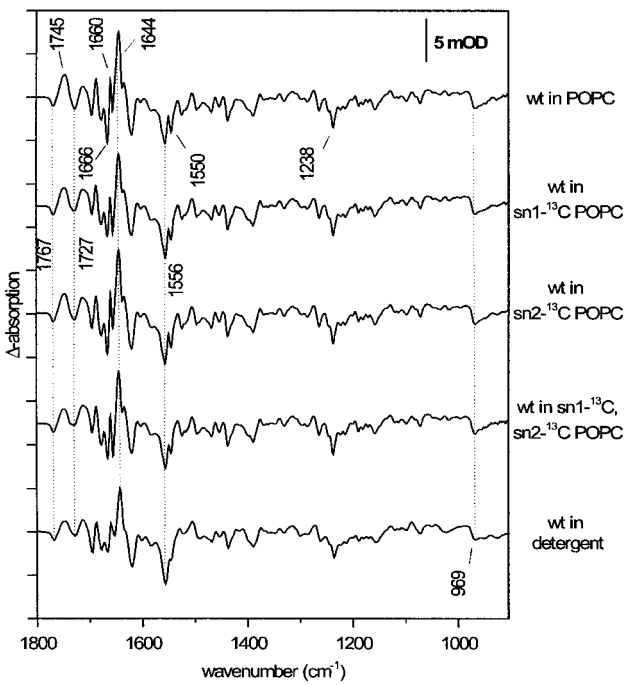

FIGURE 2 FTIR-difference spectra of the rhodopsin to MII transition were recorded for ROS rhodopsin (wt) reconstituted into synthetic phospholipid POPC, isotopically labeled POPC (*sn*1-^{13}C POPC, *sn*2-^{13}C POPC or *sn*1-^{13}C, *sn*2-^{13}C POPC) or rhodopsin solubilized in DM detergent. The vertical bar indicates the approximate absorbance scale of the spectra, which were normalized to the band at 1238 cm^{-1}. Measurements were performed as described in Materials and Methods at 5°C with 2-cm^{-1} spectral resolution.

(Fahmy et al., 1993; Rath et al., 1993). The lipid band at 1744 cm^{-1} (+)/1727 cm^{-1} (−) is superimposed onto the difference bands assigned to the protein carboxylic acid side chains when rhodopsin is reconstituted into POPC (Fig. 2). Individual ^{13}C labels at either the *sn*1 or *sn*2 positions reduce the intensity of the superimposed difference band compared with the band obtained with unlabeled POPC. The lipid band intensity is further reduced if both C═O groups in the POPC are isotopically labeled. As expected from the results with mutant D83N/E122Q, the spectrum of rhodopsin in *sn*1-^{13}C, *sn*2-^{13}C POPC is very similar to that obtained in DM detergent (Fig. 2). This clearly shows that the detergent effect described earlier (Fahmy et al., 1993) is due to the removal of the lipid C═O band.

Although the isotope shift of the ^{13}C═O stretch was expected to be ~40 cm^{-1} (Blume et al., 1988), the shifted bands are not readily apparent in the spectra of rhodopsin reconstituted in labeled POPC. Visual inspection reveals a slight increase in the intensity of the band around 1710 cm^{-1} and a decrease in the intensity of the band at 1686 cm^{-1}. These effects could be caused by the superposition of the positive and negative shifted bands. To identify clearly the shifted bands and to clarify whether both C═O groups equally contribute to the lipid band, differences between spectra taken in unlabeled and labeled POPC were calculated. To carry out such subtractions, a band is obviously needed for normalization that is not altered by the labeling. Our experience suggests that for reliable subtraction such a band should not be located too far from the bands of interest. The somewhat inhomogeneous distribution of the membranes across the infrared window often causes small but observable variations in the relative intensity of bands. This effect is especially severe in regions where the sample transmission is low. Several spectral bands were considered for normalization. As seen in the spectra in Fig. 2, the bands between 1600 cm^{-1} and 1660 cm^{-1} (amide-I modes) and those between 1510 cm^{-1} and 1570 cm^{-1} (amide-II bands and C═C stretch of the chromophore) vary somewhat from sample to sample and are not appropriate for spectral normalization. The chromophore band at 1238 cm^{-1} is also not suitable as the transmission at this frequency differs considerably from that around 1730 cm^{-1}. The negative band at 1767 cm^{-1}(−) assigned to Asp^{83}, which is reproducible and close to the lipid band, was chosen for spectral normalization.

The spectra in Fig. 3 represent the differences between the spectrum of rhodopsin in unlabeled POPC and spectra of rhodopsin in the isotopically labeled POPC (Fig. 2). It should be noted that a reasonable baseline is obtained below 1500 cm^{-1}. The small deviations are caused by the effects described above. Similarly, due to the low transmission, the bands between 1600 cm^{-1} and 1660 cm^{-1} and between 1510 cm^{-1} and 1560 cm^{-1} are not canceled, confirming that these bands cannot be used for normalization. However, above 1686 cm^{-1}, the bands attributable to the isotope label

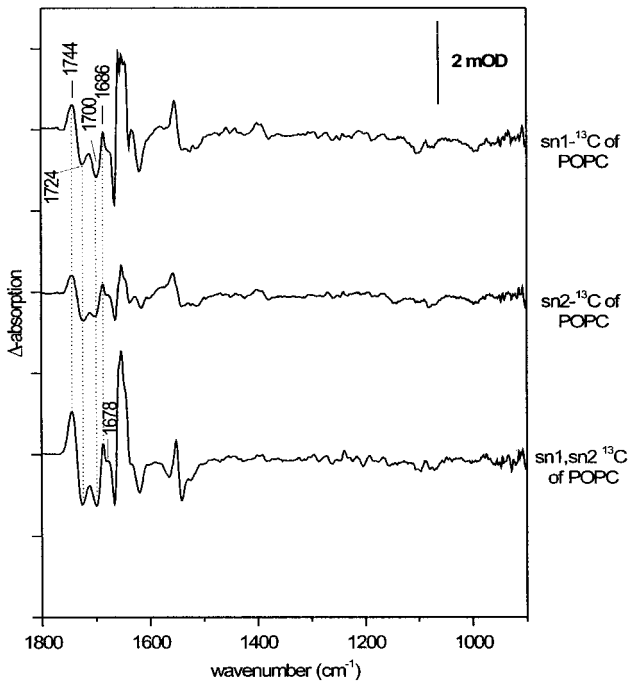

FIGURE 3 Double-difference spectra were calculated from data presented in Fig. 2. Each spectrum obtained in labeled POPC was subtracted from that in unlabeled POPC. In calculating the differences, the band at 1767 cm^{-1} was used for normalization as described in Materials and Methods. The resulting spectra represent the absorption change of *sn*1-^{13}C of POPC, *sn*2-^{13}C of POPC and *sn*1-^{13}C, *sn*2-^{13}C of POPC, respectively, during the transition of rhodopsin to MII. The vertical bar indicates the absorbance scale.

can be clearly identified. The difference band 1744 cm^{-1}(+)/1724 cm^{-1}(−) is caused by the unlabeled lipids, and the difference band 1700 cm^{-1}(−)/1686 cm^{-1}(+) is due to the labeled lipids, although the 1686-cm^{-1} band cannot be unequivocally identified. Thus, the isotopic shift is observed experimentally to be 44 cm^{-1}, which is very close to the value expected (Blume et al., 1988).

The relative contributions of the two POPC ester groups to the lipid band can be determined from Fig. 3. The isotopic enrichment was determined by mass spectrometry by the manufacturer to be 80%, 70%, and 90% for the *sn*1, *sn*2, and doubly labeled lipids, respectively. As the spectra shown in Fig. 3 are all normalized to the spectrum obtained in the unlabeled POPC, the band intensities can be directly compared. It is clear that the 1744 cm^{-1}(+)/1724 cm^{-1}(−) bands are largest for the difference obtained with the doubly labeled lipid. The smallest band is noted in the spectrum of the sample where the *sn*2-labeled lipid is used for subtraction. Quantitative evaluation of the band intensities yields a ratio of 0.53:0.40:1.0 for the contribution of the *sn*1, *sn*2, and doubly labeled lipids, respectively. From the isotopic enrichment, assuming an equal contribution of both ester groups, a ratio of 0.44:0.38:1.0 would be expected. Thus, within the accuracy determined by the subtraction proce-

dure, we conclude that both ester groups contribute approximately equally to the lipid band. This is in clear contrast to our earlier tentative assignment (Beck et al., 1998b), which was based on the observation that the intensity of the lipid band approximately amounted to one ester group of one lipid molecule. In the previous assignment, we had assumed that only one ester group of one specific lipid molecule per rhodopsin was involved in the MII transition.

The influence of rhodopsin insertion into the lipid membrane on the C═O band was measured directly by conventional infrared absorption spectroscopy. Fig. 4 compares absorption spectra of rhodopsin reconstituted into POPC lipids at varying lipid:protein ratios (39:1, 64:1, 108:1). The spectrum of a pure POPC lipid film is also displayed in Fig. 4. To avoid contributions from the water band at 1650 cm^{-1}, the samples were resuspended in ^2H_2O. The C═O stretch of pure POPC lipids produces a single absorption band at 1731 cm^{-1}. The corresponding band of the reconstituted samples appears at 1737 cm^{-1}. The upshift from 1731 cm^{-1} to 1737 cm^{-1} seems to support the hypothesis that rhodopsin incorporation creates a more hydrophobic environment in which lipids are less hydrogen-bonded to water as discussed below.

To facilitate the comparison of the spectra in Fig. 4, the spectrum of the sample with lowest lipid:protein ratio (39:1) was subtracted from that with the highest (108:1). The result is presented in Fig. 5 (bottom trace). The subtraction constant was adjusted to cancel as far as possible the lipid band. Any difference between the two samples should appear as a difference band. However, in the region where the lipid band should appear, a completely flat baseline was obtained (the different protein concentrations cause the large negative slope below 1710 cm^{-1}). The deviations from the baseline can be estimated to be less than 5×10^{-4}. This shows that even at the enlarged absorbance resolution no difference between the two samples with respect to the lipid C═O band can be detected.

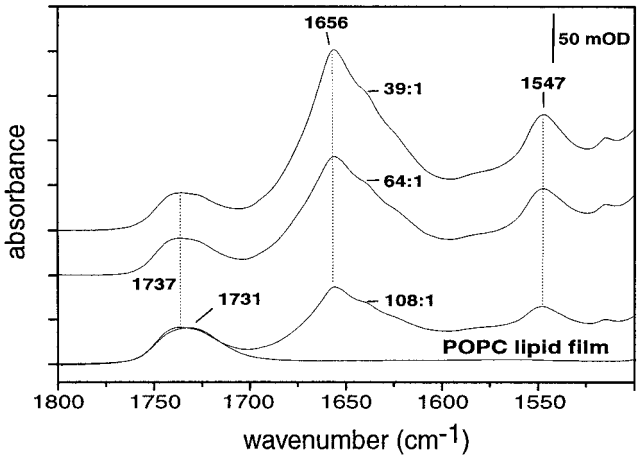

FIGURE 4 Absorption spectra were obtained from rhodopsin reconstituted into POPC in varying lipid:protein molecular ratios as indicated. The samples were hydrated with ^2H_2O as described in Materials and Methods. The spectrum of a pure POPC film hydrated with ^2H_2O is shown for comparison. The film was prepared by evaporating the solvent from a lipid-chloroform solution placed on the special CaF_2 window used for the other samples. The vertical bar indicates the absorbance scale.

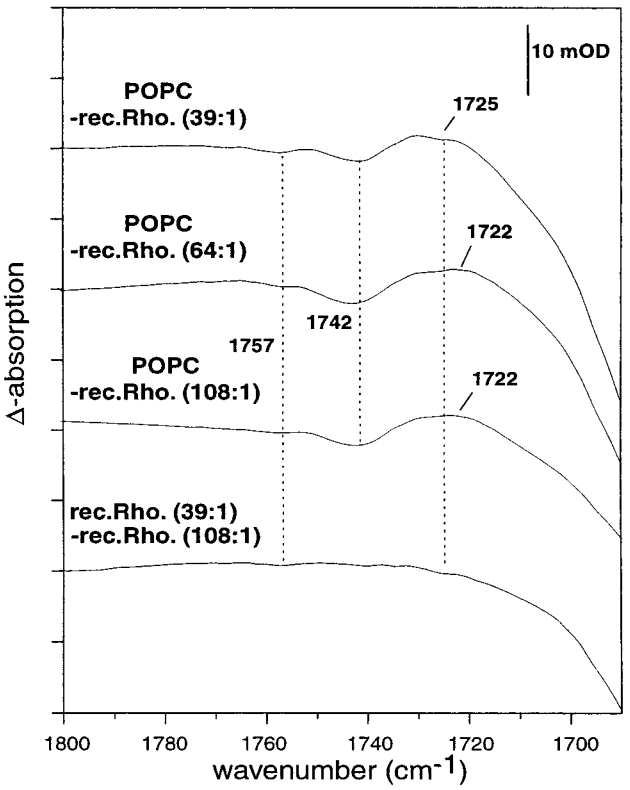

FIGURE 5 Absorption-difference spectra were obtained from the data presented in Fig. 4 for rhodopsin reconstituted in POPC (rec.Rho) at the lipid:protein molecular ratios indicated. The subtraction constant was determined to cancel as precisely as possible the lipid absorption band at 1737 cm^{-1} so that, effectively, the spectra are normalized to the amount of lipid. The vertical bar indicates absorbance scale.

In contrast, if the spectrum of a reconstituted rhodopsin sample is subtracted from that of the pure lipid sample, a flat baseline cannot be obtained irrespective of the choice of the subtraction constant. This is demonstrated for each of the molecular lipid:protein ratios (39:1, 64:1, and 108:1) in Fig. 5. A difference band at 1742 cm^{-1} (−)/1722 cm^{-1} (+) clearly shows up, which is expected from the spectra in Fig. 4. Interestingly, some smaller reproducible negative bands are present at 1757 cm^{-1} and 1725 cm^{-1}. They can be attributed to the internal deuterated carboxyl groups in the dark state, Asp^{83} and Glu^{122}, respectively (Fahmy et al., 1993). The identification of these protein bands demonstrates the sensitivity of the subtraction procedure. These bands can also be detected at reduced amplitude in the subtraction of the 108:1 minus 39:1 reconstitutions as the protein contents differ (Fig. 5). However, a precise quantification cannot be made because of the small size of the bands and the level of baseline noise.

DISCUSSION

The results presented above clearly demonstrate that a difference band at 1744 cm^{-1} (+)/1727 cm^{-1} (−) in the rhodopsin/MII spectrum arises from lipid ester groups that change environment upon conversion of rhodopsin to MII. In addition, both of the ester groups of the POPC contribute approximately equally to the band intensity. A molecular interpretation of the lipid band requires the examination of various potential explanations concerning the interaction of rhodopsin with the lipid bilayer. The lipid band could arise from the alterations of one specific lipid molecule. However, it could also be that the number of one or two ester groups per photolyzed rhodopsin is fortuitous. The lipid band could arise as well from a small alteration of the bulk lipid phase or of the so-called boundary lipids (i.e., the belt of lipid molecules surrounding rhodopsin and interacting in a dynamic equilibrium with its hydrophobic surface) (Davoust et al., 1980, 1983; Pates et al., 1985; Ryba et al., 1987; Zumbulyadis and O'Brien, 1979).

Recent work has addressed the molecular interpretation of the C═O band of phospholipids in bilayers (Blume et al., 1988; Lewis et al., 1994; Pohle et al., 1998; Selle and Pohle, 1998). Individual ester groups could not be distinguished on the basis of their absorption bands even upon selective ^{13}C labeling. This suggested that the molecular environment of the *sn*1 and *sn*2 ester groups must be very similar with respect to polarity and hydrogen bonding. However, spectroscopic analysis of the band indicated that it was not homogeneous and that it was altered by the physical state of the lipids (Blume et al., 1988; Lewis et al., 1994). Changing the temperature from above to below the lipid phase transition shifted the band position to somewhat higher wave numbers. This effect was interpreted in terms of changes in hydrogen bonding for both ester carbonyls. Above the phase transition, more water molecules can penetrate into the interface layer, increasing the number of hydrogen bonds. Below the phase transition, water is squeezed out of the interface. Thus, a change in the hydrophobic surface of rhodopsin could affect a property of the bulk lipid phase, such as fluidity, and alter in turn the number of water molecules interacting with the ester groups. However, in the current work, because the lipid band is independent of the molecular lipid:protein ratio, it is unlikely that it arises from the bulk lipid phase.

Alternatively, if the rhodopsin to MII transition caused an increase in the membrane-exposed hydrophobic surface, then a straightforward interpretation of the lipid band would be possible. Lipid molecules directly adjacent to the hydrophobic surface would experience an environment with reduced possibilities for hydrogen bonding with water molecules. Essentially, on average, the fraction of hydrophobic lipids would be increased. Implicit here is the assumption that the insertion of rhodopsin into the lipid bilayer causes already a pronounced upshift of the C═O band because a belt of boundary lipids would be formed. The lipid band observed in MII would then scale with the amount of rhodopsin as observed. To test this hypothesis, we directly measured by conventional infrared absorption spectroscopy the influence of rhodopsin insertion into the lipid membrane on the C═O band (Fig. 4).

The upshift of the lipid band from 1731 cm^{-1} to 1737 cm^{-1} seems to support the hypothesis that rhodopsin incorporation creates a more hydrophobic environment in which lipids are less hydrogen-bonded to water. Here, the lipid band could be caused by an increase of the hydrophobic surface in MII. However, one has to take into account that the pure lipid sample differs in its physical state from the reconstituted rhodopsin samples. Whereas the former consists of stacks of predominantly planar bilayers (Nollert et al., 1995), the latter are assemblies of vesicles. Ultraviolet-visible dichroic measurements showed a rather poor orientation of rhodopsin on the infrared window consistent with its incorporation into vesicles (data not shown). It is possible that the different curvatures of the membrane surfaces in the two samples influences the C═O band. Thus, to detect an influence on the C═O stretch of the boundary lipids by membrane protein insertion, two samples of the same type but with different lipid:protein ratio need to be compared. As shown experimentally by spin-labeling experiments, the number of boundary lipids is independent of the lipid:protein ratio and has been estimated to be 23 ± 2 per rhodopsin (Ryba et al., 1987). In a sample with fewer lipids, the percentage of boundary lipids (i.e., of lipids in which the C═O band might be influenced) is larger, and any effect relative to the amount of lipid present should also be larger. If reconstituted samples are normalized to the amount of lipid present, the influence of the protein on the boundary lipids should become visible (Fig. 5).

The spectrum of the sample with lowest lipid:protein ratio (39:1) was subtracted from that with the highest ratio (108:1). In the region where the lipid band should appear, a completely flat baseline is obtained. The deviations from the baseline are estimated to be less than 5×10^{-4}. This result shows that no difference between the two samples with respect to the lipid C═O band can be detected. In contrast, if the spectrum of reconstituted rhodopsin at any of three lipid:protein ratios is subtracted from that of the pure POPC lipid sample, a flat baseline cannot be obtained. A difference band at 1742 cm^{-1}/1722 cm^{-1} clearly appears (Fig. 5). Thus, the insertion of progressively more rhodopsin into the bilayer does not influence the lipid C═O band, and it appears unlikely that the lipid band can be explained by an increase of the hydrophobic surface in MII.

These qualitative arguments can be expressed more quantitatively. If we denote the two lipid:protein ratios as r_1 and r_2, the total lipid concentration (mol/area) as L_0, the concentration of protein bound lipids in the two samples as L_p^1 and L_p^2, then under the assumption that the absorption band of protein-associated lipids, ϵ_p, differs from that of the bulk

lipids, ϵ_b, then the spectral difference represented by the lower trace of Fig. 5 can be expressed as $(L_p^1 - L_p^2)(\epsilon_p - \epsilon_b)$, which in turn, can be expressed as $23L_0(r_2 - r_1)(\epsilon_p - \epsilon_b)/(r_1r_2)$ (23 being the number of boundary lipids). With the lipid:protein ratios used for the lower trace in Fig. 5 and the estimate of 5×10^{-4} for the deviation from baseline, the maximum difference in absorption for the bulk and boundary lipids is as follows:

$$L_0|\varepsilon_p - \varepsilon_b| \leq 1.3 \times 10^{-3}.$$

This value has to be correlated with the observed magnitude of the lipid band for the reconstitution with the lipid:protein ratio of 108, which was determined for this case to be 5.7×10^{-4}. If the membrane exposed surface area of the protein would be doubled in the transition to MII, then the difference band would amount to $23 \times 1.3 \times 10^{-3}/108 = 2.8 \times 10^{-4}$. Thus, even a twofold increase in surface area should cause a smaller band than that observed. Such an increase in surface area, however, is not a reasonable assumption as the number of additional bound lipid molecules in MII increases at most by three (Baldwin and Hubbell, 1985). This clearly confirms that the observed lipid band arising in MII cannot be simply explained by an increase of the protein hydrophobic surface. As an effect on the bulk lipid phase has been excluded because of the independence of the lipid band on the reconstitution ratio (see above), our previous conclusion that one or a few specific lipid molecules undergo a molecular change is supported by the current study.

As both *sn*1 and *sn*2 ester groups contribute to the MII lipid band, it is important to comment on its size in relation to the total absorbance of the lipids. Previously, we estimated the band amplitude to represent 0.5 lipid molecules per rhodopsin. However, in view of a better estimate of the lipid content based on native disc membranes, a reevaluation seems appropriate. Furthermore, the overlap between the positive and negative components of the difference band has not been adequately treated. Two procedures were used, and both yielded approximately the same number of lipid molecules/rhodopsin involved in the difference band. For both methods, we evaluated the spectra of the D83N/E122Q mutant, where the MII lipid band can be directly measured.

In the first estimate, we assumed that the heterogeneity of the lipid bands in Fig. 4 was caused by two populations and correspondingly that the lipid bands could be fitted to two Gaussian bands with equal widths. Although the fit was not perfect, it provided a reasonable approximation. The areas were proportional to the amount of lipid in each population. The MII difference band was approximated by a weighted subtraction of the two Gaussian bands, and from this an estimate of the number of involved lipids/rhodopsin was obtained. The number was 0.35 based on measurements with the lipid:protein ratio determined in this case to be 50.

In the second estimate, still assuming two populations of lipid molecules, the difference band was directly fitted to a positive and negative band with equal half-widths. Their areas could be correlated with the area of the total lipid band, and from this the number of lipid molecules/rhodopsin was obtained. Due to the overlap of the positive and negative bands, a range of possible band positions and bandwidths could approximate the difference band. The estimation of the quality of the fit was mainly based on deviations in the high-frequency part. The number of lipid molecules/rhodopsin determined with this second method ranged from 0.25 to 0.43. As expected, the bands calculated from a fit with a value of 0.35 very closely resembled the bands used in the first method. Thus, it is clear that under the assumption of two populations of lipid molecules, the band size would correspond to less than one lipid molecule/rhodopsin and the model of lipid molecule(s) changing from one population to the other in the MII transition is not appropriate.

For an alternative description of the MII lipid band one has to consider that the nature of the heterogeneous lipid band is not well understood. Our own measurements showed that a lipid film dried under phosphorous pentoxide still shows a heterogeneous band with increased intensity of the high-frequency part as compared with the hydrated film (not shown). Thus, hydrogen bonding to water cannot be the only cause for the heterogeneity, but we have to assume that it is, at least partially, an intrinsic property of the lipid bilayer (i.e., each lipid molecule is causing a heterogeneous band shape). Under this assumption, the small size of the MII lipid band can be explained by a molecular change of one or a few lipid molecules affecting the band shape. As the high-frequency part is increased, the band can be explained by a transition from a stronger to a weaker hydrogen-bonding environment. This could be caused by the release of water molecules in MII, deduced from the dependence of the MI/MII equilibrium on the osmolality of neutral solutes (Mitchell and Litman, 1999). The responsible water molecules would be at the interface between the protein and the lipid phase. In addition, the release of the lipid molecule(s) from a specific protein site could change the hydrogen bonding and thus explain the lipid band. Alternatively, the band shape could be altered by a change in the structural arrangement of lipid molecule(s), which might be induced by helix movement. As it is not reasonable to assume that all the boundary lipid molecules are affected in the same way, we conclude that the 1744 $cm^{-1}(+)$/1724 $cm^{-1}(-)$ lipid band is caused by a specific effect of part of the membrane-exposed surface of the protein on the interacting lipid phase. Preliminary experiments examining the influence of MII formation on the phase transition of the lipids also indicate a small change in the lipid-protein interaction (T. Heimburg and F. Siebert, in preparation). As MII can activate transducin in detergent solution, specific lipid moloecules are probably not important for this function. However, the infrared lipid band associated with MII formation serves as a monitor for the active state of the

receptor in membranes and therefore bears on the changes in lipid-rhodopsin interactions upon MII formation.

It should be possible to test whether helix movement in other membrane proteins influences the lipid-protein interaction. Corresponding experiments with bacteriorhodopsin and sensory rhodopsin II reconstituted into ^{13}C-labeled lipids are in progress. However, if the lipid band is specific for rhodopsin activation, its functional significance should be clarified. One could speculate that it is caused by the alteration of the fourth cytoplasmic loop, which is involved in rhodopsin-transducin interaction (Marin et al., 2000), and which is thought to be anchored to the lipid phase by the two palmitic acids bound to Cys^{322} and Cys^{323}. Indeed, preliminary experiments seem to indicate that mutation of these two Cys residues considerably reduces the size of the lipid band, although it is not abolished (J. Isele, F. Siebert, and T. P. Sakmar, in preparation).

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