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Evidence for the specific interaction of a lipid molecule with rhodopsin which is altered in the transition to the active state metarhodopsin II

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Abstract Comparing the FTIR difference spectra of the rhodopsin → metarhodopsin II transition in membranes and in dodecylmaltoside detergent, characteristic variations are observed between 1715 and 1750 cm^{-1}. By repeating the measurements with the rhodopsin mutant D83N/E122Q, the spectral variation between the samples in membranes versus detergent could be assigned to a difference band at 1743(+)/1724(−) cm^{-1}, which does not exhibit a deuteration-induced downshift. We provide evidence that this band is probably caused by the C=O stretch of only one ester group of one lipid molecule. This group interacts with the dark state of rhodopsin, whereas in metarhodopsin II, the lipid molecule behaves as if it were in the bulk lipid phase.

Key words: Rhodopsin; G protein coupled receptor; Lipid; Lipid-protein interaction

1. Introduction

Rhodopsin, a typical member of the family of G protein coupled receptors (GPCRs) with seven α-helices spanning the membrane, is the visual pigment of retinal rod cells, and is located in the disc membranes of their outer segments. The absorption of light by the 11-*cis*-retinal chromophore bound to the apoprotein opsin via a protonated Schiff base causes isomerization to the all-*trans* isomer. Thermal relaxation of the irradiated pigment via several spectroscopically distinct intermediates leads to the activated state, metarhodopsin II (MII). MII triggers the enzymatic cascade of vision by activating the G protein, transducin (see [1] for review). Since transducin is a cytosolic peripheral membrane protein, its activation by MII requires conformational changes at the cytosolic surface of the receptor. Theoretical stochastic modelling of the enzymatic cascade has demonstrated that the activation of transducin molecules evolves effectively along the two-dimensional membrane surface [2,3]. Thus, knowledge of the organization of rhodopsin and of GPCRs in general into membrane bilayers is relevant to understanding the dependence of transmembrane signaling on specific protein-protein interactions that occur at the membrane surface (e.g. membrane docking processes, two-dimensional diffusion of receptors, myristylated G protein α subunits, or isoprenylated G protein βγ subunits, etc.).

However, it also appears possible that the lipid phase of the membrane that contains rhodopsin exerts an influence on the receptor to optimize its function in the dark (inactive) and illuminated (active) states. Although illuminated rhodopsin in detergent is a potent activator of transducin, assuming that the detergent concentration is low enough [4,5], it is known that a more fluid lipid bilayer (e.g. [6–10]) or fluid detergents [11–13] favor MII, and that the charge of the lipid head groups modifies the pH dependence of the MI/MII equilibrium by altering the surface charge of the membrane [9,14]. The increased fluidity of the environment is thought to enhance MII formation by facilitating a volume increase in MII [11,15]. Furthermore, especially in more fluid detergents, the decay of MII results in an unstable apoprotein product, which is difficult to regenerate with 11-*cis*-retinal [16,17]. Finally, the lipid membrane may be required for formation of a cytoplasmic C-terminal loop. Transmembrane helix 7, the attachment site of the chromophore, extends nearly to Cys-322 and Cys-323, which are palmitoylated [18,19]. The two fatty acids presumably insert into the membrane to serve as an anchor that defines a putative fourth cytoplasmic loop [20]. It has been reported that this loop takes part in the interaction with transducin [21]. However, another report using site-directed mutagenesis strategies failed to demonstrate a clear functional role for the loop [41]. In addition, as mentioned above, transducin can also be activated in detergent, where the fatty acids are probably not anchored as in the bilayer, leading to conflicting opinions about the role of the palmitoylation (see [22] for review). However, even if this loop does not take part in the light-activated interaction with transducin, several other proteins, including rhodopsin kinase and arrestin, may interact with this region of the receptor. One would expect that it undergoes some conformational changes which may have, possibly because of the palmitoylation, a direct or indirect effect on the lipid bilayer. In this report, by analyzing the rhodopsin → MII transition with FTIR difference spectroscopy under a variety of conditions, we provide direct evidence for changes in the molecular environment of probably a single lipid molecule in the transition of rhodopsin to MII. As judged from its ester C=O stretching vibration, the lipid specifically interacts with the dark state of rhodopsin, but behaves as if it were a bulk lipid in MII.

2. Materials and methods

Purification of bovine rhodopsin and its reconstitution into egg

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Abbreviations: FTIR, Fourier transform infrared; GPCR, G protein coupled receptor; MII, metarhodopsin II; PC, phosphatidylcholine

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lecithin (phosphatidylcholine, PC) vesicles as well as the reconstitution of expressed recombinant rhodopsin mutants have been described [23]. The approximate lipid:rhodopsin ratio after reconstitution was 100:1. The construction of the double mutant D83N/E122Q and its characterization have been reported [24]. Samples for infrared spectroscopy were prepared by drying approximately 1.5 nmol of pigment (in disc membranes, dodecylmaltoside detergent, or reconstituted vesicles) containing 500 nmol of phosphate buffer, pH 5.5, onto a BaF_2 window. The approximate amount of the detergent was 20–30 nmol. Afterwards, the samples were rehydrated to allow full conversion to MII. The procedure to obtain FTIR difference spectra of the transition to MII has been described [24]. Spectra were obtained at 0°C, using a Bruker IFS 28 FTIR spectrophotometer. Resolution was 2 cm^{-1}, and 512 scans were recorded for each of the single beam spectra of the dark and illuminated states. For measurements in ^2H_2O, the rhodopsin film was exposed four times to ^2H_2O for 20 min after completely drying under a stream of nitrogen gas. Subsequently, the film was hydrated with ^2H_2O to the extent necessary for MII generation.

3. Results and discussion

In order to identify bands due to lipid molecules undergoing changes in the transition to MII, we compared MII difference spectra of bovine rhodopsin in disc membranes, reconstituted into egg PC vesicles, and solubilized in dodecylmaltoside detergent (Fig. 1). By convention, negative bands

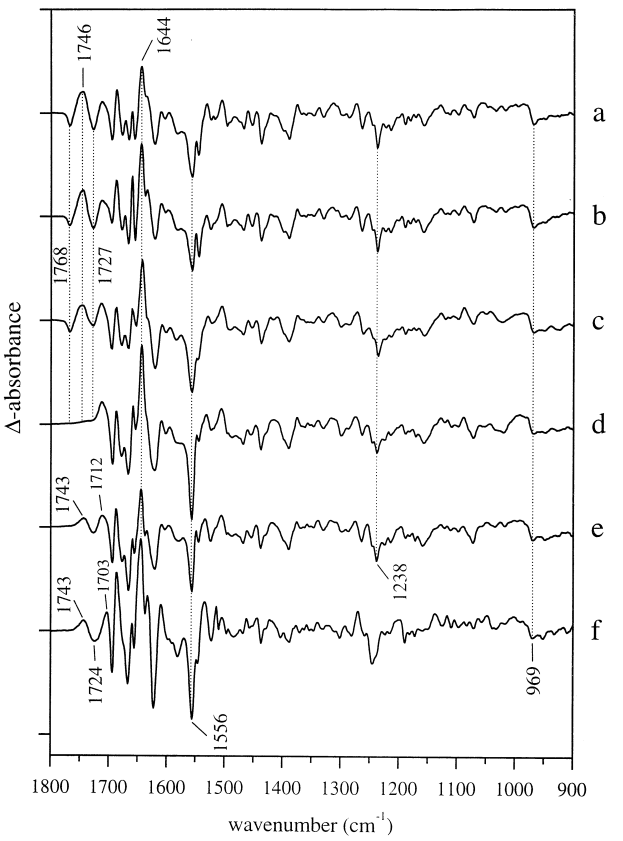

Fig. 1. FTIR difference spectra of the transition of rhodopsin to metarhodopsin II. a: Rhodopsin in disc membranes; b: rhodopsin reconstituted into egg PC; c: rhodopsin in the detergent dodecyl maltoside; d: rhodopsin mutant D83N/E122Q in dodecyl maltoside detergent; e: D83N/E122Q reconstituted into egg PC; f: as in e, but measurements performed in ^2H_2O. Details of the measurements are given in Section 2. Spectral intensities are approximately normalized to each other taking the negative band at 1238 cm^{-1} as reference.

are caused by the initial (dark) state, positive bands by the photoproduct, MII in this case. It has been reported that for rhodopsin reconstituted into egg PC, the transition to MII is largely blocked at pH 7 below 18°C [7]. However, the low pH used in our investigations strongly favors MII in the reconstituted system [25]. In addition, since we did not explicitly attempt to remove all octylglucoside detergent during the reconstitution procedure, some residual detergent in the vesicles may also shift the MI/MII equilibrium towards MII. In any case, when comparing the difference spectra of the reconstituted pigments to that of rhodopsin in disc membranes, their almost identical shape below 1700 cm^{-1} demonstrates that uniformly the transition to MII has been monitored (note the lack of even the smallest indication of a positive band at 1662 and 950 cm^{-1}, which are characteristic features of the MI state [4]).

The difference spectrum of solubilized rhodopsin (Fig. 1c) also reveals below 1700 cm^{-1} all the characteristics of MII in disc membranes. Relative band intensities are somewhat different due to lack of the orientational effect present in the membrane systems [24]. Furthermore, as has been discussed earlier, in the difference spectra of the double mutant D83N/E122Q (Fig. 1d,e), the C=O stretching vibrations of the Asn and Gln side chains introduced by the mutation cause some spectral changes between 1650 and 1685 cm^{-1} [24]. In addition, the blue-shifted absorption maximum resulting in an upshifted C=C stretching vibration of the retinal in the dark state causes spectral alterations around 1550 cm^{-1} [24].

Characteristic infrared lipid bands below 2000 cm^{-1} are located at 1740 cm^{-1} (ester C=O stretch), around 1467 cm^{-1} (CH_2 scissoring), 1255 cm^{-1} (asymmetrical PO_2^- stretch), 1179 cm^{-1} (CO-O stretch), 1092 cm^{-1} (symmetrical PO_2^- stretch), and around 1065 cm^{-1} (C-O-PO_2^- stretch). The $N^+(CH_3)_3$ stretch of PC, the major lipid component in disc membranes, is located at 970 cm^{-1} [26]. The very good agreement of the difference spectra below 1500 cm^{-1} demonstrates that, with the exception of the C=O stretch, the other lipid bands apparently do not contribute to the spectral changes occurring in the transition to MII.

The environment of rhodopsin seems to exert an influence on the spectral range above 1700 cm^{-1} (Fig. 2). The comparison of the MII difference spectra of rhodopsin in disc membranes (Fig. 2a) and of rhodopsin in detergent (Fig. 2c) shows that in the spectrum of the latter both the negative band at 1727 cm^{-1} and the positive band at 1746 cm^{-1} have reduced intensities. This is especially clear for the band at 1727 cm^{-1}, which in Fig. 2c remains above the baseline (the unchanged band at 1712 cm^{-1} due to protonation of Glu-113 in MII [27] may serve as a standard). These intensity differences are beyond those expected to arise from orientational differences. In the MII difference spectrum of rhodopsin reconstituted into egg PC (Fig. 2b), the effects caused by the detergent are not observed, corroborating our assumption that these alterations are caused by the different environment and suggesting that the C=O stretching frequency of the lipids is involved. However, the C=O stretches of buried protonated carboxylic acid groups of the protein, namely Glu-122 and Asp-83, contribute to this spectral region. As previously shown with recombinant pigments, Asp-83 causes the negative band at 1768 cm^{-1} and part of the positive band around 1745 cm^{-1} [24,28], whereas Glu-122 causes two negative bands at 1734 and 1727 cm^{-1}

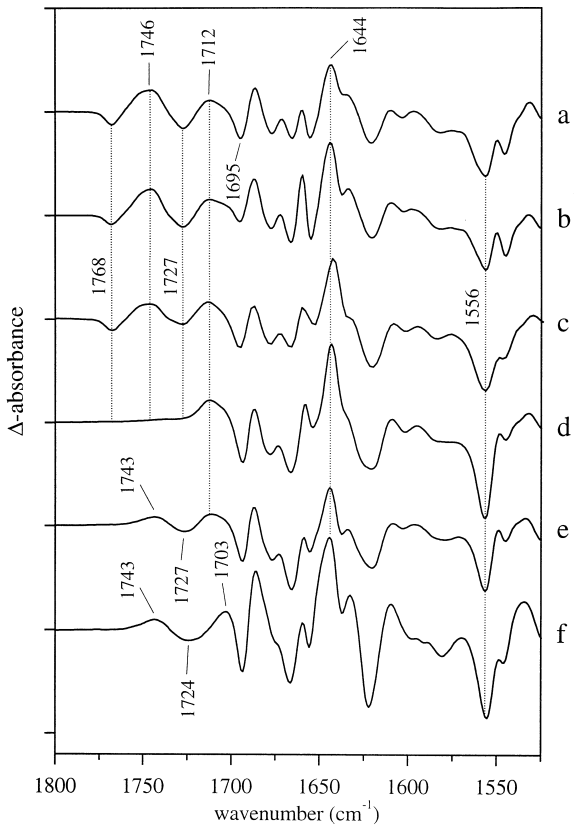

Fig. 2. FTIR absorption changes in the spectral range of 1500–1800 cm^{-1}. The expanded scale provides more detail in the spectral range of C=O stretching vibrations. Data are identical to those shown in Fig. 1. See the legend to Fig. 1 for identification of spectra a–f.

and a positive band at 1745 cm^{-1} [23,24]. Any potential contribution of the C=O stretch of lipids superimposes on these rather strong spectral features, and is, therefore, not easy to identify.

We have previously shown that all bands above 1712 cm^{-1} disappear in the MII difference spectrum of the double mutant D83N/E122Q measured in detergent [24]. This spectrum is shown in Fig. 2d. However, if the MII difference spectrum of this mutant is obtained after reconstituting the pigment into egg PC (Fig. 2e), a clear difference band at 1743 cm^{-1} (+) and 1727 cm^{-1} (−) is present. If the same measurement is repeated after extensive $^1H/^2H$ exchange in ^2H_2O, the positive band at 1743 cm^{-1} remains at the same position, whereas the negative band is now located at 1724 cm^{-1}. In addition, as reported earlier, the positive band due to protonation of Glu-113 is shifted from 1712 cm^{-1} to 1703 cm^{-1} [4,27]. This downshift explains the slight apparent downshift of the negative band from 1727 cm^{-1} to 1724 cm^{-1}. For measurements in H_2O, there is a partial overlap between the positive band of Glu-113 and the negative band at 1724 cm^{-1}, shifting the band position in the difference spectrum to 1727 cm^{-1}. For measurements in ^2H_2O, the two bands no longer overlap, and the negative band shows up at its undistorted position at 1724 cm^{-1}. Thus, we can conclude that the vibration causing the 1743/1724 cm^{-1} difference band is not sensitive to deuteration. Therefore, it must originate from a chemical species different from a carboxyl group. This newly discovered band also explains the difference mentioned above between the spectra of wild type rhodopsin membranes (native or reconstituted in egg PC), and of wild type rhodopsin in detergent. Since the additional band is missing, both the negative band at 1727 cm^{-1} and the positive band at 1746 cm^{-1} have lower intensities in detergent-solubilized samples. Its insensitivity to ^2H_2O and its spectral position make it highly likely that this band is caused by the C=O stretching vibration of an ester group. Under the conditions where the band is observed, two general ester functions are present: one representing membrane lipids, the other from the thioester bond of palmitoylated Cys-322 and Cys-323. According to the literature, the C=O stretch of thioesters is between 1700 and 1680 cm^{-1} [29], rendering it unlikely that the described difference band is caused by such a group. Therefore, the most plausible explanation for the band is an environmental change of one or several lipid molecules. In the dark state, the lipid experiences an unusual environment with hydrogen bonding to the ester group(s) (band position 1724 cm^{-1}). The band position (at 1739 cm^{-1}) in MII is approximately that of the bulk lipid phase.

It is important to make an estimate of how many lipid molecules may account for the spectral features described above. This can be determined in an approximate way by relating the integrated absorbance of the C=O band of the mutant sample reconstituted into egg PC (not shown) to the integrated absorbance of the positive band at 1743 cm^{-1} in Fig. 2e of the same sample. In order to determine the absorbance of the total lipids, their C=O band has to be corrected for the overlap with the amide-I band of rhodopsin which causes some uncertainty of approximately 10%. Furthermore, to eliminate contributions from the water absorbance, the absorbance of the total lipid band was determined of the dried sample after the MII difference spectrum was measured. We obtain a ratio of 350:1 (total lipid absorbance:special lipid absorbance). Taking into account the lipid to rhodopsin molar ratio of 100:1 (from the reconstitution procedure), we can estimate that per rhodopsin molecule the absorbance strength of only 0.29 times that of a single lipid molecule would be involved. However, this value has to be increased, since the absorbance of the positive part of the lipid band in Fig. 2e has been underestimated due to the overlap with the negative band. This overlap can be deduced from the steeper slope of the low-frequency part of the band. It also explains the higher band position as compared to that of the bulk lipid (1743 vs. 1739 cm^{-1}), and it reduces the area under the positive band. Thus, a value of 0.5 is probably more appropriate. Moreover, a value of 1 can be most likely excluded. This would suggest that only one of the two C=O groups of one single lipid molecule undergoes the drastic environmental change as demonstrated in the infrared difference spectra. However, since we have not determined the lipid:protein ratio experimentally after the reconstitution, the actual value may be somewhat different from the theoretical value of 100 given by the reconstitution method. Thus, we cannot completely exclude the possibility that both C=O groups of the lipid molecule are involved. However, we regard it as highly unlikely. Furthermore, since the two ester groups have completely different geometrical arrangements [26,30], it appears difficult to explain the same downshift of the C=O band upon interaction with the dark state of rhodopsin. Nevertheless, only experiments with isotopically labelled lipids will provide unequivocal answers. Such investigations are planned.

NMR and EPR spin label experiments on rhodopsin in disc membranes and in artificial lipid vesicles have indicated that the protein is surrounded by a dynamic boundary layer of lipids (ca. 24) which are slightly immobilized as compared to those of the bulk phase [31–35]. The exchange rate is fast and is essentially determined by the free diffusion of the lipid molecules. NMR studies indicate that the saturated fatty acid at the *sn*-1 position is more strongly immobilized [31]. One could assume that a major fraction of the boundary lipids causes the difference band by exhibiting only a small shift. However, whereas this could explain the size of the band, the large shift from 1724 to 1743 cm^{-1} excludes such an explanation (the overlap between the positive and negative bands is small). It also could be that, on average, the number of boundary lipids is approximately reduced by one in MII. This would suggest that the hydrophobic surface is reduced. However, the volume increase observed in MII [11,15] rather suggests an increase of the hydrophobic surface. Thus, taking all arguments together, the estimate of the absorbance of the special lipid band strongly suggests that only one single lipid molecule, which differs from the dynamic boundary lipids by a special interaction with the dark state of rhodopsin, changes its environment in the transition to MII. We cannot yet predict which of the two ester groups at positions *sn*-1 or *sn*-2 is involved. Since spin label measurements using pairs of ^{14}N and ^{15}N labels have excluded the presence of lipids close to the chromophore, we conclude that the respective lipid molecule is located at the protein-bilayer interfacial surface [36]. It is interesting to note that upon hexane extraction of lipids from disc membranes, especially lipids with long-chain and polyunsaturated fatty acids seem to remain associated with rhodopsin [37]. It should also be noted that the involvement of lipids has been suggested earlier, based on reconstitution of rhodopsin in ether lipids [38,39]. However, due to the overlap with the bands of Asp-83 and Glu-122, no clear-cut spectral identification could be made, and the band position was inaccurately determined. Only experiments with the double mutant in connection with $^{1}H/^{2}H$ exchange could clearly resolve the spectral identification of the lipid molecule.

We can only speculate on the functional role of such a conformationally dependent change of the lipid-protein interaction. Since the C-terminal loop may be involved in the interaction of MII with transducin, it appears intriguing that such a change could be invoked via the two palmitic acids connected to Cys-322 and Cys-323. Experiments to address this possibility are in progress. The lipid binding site also may play a role in the binding of peripheral membrane proteins to rhodopsin via their aliphatic post-translational modifications (e.g. myristylation of the α subunit and farnesylation of the γ subunit of transducin). Furthermore, such a structurally distinct lipid molecule may be an important consideration for crystallization of rhodopsin. To our knowledge, such a specific lipid-protein interaction, which changes during receptor activation, has not been identified before by spectroscopic techniques. In the case of the purple membrane, it has been demonstrated by neutron scattering that glycolipids are arranged at specific positions of the two-dimensional crystal and have specific contacts with the protein bacteriorhodopsin [40]. In addition, the recently published crystal structure of bacteriorhodopsin contains long sections of electron density at various positions which are interpreted as native dihydrophytyl lipids carried along through solubilization and crystallization [42].

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