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Carboxyl group involvement in the meta I and meta II stages in rhodopsin bleaching. A Fourier transform infrared spectroscopic study

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Structural changes due to photoreceptor membrane bleaching can be studied by Fourier transform infrared difference spectroscopy [1,2]. In this paper we focus on the differences between rhodopsin and metarhodopsin I or II. Peaks in the 1700–1770 cm^{-1} region are observed, which may be produced by carbonyl groups in either carboxyl (COOH) or ester carbonyl (COOC) groups, the latter being found exclusively in membrane lipids. In order to distinguish between these two types of carbonyl groups, we have studied reconstituted membranes of rhodopsin in a synthetic phosphatidylcholine that lacks ester carbonyl groups. On this basis, we conclude that the major changes in this region are due to rhodopsin carboxyls which undergo either a change in local environment or a protonation/deprotonation reaction. Additional small changes in this region may reflect a direct involvement of phospholipids in the metarhodopsin I-to-II transition. One or more groups responsible for peaks near 1727 and 1702 cm^{-1} are inaccessible to the outside medium according to hydrogen/deuterium exchange. In contrast, carboxyl group(s) producing peaks near 1710, 1745 and 1768 cm^{-1} exchange freely with the outside medium and are therefore likely to be located near the membrane surface. Removal of a portion of the C-terminal tail region using proteinase K demonstrates that the carboxyl groups in the C-terminal sequence 248–348 are not involved directly in the rhodopsin to metarhodopsin II transition. At the meta I stage, only carboxyl peaks associated with buried groups appear, suggesting that the initial bleaching events, leading to the formation of this intermediate, produce structural rearrangements in the interior region of rhodopsin. These changes then spread to the peripheral surface regions during the metarhodopsin I-to-II transition.

Introduction

The reaction cascade following light activation of the membrane-protein rhodopsin, the primary photoreceptor in rod vision, has been the subject of extensive biochemical and biophysical investigation (cf. for example Ref. 3). The early pioneering studies of Yoshizawa and Wald [4] established the existence of distinct intermediates which can be stabilized at low temperature. At room temperature the bleaching kinetics range from picoseconds for the formation of bathorhodopsin to milliseconds for the formation of meta II [3,5]. While a function in transduction has not yet been attributed to the early intermediates, the formation of metarhodopsin II has been shown to activate the

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Abbreviation: Pipes, 1,4-piperazinediethanesulphonic acid; PMSF, phenylmethylsulfonyl fluoride; ether-PC, 1-hexadecyl-2-octadecenyl(ω9)-glycero-3-phosphocholine.

binding of transducin (G-binding protein) which in turn triggers the cyclic nucleotide cascade by activation of a c-GMP phosphodiesterase [6,10]. The release of Ca^{2+} from the intact rod outer segment upon bleaching may also be triggered directly by photoexcitation of rhodopsin [11,12].

Recently, it has been demonstrated that it is possible to detect the structural changes which occur during bleaching of rhodopsin by using Fourier transform infrared difference spectroscopy [1,2]. The sensitivity of this method is sufficient to detect single group alterations in the chromophore, protein and lipids. We previously reported difference spectra for the rhodopsin-to-bathorhodopsin and -meta II transitions. In this paper, we will focus primarily on the rhodopsin-to-meta I and II transitions and restrict our analysis largely to the 1675–1800 cm^{-1} region which includes contributions from the protein and lipids. On the basis of lipid reconstitution, proteolysis and D/H exchange we are able to assign several peaks in this region. Major conclusions include identification of rhodopsin carboxyl groups which are involved in the formation of metarhodopsin I and II. One or possibly two of these groups are inaccessible to water and are therefore likely to be located towards the interior of the protein. On the basis of the amino acid sequence and 2-dimensional hydrophobicity maps, it is likely that these groups are located on helix 2 and 3 and are due to Asp 78, Glu 122 or Glu 134. Furthermore, these buried groups are already altered at the metarhodopsin I stage in contrast to the water accessible groups which undergo a change between metarhodopsin I and II. Further, we find evidence for either the direct or indirect involvement of phospholipid ester carbonyl groups in the formation of metarhodopsin II.

Materials and Methods

All manipulations involving rhodopsin were performed in dim red light (RG655 filter, Schott AG, Mainz, F.R.G., which has a 1% T cut-off at 659 nm).The buffer used throughout contained: 20 mM Pipes/2 mM $CaCl_2$/3 mM $MgCl_2$/ 0.1 mM EDTA/120 mM NaCl/5 mM KCl/1 mM DTE (pH 6.5).

Isolation of rod outer segment membranes

Rod outer segments were isolated from fresh bovine eyes obtained at the local slaughterhouse. Eyes were dark-adapted within 15 min after the death of the animal and processed within two hours. The rod outer segments were purified by sucrose density centrifugations and analysed by difference spectroscopy as described before [13]. The rod outer segments-band was collected from the gradient, diluted with half a volume of buffer and sedimented at low speed (10 min, $5000 \times g$, 4°C). The membrane pellet obtained was washed twice with double distilled H_2O, sedimented after each washing (30 min, $80000 \times g$; 4°C) and stored in light-tight containers at −70°C. The yield of rod outer segment membranes varied between 10–15 nmol of rhodopsin per eye, with a 280/500 ratio of 2.0–2.2 and less than 5% of the visual pigment in the opsin form.

Purification of rhodopsin

Rhodopsin was purified by chromatography over Con A-Sepharose (Pharmacia, Uppsala, Sweden), as described before, using 20 mM nonylglucose as a detergent [14]. The column-bound material was eluted with 200 mM methylmannoside, concentrated if required to about 100 μM in rhodopsin by pressure-filtration (Amicon Ultrafiltration cell) and gel-filtrated through a small bed of Sephadex-G50 in order to remove methylmannoside and excess detergent. The final solution has an $A_{280/500}$ ratio of 1.6–1.7. Contamination with Concanavalin A is less than 0.5% (w/w).

Preparation of reconstituted membranes

The alkyl analogue of PC, 1-hexadecyl-2-octadecenyl(ω9)-glycero-3-phosphocholine (ether PC), was obtained from Senn Chemicals (Dielsdorff, Switzerland). It was pure by TLC analysis. Reconstituted membranes of this ether-PC with rhodopsin were prepared in a similar way as rhodopsin-phosphatidylserine membranes described before [15]. All manipulations were done under argon. Briefly, the required amount of ether-PC (60–80-fold molar excess over rhodopsin) was dried under high vacuum (30 min, room temperature), wetted by addition of 10–20 μl ethanol, dissolved in 20 mM nonylglucose (10-fold excess of micellar detergent to lipid) and mixed

with the required amount of purified rhodopsin in the same detergent. The resulting solution, which should be clear, is sonicated on a bath sonicator (10 min; room temperature) and left standing at room temperature for 30–60 min. Subsequently, the solution is stepwise diluted by six consecutive additions of the same volume of buffer without detergent. After the second addition, the solution becomes turbid due to the formation of liposomal structures. The latter are precipitated (16 h; 80000 $\times g$; 5°C), washed twice with buffer and stored at −70°C. The recovery of rhodopsin and lipid is 75–90% and 85–95%, respectively. Analysis by electronmicroscopy indicates the presence of large multilamellar structures, with dispersed particles of similar size (8–10 nm) as observed in photoreceptor membranes and other rhodopsin-reconstituted membranes [15]. Intactness of rhodopsin function was checked by its photolytic behaviour as described before [15] by means of a Pye-Unicam PU 8800 spectrophotometer with end-on photomultiplier and cuvettes in the front-position to minimize loss of scattered light. On the average $75 \pm 10\%$ ($n = 3$) of photolysed rhodopsin proceeded to the meta II stage, which subsequently decayed normally to meta III. Hence, this minimally unsaturated ether-PC preserves the photolytic functionality of rhodopsin reasonably well.

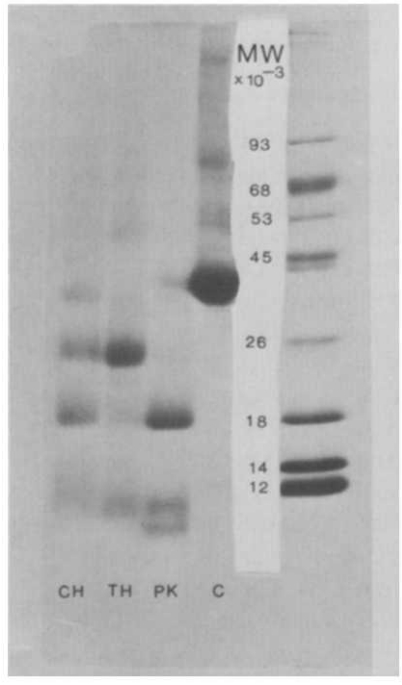


Fig. 1. Fragmentation pattern of rhodopsin obtained after treatment with chymotrypsin (CH), thermolysin (TH) or proteinase K (PK). Samples are analysed by SDS polyacrylamide gradient (8–20% acrylamide) gel electrophoresis. Lane 4 shows the untreated control (C). Lane 5 shows calibration proteins with molecular weight indicated.

Proteolysis

For proteolytic treatment rod outer segment membranes were dissolved under nitrogen in buffer containing 20 mM nonylglucose to a final rhodopsin concentration of 30–40 nmol/ml. Proteinase K (Boehringer, Mannheim, F.R.G.) was added to a 20% (w/w) ratio with respect to rhodopsin. After 30 min incubation at room temperature, the reaction is stopped by addition of PMSF to a final concentration of 1 mM. The proteolyzed rhodopsin is then reconstituted into its own lipids as described above by stepwise dilution with buffer containing 0.1 mM PMSF. The reconstituted membranes are recovered by centrifugation (100 000 $\times g$; overnight; 4°C), washed twice with buffer and stored at −70°C. This procedure effectively removes the enzyme and soluble fragmentation products. The final fragmentation pattern is shown in Fig. 1. It is comparable to that obtained with chymotrypsin, but proteinase K acts more rapidly and completely. It removes 23 amino acids from the C-terminal of rhodopsin, clips and removes a small fragment from the third cytosolic loop (residues 231–244) and clips the second cytosolic loop (between 135 and 150) (De Grip and Bovee-Geurts, unpublished results). Three fragments of approx. 18, 12 and 8 kDa result (Fig. 1). The 18 kDa fragment contains the carbohydrate and therefore derives from the N-terminal part of rhodopsin, while the 12 kDa fragment contains the retinal binding lysine and derives from the C-terminal part [16]. In the membrane the three fragments 'stick together' and form a functional unit, both in darkness and after illumination. This is demonstrated by the facts that (i) the photolytic behaviour of the proteolysed rhodopsin does not differ significantly from rhodopsin itself and (ii) its capacity to regenerate following illumination and addition of 11-*cis* retinal remains intact (De

Grip, unpublished results). In detergent solution, the three fragments fall apart upon illumination or denaturation.

Preparation of membrane films

All manipulations involving membrane samples were performed under argon. For the preparation of oriented membrane films the isopotential spin-dry procedure, developed by Clark et al. [17], was used. Membrane samples were washed twice with double-distilled water, each time collected by centrifugation (100 000 × *g*, 30 min, 4°C) and finally suspended in double-distilled water to a rhodopsin concentration of 30–60 nmol/ml. Aliquots containing 4–8 nmol rhodopsin were pipetted into isopotential cells [17] fitted with an AgCl window (Fisher Sci., U.S.A.) for film deposition. Subsequently, double distilled water was added to the samples to a final volume of 500–800 μl. Samples were spun overnight (10 000 rpm; Beckman SW-25-2 rotor; 4°C), after which AgCl windows and film were removed and stored in light-tight containers at 4°C over Drierite (Fluka AG, Buchs, Switzerland).

Fourier transform infrared difference spectra measurements

Films were humidified by placing a drop of distilled water over the film surface for 1 min and then partially drying with a stream of dry air. The film is immediately sealed in a cell formed by adding a second AgCl window and mounted on the tail of an Air Products HeliTran cryostat positioned inside the compartment of an Nicolet MX-1 Fourier transform infrared spectrometer. The actual amount of humidification of the sample is monitored by observing the water band at 3400 cm^{-1}. Since we have found that the photoreceptor film must be exposed to at least 90% relative humidity in order to observe bleaching to meta II, it is essential that the height of this band be at least twice the intensity of the CH_2 and CH_3 symmetric and asymmetric CH stretching peaks between 2800 and 3000 cm^{-1}.

All measurements of the rhodopsin-to-metarhodopsin II transition were made at 283 K which slows the half-time of formation metarhodopsin III to over 2 h in photoreceptor membranes. For measurements of the rhodopsin to meta I transition samples were cooled to 263 K in order to block the formation of metarhodopsin II. In a typical bleaching experiment ten 5-min spectra of the sample, each computed from 160 interferograms, were made in the dark. The sample was then bleached for 5 min using light from an annular fiber optic illuminator filtered by a 500 nm broad band interference filter (Ditric Optics, U.S.A.), and ten additional 5-min spectra are then recorded in the dark. All interferograms were Fourier transformed using triangular apodization. The effective resolution of all spectra shown is 2 cm^{-1}. Since we observed that upon illumination at 283 K the infrared spectrum of the sample slowly changed over a 20 min period subsequent to bleaching, the difference spectrum was computed from a 5 min spectrum recorded immediately before and after bleaching, so as not to include spectra obtained later in time. In order to increase the signal-to-noise ratio, in some cases several difference spectra obtained from different samples were averaged together.

Results

Carboxyl group involvement in meta II formation

As shown in Fig. 2A, the rhodopsin to metarhodopsin II transition produces several positive and negative peaks in the region from 1700–1770 cm^{-1} in agreement with spectra reported before [1,2]. This region is generally considered at too high a frequency for the C=O stretch mode of amide carbonyl groups (1630–1690) cm^{-1}, but is within the range of the carbonyl stretching frequency of both ester carbonyls (COOC) which are present in membrane lipids and of protonated carboxyl groups (COOH) which are found in the protonated state of the protein side chain residues aspartate and glutamate as well as in the lipid phosphatidylserine. In order to distinguish between carboxyl and ester carbonyl groups one can utilize hydrogen/deuterium exchange which results in a 5–10 cm^{-1} downshift only for carboxyl groups which contain an exchangeable hydrogen [18].

As seen in Fig. 2A and B, the peaks at 1768, 1747 and 1709 cm^{-1} are all downshifted by $^1H/^2H$ exchange, and can therefore be assigned to exposed carboxyl groups. The negative peaks which

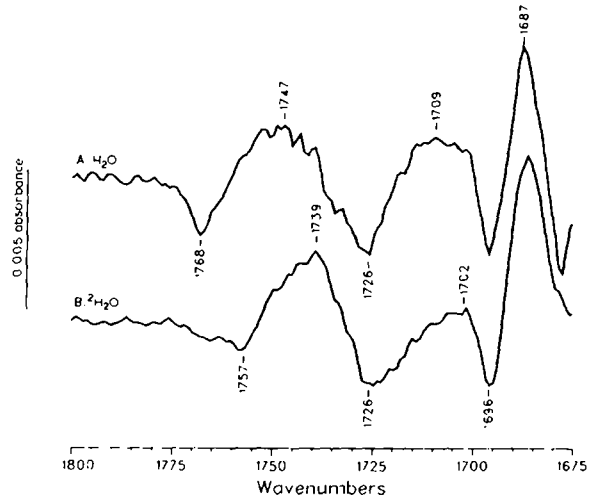


Fig. 2. Difference spectra between rhodopsin and metarhodopsin II in H_2O (A) or ^2H_2O (B), respectively. The bar represents 0.005 A. Membrane samples were humidified with H_2O or ^2H_2O prior to illumination. For clarity, only the carboxyl region of the spectrum is shown (1675–1800 cm^{-1}). Note the shift in 1768, 1747 and 1709 cm^{-1} peaks upon deuteration. For complete Fourier transform infrared spectra of photoreceptor membranes, see Ref. 36. In this and subsequent figures, negative peaks represent rhodopsin vibrations, which shift during the transition, and positive peaks represent newly appearing photoproduct vibrations.

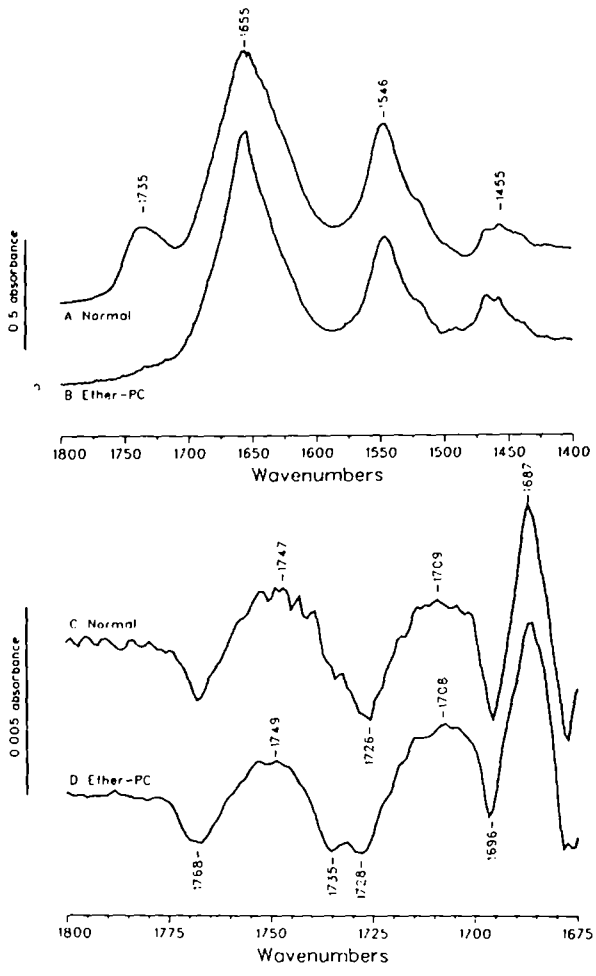


Fig. 3. Comparison of photoreceptor membranes (A, C) and reconstituted membranes of rhodopsin in ether-PC (B, D). A and B show the Fourier transform infrared difference spectrum of unilluminated membrane samples, humidified with H_2O. C and D show the rhodopsin-metarhodopsin II difference spectrum of the same samples. The bar represents 0.5 A (A, B) and 0.005 A (C, D), respectively.

shift must reflect COOH → COO^2H exchange occurring in the rhodopsin stage, i.e., prior to bleaching. On the other hand, the positive peaks that shift might also be due to fast exchange occurring during metarhodopsin II formation, which is too rapid to be detectable on the time-scale of minutes. In contrast to the peaks which shift upon $^1H/^2H$ exchange, the negative peaks at 1726 and 1696 cm^{-1} and the positive peak at 1687 cm^{-1} remain fairly constant in frequency. The broadness of the 1710 cm^{-1} peak and the residual peak at 1702 cm^{-1} in ^2H_2O may also indicate a positive peak at this position which is insensitive to ^2H_2O. These peaks are more difficult to assign, since $^1H/^2H$ exchange cannot distinguish between ester carbonyl groups and buried carboxyl groups.

Lipid carbonyl groups may be involved in the rhodopsin to metarhodopsin II transition

The possibility that the 1687, 1696, 1702 and 1726 cm^{-1} peaks, which do not show $^1H/^2H$ exchange, originate from lipid ester carbonyl(s) or from carboxyl groups in phosphatidylserine, can be eliminated on the basis of bleaching data obtained from rhodopsin reconstituted in a synthetic phosphatidyl-choline containing ether linkages instead of the normal ester carbonyls. As seen in Figs. 3A and B, the prominent lipid ester peak near 1740 cm^{-1} is indeed completely absent in this reconstituted membrane. However, Fig. 3C and D clearly show that the rhodopsin to metarhodopsin II transition for the reconstituted membrane is very similar, although not identical (see below), to

the normal photoreceptor membrane difference spectrum. If any peaks between 1700 and 1770 cm^{-1} originated in phosphatidylserine or ester carbonyl groups, we would expect them to be absent in the spectrum of the rhodopsin/ether-PC reconstituted membrane. In fact, none of these peaks are absent, demonstrating that all changes in the normal spectrum between 1700 and 1770 cm^{-1} arise in protein carboxyl groups. However, an extra peak is reproducibly observed at 1735 cm^{-1} in all ether-PC recombinant membranes studied. This peak cannot be due to the approx. 20–30% of the sample trapped at the metarhodopsin I stage of bleaching, since this intermediate does not display a peak at this frequency as discussed below. Since ester carbonyl groups from the lipids exhibit a vibrational frequence near 1735 cm^{-1}, one possible origin of the new 1735 cm^{-1} peak is the contribution of a small positive ester C=O peak near 1735 cm^{-1} in the normal spectrum which obscures a negative peak near the same frequency originating in a carboxyl group. In the case of the ether-PC lipids which lack ester carbonyl groups, the positive peak is absent revealing the negative component. A second possibility is that the lipid ester carbonyl groups produce a negative peak near 1730 cm^{-1}, which masks the 1735 and 1728 cm^{-1} doublet. A careful comparison of several normal and recombinant membrane difference spectra does consistently show that the 1728 cm^{-1} peak is more negative in the normal sample. This supports the latter interpretation. Either case would indicate a change in the stretching vibration of lipid ester carbonyl groups upon metarhodopsin II formation. We cannot, however, exclude the possibility that all of the peaks in this region, including the 1735 cm^{-1} peak, arise from protein carboxyls and that the additional change observed is due to an indirect effect on the conformation of rhodopsin induced by the lipid substitution.

The peaks at 1696 and 1687 cm^{-1} are more difficult to assign, since they fall in a region which is unusual for both an amide C=O vibration (1630–1690 cm^{-1}) or a carboxyl C=O stretching vibration (1725–1745 cm^{-1}). Since the peak at 1768 cm^{-1} is located 20–40 cm^{-1} higher than is normally found for carboxyl groups, it is also possible that the peaks at 1687 and 1696 cm^{-1} are

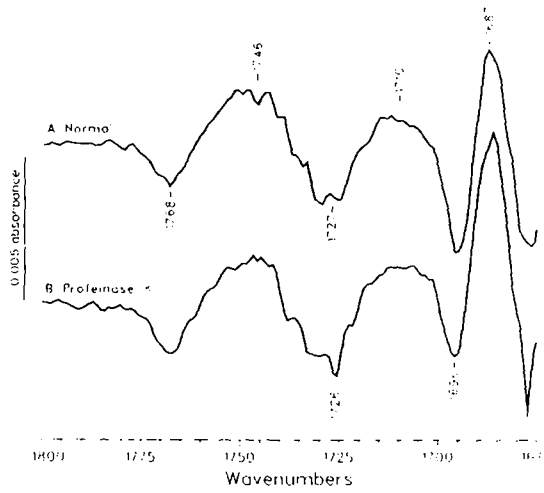


Fig. 4. Comparison of rhodopsin-metarhodopsin II difference spectrum in normal photoreceptor membrane (A) and samples treated with proteinase K (B). The bar represents 0.005 A.

correspondingly shifted down from their normal frequency. On the other hand, the amide I carbonyl stretching vibration in beta-structure is often found at 1680–1695 cm^{-1} [19]. In the case of the β_{II}-turn an amide frequency at 1687 cm^{-1} has been reported in the model peptide Gly-L-Pro-Gly-Gly associated with the carbonyl of the prolyl residue [20].

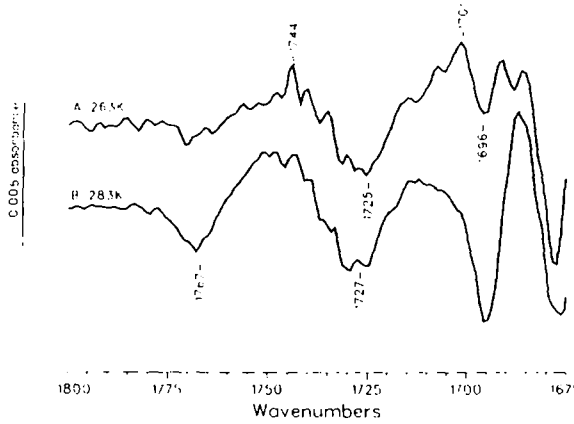


Fig. 5. Comparison of rhodopsin → metarhodopsin I (A) and rhodopsin → metarhodopsin II (B) difference spectra in the photoreceptor membrane. Note the strong reduction of the 1768, 1747 and 1710 cm^{-1} peaks and the much smaller size of the 1696 and 1687 cm^{-1} peaks in the metarhodopsin I spectra. The bar represents 0.005 A.

Altered carboxyl groups do not reside in the C-terminal region of rhodopsin

It is possible to remove the last 23 amino acid in the rhodopsin sequence as well as the sequence 231–244 by using proteinase K (cf. Materials and Methods). As shown in Fig. 4A and B, this procedure does not significantly change the region between 1675 and 1800 cm^{-1} in the rhodopsin → metarhodopsin II transition. We can therefore conclude that the carboxyl groups which are involved in this transition are not part of the C-terminal region of the protein. There is also a remarkable similarity between normal and proteinase-K-treated photoreceptor membrane difference spectra in other regions of the spectrum (not shown). A major exception is the 1687 cm^{-1} peak which appears to become more intense. This may indicate an indirect effect of proteolytic treatment on this group(s), which as discussed above may represent amide carbonyl(s) involved in β-type hydrogen binding. We have further observed that addition of Ca^{2+} ions to the film has a similar effect (Rothschild and De Grip, unpublished results). Other frequencies which exhibit small differences are indicated in Fig. 4.

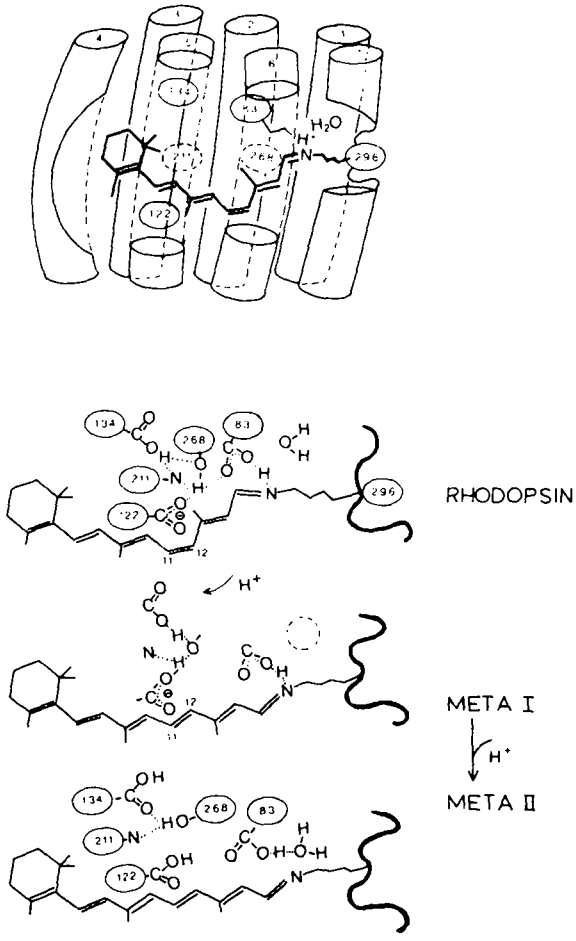


Fig. 6. Schematic diagram visualizing a possible arrangement of some amino acid residues lining the chromophoric centre of rhodopsin, which could explain our experimental results. Asp-83 protonates the Schiff-base between 11-*cis* retinal and Lys-296; Glu-122, Glu-134 and Asp-83 or Asn-78 might link helix 2 and 3 through a hydrogen bond. One or more tyrosine residues and a water molecule might also be involved and extend the hydrogen-bond belt toward a β-type of structure in the vicinity of Lys-296 in helix 7. This belt might be involved in wavelength regulation. Upon illumination, isomerization of 11-*cis* retinal into a strained all-*trans* isomer (bathorhodopsin) will induce small shifts around the Lys-296 binding site. Further relaxation of the chromophore will break or rearrange the 'hydrogen bond belt'. This will induce a positional change of helix 2 with respect to helix 3, larger changes around the Lys-296 binding site with concomitant deprotonation and specific changes in the exterior part of the protein, resulting in formation of metarhodopsin II.

Buried carboxyl groups are involved in the formation of metarhodopsin I

It is possible to block the bleaching sequence at metarhodopsin I by cooling the sample below 0°C [3]. Under these conditions the peaks at 1768, 1747 and 1710 cm^{-1}, which appear in samples proceeding to metarhodopsin II are strongly reduced (Fig. 5A and B). As discussed above these peaks can all be assigned to exposed carboxyl groups. In contrast peaks at 1727 and 1702 cm^{-1} are already present at the metarhodopsin I stage (and actually appear between lumirhodopsin and metarhodopsin I; not shown), and these peaks are assigned to buried carboxyl groups (see above). Thus, the picture emerges that carboxyl groups in the buried portion of the protein are the first to become altered in the photolytic sequence of rhodopsin.

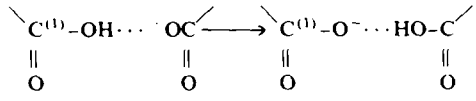
Discussion

In the present study we have restricted our measurements to the bleaching of rhodopsin to the metarhodopsin I and II intermediates. In addition, we have focused our analysis primarily on the region between 1675 and 1800 cm^{-1}. On the basis of these data the following four conclusions can be reached.

The transition from rhodopsin to metarhodopsin II involves several carboxyl groups which have a variety of different local environments

We are able to assign on the basis of reconstitution of rhodopsin with ether-PC lipids and hydrogen/deuterium exchange, all of the peaks above 1700 cm^{-1} to rhodopsin carboxyl groups. However, the actual changes which these carboxyl gruops undergo is uncertain. For example, the peaks at 1768 cm^{-1} (negative) and 1747 cm^{-1} (positive) may be due to a downshift in frequency of a single carboxyl group, which undergoes a change in its local environment. An increase in hydrogen bond strength could, for instance, produce such a downshift in frequency.

Alternatively, the 1768 cm^{-1} and 1745 cm^{-1} peaks might reflect the deprotonation of one carboxyl group ($^{(1)}OOH \rightarrow C^{(1)}OO^-$) and the protonation of a second carboxylate group ($C^{(2)}OO^- \rightarrow C^{(2)}OOH$). This event would occur for example if a proton is exchanged directly from one group to another as shown below:



Scheme I

Such a tautomerism is well known and has been found in several enzyme catalytic mechanisms. One example is penicillopepsin, which contains a charge relay system near the active site involving two aspartates (Asp 215 and Asp 32) which are bonded to each other as well as to a serine and peptide amide group [21].

If scheme I is correct, we would also expect to observe changes in C=O stretching vibrations from carboxylate groups which fall at approx. 1575 and 1400 cm^{-1} [22]. Several peaks do appear in this region, but these might also derive from chromophore vibrations (unpublished data) and have not yet been definitely assigned.

We further note that the intensity of the carboxyl peaks above 1700 cm^{-1} (0.003 A) is roughly consistent with the intensity expected for a single carboxyl group which either is newly formed or undergoes a large frequency shift (> 20 cm^{-1}). The formation of an exposed carboxyl group was also deduced from the appearance of a positive 1760 cm^{-1} peak in the bacteriorhodopsin$_{570}$ → M412 difference spectrum [23–25]. Smaller shifts would create overlap of the negative and positive components producing less intensity. Such shifts would be consistent, for example, with the small carboxyl peaks ($\leqslant 0.0005$) found in the rhodopsin to bathorhodopsin transition (unpublished data, cf. also Ref. 1, 2) and bacteriorhodopsin$_{570}$-to-K transition [26,27].

Only buried carboxyl groups are involved in formation of metarhodopsin I

Two peaks at 1725 cm^{-1} (negative) and 1701 cm^{-1} (positive) appear at the metarhodopsin I stage but are not present in the rhodopsin to bathorhodopsin or rhodopsin to lumirhodopsin difference spectrum (unpublished). The results of hydrogen/deuterium exchange as well as reconstitution into ether-PC leads to the conclusion that these peaks originate in one or more rhodopsin carboxyl groups which do not exchange freely with the external medium. As discussed above, these peaks may actually reflect a single COOH-group which undergoes a downshift in frequency or, alternatively, two different groups undergoing a concerted protonation and deprotonation reaction. If one examines the reported 2-dimensional folding patterns of rhodopsin based on the amino acid sequence there are 19 Asp and Glu groups located outside the core region of the protein [28,29,30]. In contrast, there are only three COOH- or COO^--containing amino acids, Asp 83, Glu 122 and Glu 134 on helices 2 and 3 which are located in the membrane interior and significantly removed from the membrane surface. It is well known from X-ray crystallographic studies that charged groups located within hydrophobic domains, normally pair with other hydrophilic groups forming all possible hydrogen bonds [31]. Since Asp 83, Glu 122 and Glu 134 are located close to each other, it is possible that two of them interact in a manner similar to Scheme I. Other groups in the region capable of hydrogen-bonding such as Tyr 43 or 136 might also participate in the interaction. The breakage or rearrangements of these bonds involving proton exchange could then lead to more delocalized alterations during the metarhodopsin

I → II transition. One possibility is that an ionic bridge is formed between Asp 83 on helix 2 and either Glu 122 or Glu 134 on helix 3. The disruption of this bridge at the metarhodopsin I stage might then trigger a rearrangement in the overall position of these two helixes relative to each other (cf. Fig. 6).

Structural changes spread to water-accessible regions by metarhodopsin II

We have found evidence that several rhodopsin carboxyl groups exposed to water undergo a change between metarhodopsin I and II. Hence, most of these groups are, in contrast to those altered by metarhodopsin I, likely to be located in the surface regions of rhodopsin. There are approx. 19 Asp and Glu residues in a position to easily undergo $^1H/^2H$ exchange [28–30]. We can eliminate all Glu and Asp after Asp 282, and Glu 232 and 239, since these are removed by proteinase K (6 in total). The remaining 13 carboxyl groups are distributed among 3 loop regions on each surface and on the N-terminal region. The 5 Glu residues in the 2nd and 3rd cytosolic loops are of particular interest, since the latter are involved in the binding of G-protein [32]. It would be interesting to determine whether the 'exposed' carboxyl peaks are affected by binding of G-protein. In addition, another candidate for responsible for one of the carboxyl peaks appearing in metarhodopsin II, might actually be a buried group which is at least 'indirectly' accessible to water. It has been deduced that at least one molecule of water is involved in the chromophoric centre of rhodopsin [33], and that the Schiff-base proton easily undergoes $^1H/^2H$-exchange [34]. If the Schiff-base is protonated by a buried carboxyl group, transition of metarhodopsin I to II, which depronates the Schiff base, will reprotonate this carboxyl group or at least strongly changes its hydrogen bond character. Hence, such a group would both appear at the metarhodopsin II stage and be subject to $^1H/^3H$ exchange, and could give rise to the 1745 or 1710 cm^{-1} peak.

Several other new peaks appear or intensify at the metarhodopsin II stage. The 1696 and 1686 cm^{-1} peaks appear already at the rhodopsin → batho-, lumi- or metarhodopsin I transition, but intensify in the transition metarhodopsin I → II. These peaks are produced by amide carbonyls or by carboxyl groups, which are apparently buried, since they do not show $^1H/^2H$ exchange. Although we cannot as yet exclude carboxyl groups with an abnormally low stretching frequency, they are best assigned to amide carbonyl groups involved in β-type hydrogen bonding as discussed above. The location of these groups can at present be only a matter of speculation. One interesting possibility would be the region around the lysine 296 retinal binding site. The residues in this region, including Pro 303 and Pro 291, do not very well fit in a typical α-helix [cf. also Refs. 30 and 35]. Further, helix 2 and 3 probably fold back close to helix 7 [35]. This might place the region around Lys 296 in close enough proximity to the Glu and Asp in helix 2 and 3, so that retinal isomerisation following illumination could trigger a change in the interaction of these groups (cf. Fig. 6).

Lipid ester carbonyl groups may be involved in the formation of metarhodopsin II

The finding of a small additional negative peak at 1735 cm^{-1} in ether-PC rhodopsin recombinants raises the possibility that some lipids are changing during the formation of metarhodopsin II. Additional evidence for such a change is the appearance of features at 1468 cm^{-1} (negative) and 1460 cm^{-1} (positive) in the rhodopsin to metarhodopsin II difference spectrum. These peaks are in the region of CH_2 scissoring and are also observed to change when the photoreceptor membrane is warmed from 250 to 310 K, most likely reflecting a transition from the L-beta (gel) to L-alpha (liquid crystalline) phase of lipids in a bilayer configuration (unpublished data). It is unclear, however, if these features in the difference spectrum arise from lipids or derive from changes in the chromophore or protein. We again note that the changes near 1735 cm^{-1} might also arise from an indirect effect of lipid substitution on the rhodopsin conformation and not to lipid ester carbonyl vibrations. This aspect will be resolved by using specifically labeled phospholipids for reconstitution.

Conclusions

In conclusion, Fourier-transform infrared difference spectroscopy is able to give new informa-

tion on the structural changes occurring upon illumination of rhodopsin. Future studies will be aimed at further localizing the position of the different carboxyl groups involved in the various stages of the rhodopsin photolytic sequence. In addition, specific labeling studies will be attempted in order to investigate whether other groups capable of hydrogen bonding (Tyr, Asn, Ser, Thr) might participate, whether the 1696 and 1686 cm^{-1} peaks indeed represent β-type carbonylamide groups and where they are located. In this context it should be pointed out, that hydrogen bonds, with their high degree of polarisability, could play an important role in the mechanism which governs the dynamic character of a membrane protein like rhodopsin, and possibly of proteins in general.

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