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FOURIER TRANSFORM INFRARED STUDY OF PHOTORECEPTOR MEMBRANE

I. GROUP ASSIGNMENTS BASED ON RHODOPSIN DELIPIDATION AND RECONSTITUTION

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Summary

Fourier transform infrared spectroscopy has been used to study the structure of bovine photoreceptor membrane. Rhodopsin appears to contain an extensive alpha-helical structure which is arranged predominantly perpendicular to the membrane plane. Spectra of delipidated rhodopsin and rhodopsin membranes reconstituted from dioleyl-phosphatidylcholine were compared with native photoreceptor membrane from rod outer segments in order to facilitate peak assignments. It is concluded that spectroscopic peaks characteristic of several protein and lipid groups can be assigned. We also find delipidation leads to alteration of the rhodopsin structure which is restored upon reconstitution. Membranes both suspended in ^2H_2O and dehydrated were compared in order to detect possible conformational differences. Dehydration does not appear to grossly alter rhodopsin structure, although it may affect delipidated rhodopsin.

Introduction

Elucidating the mechanism by which the photoreceptor membrane responds to light remains a key goal in vision research. Rhodopsin, the major protein component of the disc photoreceptor membrane is the primary light receptor [1—8]. It is known that the absorption of light by the rhodopsin chromophore, 11-*cis*-retinal, triggers a series of well defined visible absorption changes called

bleaching. However, little is known about the structure of rhodopsin or the molecular changes accompanying bleaching. In fact ultraviolet spectroscopy so far has only revealed very minor changes to accompany bleaching [4]. Infrared spectroscopy might be more sensitive in this respect since it probes all protein components instead of only the ultraviolet-absorbing ones.

The recent application of Fourier transform infrared spectroscopy to purple membrane in films [5] and solution [6] has demonstrated that this technique can indeed be a sensitive probe of membrane structure. In the case of oriented membranes, information can be obtained about the three-dimensional structure of membrane proteins and lipids. For example, the average spatial orientation of bacteriorhodopsin alpha-helices has been determined by measuring the infrared dichroism of oriented purple membranes [5]. Previous measurements of the photoreceptor membrane vibrational spectrum have been restricted to resonance Raman light-scattering which is sensitive to the rhodopsin chromophore vibrations [7,8] and non-resonance Raman light-scattering on the opsin and lipid components of the photoreceptor membrane after the rhodopsin chromophore was removed by extensive bleaching of the retina [9]. The rate of rhodopsin deuteration, and the effects of bleaching rhodopsin in detergent have also been measured recently by monitoring infrared absorption changes [10,11].

We describe here the first detailed infrared absorption study of photoreceptor membrane. Fourier transform infrared spectroscopy was utilized primarily because of its capability to obtain high signal-to-noise-ratio spectra in a few seconds [12]. In order to assign lipid and protein peaks in the photoreceptor membrane spectrum, we have also studied completely lipid-stripped rhodopsin and rhodopsin recombinant membranes reconstituted with a single species of phospholipid (dioleyl-lecithin). The results of this study demonstrate the sensitivity of infrared to the different components of the membrane and the ability to detect specific molecular changes. In a future paper we shall report in detail on the effects of bleaching, reduction and deuteration kinetics as determined by Fourier transform infrared spectroscopy.

Methods and Procedures

Rod outer segment isolation

Rod outer segments were isolated under dim red light from either calf or cattle eyes using sucrose density centrifugation. The procedure we followed has been reported by DeGrip et al. [13] and consists of the following major steps: (1) Removal of retinas from freshly excised cattle eyes within two to three hours after slaughtering. (2) Gentle homogenization of retinas using a loosely fitting Teflon pestle in a 20 mM Mops(3-(*N*-morpholino)propanesulfonic acid) buffer, pH 7.2, containing 3 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 mM dithioerythritol and either sucrose or NaCl and KCl to attain isotonicity. (3) Filtration of the homogenate through a Teflon screen (70 μm). (4) Linear sucrose centrifugation of filtered homogenate (sucrose gradient 1.09—1.15 g) for 1.5 h at 80 000 $\times g$, 10°C. (5) Incubation of photoreceptor membranes in the presence of NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) and 11-*cis*-retinal to regenerate opsin. This isolation procedure can be performed in less

than 4 h and yields 20—30 nmol of rhodopsin per retina with $A_{280}/A_{500} \cong 2$. In addition to ultraviolet-visible absorption spectroscopy, we checked the purity of our preparation using SDS (sodium dodecyl sulphate) polyacrylamide gel electrophoresis and electron microscopy.

Delipidation and reconstitution of rhodopsin

We preferentially purify rhodopsin by affinity chromatography on commercially available concanavalin A Sepharose after solubilization of rhodopsin with the detergent nonylglucose [14]. The photoreceptor membrane is solubilized in the same Mops buffer containing in addition 20 mM nonylglucose and 1 mM $MnCl_2$ (detergent-buffer). The clear solution is applied to the column. Non-bound material is then washed away with detergent-buffer. This removes lipids and contaminating protein which do not bind specifically to concanavalin A. Bound rhodopsin is then eluted using α-methylglucose (200 mM in detergent-buffer) which competes with the rhodopsin carbohydrate moiety for the concanavalin A binding sites. The entire procedure takes less than 10 h and recovery of rhodopsin is 90—100%. The purified rhodopsin has an A_{280}/A_{500} ratio of 1.6—1.8. Phosphate assay indicates almost complete delipidation with less than 0.5 mol of phosphate present per mol of rhodopsin.

Reconstitution is performed by addition of a 100-fold excess of phospholipid to purified rhodopsin solution prior to dialysis of nonylglucose against Mops buffer. After repeated changes of the buffer, recombinant membranes are formed which consist of predominantly single-walled vesicles. Electron microscopy studies show that rhodopsin is incorporated in the lipid bilayer (DeGrip, W.J. and Olwe, J., unpublished data). This procedure allows us to select different lipids for specialized infrared study.

Sample preparation

All procedures prior to bleaching are made in dim red light or in darkness. Membrane samples were prepared for infrared study by washing three or more times in distilled H_2O and then freeze-dried on AgCl or KRS-5 windows. Samples were also partially oriented on AgCl windows with an isopotential centrifugation cell (Rothschild, K.J., DeGrip, W.J. and Sanches, R., unpublished data) based on the spin-dry method of Clark and Rothschild [15]. Recent studies performed by us on isopotential spin-dried photoreceptor membranes reveal that rhodopsin is structurally intact and photochemically active. Visible absorption spectra of the samples were checked before and after infrared measurements on a Cary 14 spectrophotometer. Normally infrared spectra were taken of dried membrane samples, which were suspended in 99.96% ^2H_2O and the measurements repeated. In order to avoid H_2O exchange, ^2H_2O suspensions were made in an N_2 purged dry-box. A measured amount of ^2H_2O is added to the dried membrane and then a second AgCl window is used to form a sealed cell. The infrared spectrum of ^2H_2O-suspended samples was normally recorded within 5—10 min after addition of ^2H_2O. Bleaching of samples was made with five flashes from a Honeywell flashgun using a No. 12 Wratten filter (cut off below 500 nm) and gel infrared heat filter.

Infrared measurements

Infrared measurements were made with a dual beam Fourier transform infra-

red spectrometer (Digilab FTS-14, Cambridge, MA). The sample was placed in an N_2-purged compartment. Measurements were made from 400—4000 cm^{-1} at 4 cm^{-1} resolution using 50—100 scans of both the reference and sample beam. It takes approx. 2 min to measure an infrared spectrum under these conditions. This allowed peak assignments to be made with an accuracy of better than 1 cm^{-1}. In addition signal-to-noise ratio was better than 200 : 1 over most regions of interest except in the case of delipidated rhodopsin where higher noise was due to increased light scattering. Frequency calibration was made automatically with an internal He-Ne laser and was also checked using polystyrene film. Absorption spectra were determined by subtracting the logarithm of the sample beam intensity from that of the reference beam intensity to correct for the wavelength dependence of the output power of the infrared source. In some cases the absorption spectrum was then corrected by subtracting the absorption spectrum of the solvent (^2H_2O).

Results and Discussion

Rhodopsin structure and the effects of dehydration

In Fig. 1A (600—1800 cm^{-1}) and Fig. 2A (2700—3500 cm^{-1}) is shown the spectrum of dehydrated photoreceptor membrane prepared using the isopotential spin-dry technique. Many of the prominent peaks can be assigned to specific group vibrations on the basis of previous infrared studies of protein and lipid [16—22]. In particular, the amide II (1545 cm^{-1}), amide I (1675 cm^{-1}) and amide A (3295 cm^{-1}) vibrations of the protein peptide groups as well as the lipid ester carbonyl-stretch-vibration (1740 cm^{-1}) can be identified. Other assignments are listed in Table I. We will discuss some of these assignments on the basis of rhodopsin delipidation and reconstitution in a later section.

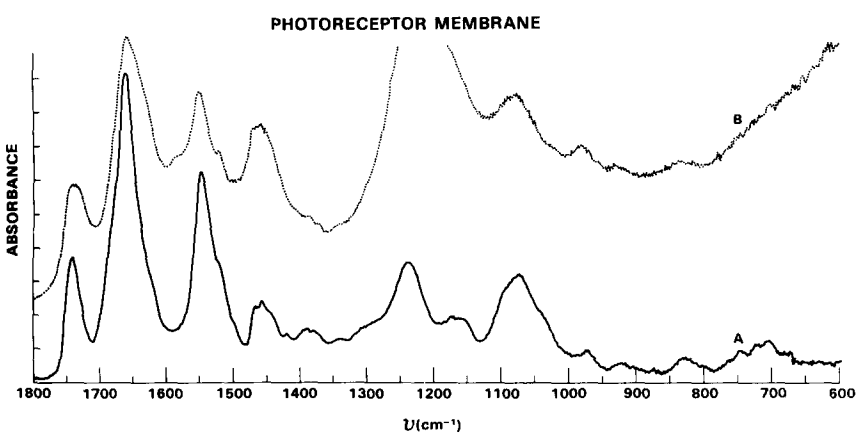


Fig. 1. Fourier transform infrared absorption spectrum of cattle photoreceptor membrane from 600—1800 cm^{-1} recorded at room temperature with spectral resolution of 4 cm^{-1}. Spectra were obtained with 100 scans of sample and 50 scans of reference beam (~2 min). (A) dehydrated sample prepared on AgCl windows using the isopotential spin-dry method. Sample had an optical density at 500 nm of 0.5 and at 1657 cm^{-1} of 0.53. (B) sample suspended in ^2H_2O and sealed between 2 AgCl windows. Spectrum was recorded 10 min after mixing with ^2H_2O in a N_2-purged dry-box. The large peak near 1260 cm^{-1} is due to ^2H_2O absorption.

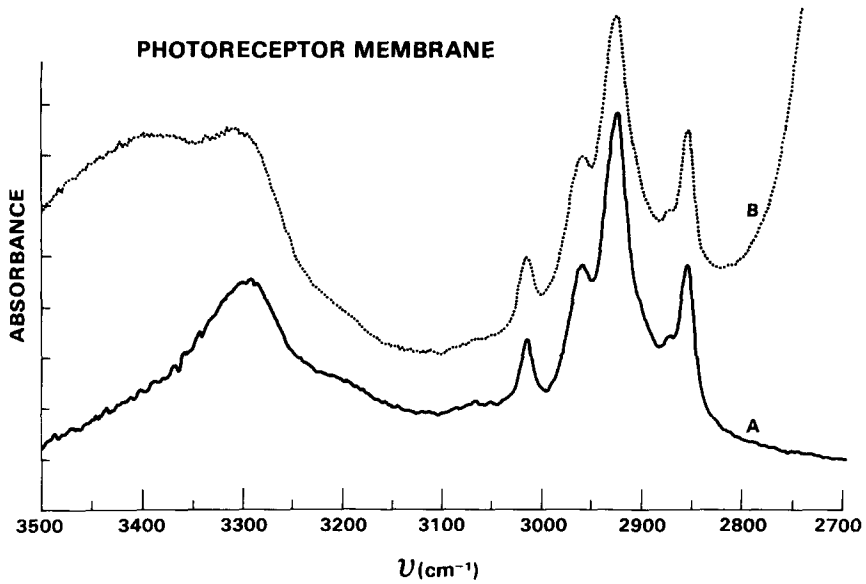


Fig. 2. Same as Fig. 1 from 2700—3500 cm^{-1}.

The outstanding feature of the spectrum of most preparations of isopotential spin-dried photoreceptor membrane is a relatively high amide II/amide I ratio which varies between 0.7 and 1.0 (cf. Fig. 3A and B). This contrasts with a ratio of 0.45 ± 0.05 found for most proteins and polypeptides [20], and 0.6 for rod outer segment membranes only partially oriented by sedimentation followed by lyopholization (cf. Fig. 3C). In the case of dried purple membrane a high amide II/amide I ratio (~1) is also found [5]. This has been shown to be a consequence of three factors: (1) the intrinsic infrared linear dichroism of the amide I and II vibrations [17]; (2) the predominance in bacteriorhodopsin of alpha-helices oriented perpendicular to the bilayer plane [23], and (c) the lamellar stacking induced by drying of purple membrane. In the case of photoreceptor membrane, we have demonstrated recently by measuring the visible dichroism at 500 nm that the isopotential spin drying technique leads to highly oriented smectic liquid-crystal films of photoreceptor membrane (Rothschild, K.J., DeGrip, W.J. and Sanches, R., unpublished data). Hence it is likely that the high amide II/amide I ratio observed in oriented photoreceptor membrane is due to a predominance of rhodopsin alpha-helices oriented perpendicular to the membrane plane.

A second indication that rhodopsin contains extensive alpha-helices is the peak frequency of the amide I vibration. Numerous studies indicate that a correlation exists between the frequency of the amide I vibration and the skeletal structure of polypeptides and proteins [16—20]. In particular, β-conformation produces an amide I peak near 1630 cm^{-1} and a weaker vibration near 1685 cm^{-1}. It is thus likely that there exists little β-conformation in rhodopsin. (The weak shoulder at 1680 cm^{-1} is probably due to Arg). In contrast, alpha-helical polypeptides and proteins have amide I frequencies

TABLE I

TENTATIVE ASSIGNMENT OF MAIN PEAKS

Wavenumber (cm^{-1})	Assignment
3295	N-H stretch associated (CONH amide A)
3200	N-H stretch associated (CONH amide B)
3015	C-H stretch (in -C=C-)
2960	C-H stretch (CH_3, antisymmetric)
2925	C-H stretch (CH_2, antisymmetric)
2872	C-H stretch (CH_3, symmetric)
2855	C-H stretch (CH_2, symmetric)
1740	C=O stretch (in esters)
	Glu, Asp
1680	Arg (guanidium modes)
1657	C=O stretch (CONH amide D)
	C=C stretch (*cis* only)
1545	N-H bend (CONH amide II)
1520	Tyr (modes of *p*-disubstituted benzene ring)
1500	Lys (NH_3^+ deformation modes)
	Phe (mode of monosubstituted benzene ring)
1467	C-H scissor (in CH_2)
1457	C-H bend (CH_3, antisymmetric)
1445	C-H scissor (in CH_2)
	C-H bend (CH_3, antisymmetric)
	Ala
1420	C-H scissor (in CH_2 next to C=O)
1390	CO_2^- stretch (symmetric)
	C-H bend (CH_3 symmetric)
	Asp
1310	C-H wag (in CH_2)
	C-H in plane deformation (in *trans* -C=C-)
1245	N-H bend (CONH amide III)
1237	P=O stretch (PO_2^-, antisymmetric)
	C-O-C stretch (antisymmetric)
1172	C-O-C stretch (symmetric)
1165	$(CH_3)_2$-C skeletal vibration
	Val, Leu
1157	C-C skeletal stretch
1112	N-H bend (?)
1095	P=O stretch (PO_2^-, symmetric)
1070	P-O-C stretch
1045	C-N stretch
1040	C-O stretch (in hydroxyl groups)
970	C-H bend (in *trans* -C=C-)
	$C\text{-}C\text{-}N^+$ stretch
955	
920	
875	
825	
775	C-H rock (in CH_2)
742	
720	
700	N-H bend (out of plane; amide V)
	C-H bend (in *cis* -C=C-)

ranging from 1650—1657 cm^{-1} [16—20]. Hence, the 1657 cm^{-1} peak in rhodopsin may be due to alpha-helices. However, caution must be taken, since unordered conformations such as found in α_s-casein and denatured β-lacto-

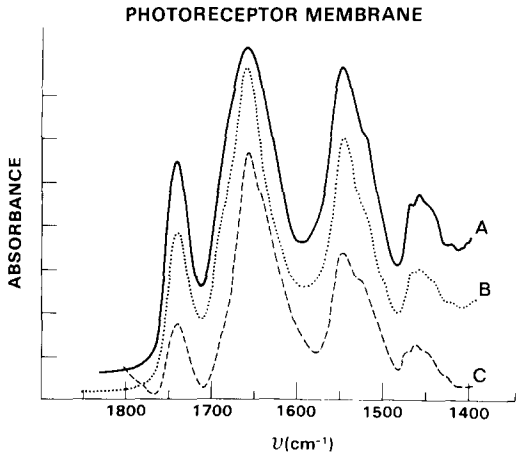


Fig. 3. Fourier transform infrared spectra of photoreceptor membrane prepared using (A and B) the isopotential spin-dry technique and (C) by sedimentation followed by lyophilization. The highest amide II/amide I ratio (~1) is found for sample A. Recording conditions were the same as in Fig. 1. The frequency of the amide I and II bands are 1657 and 1545 cm^{-1}, respectively.

globulin A also display an amide I frequency near 1657 cm^{-1} [17,18].

One means of distinguishing between unordered conformation and tightly folded alpha-helical conformation such as in myoglobin is to measure the amide I frequency shift when the protein is exposed to ^2H_2O. Upon deuteration the amide I frequency of myoglobin shifts down 2—3 cm^{-1} whereas deuterated α_s-casein exhibits a shift to 1643 cm^{-1}. We find that when bleached photoreceptor membrane is exposed to ^2H_2O buffer solution for 12.5 h, a marked reduction occurs in the intensity of the amide II vibration indicating 75% of the amide groups are deuterated. However, the maximum of the amide I peak shifts only 3 cm^{-1} from its original frequency. Deuteration of unbleached photoreceptor membrane results also in a small amide I shift. These measurements are consistent with a predominantly alpha-helical rhodopsin conformation in agreement with a previous non-resonance Raman scattering study [9] and an infrared study [10].

It is possible using Fourier transform infrared spectroscopy to observe the initial deuteration of rhodopsin. Fig. 4 shows that within 6 min of the addition of ^2H_2O, a shoulder is present at 1630 cm^{-1}. The presence of this shoulder can be seen in Fig. 1A and B by comparing the amide I peaks in the dehydrated sample and ^2H_2O suspension, respectively. Based on the reduction of the amide II peak intensity we estimate appox. 10% of the amide hydrogens have exchanged in this time period. It is likely that this 10% represents rapidly deuterating unordered rhodopsin structure. In contrast, continued deuteration leads to further reduction of the amide II peak, but little additional change is found in the amide I peak except for a small shift of 2 cm^{-1} to lower frequency (cf. Fig. 4B). This indicates that the bulk of the rhodopsin structure is more characteristic of alpha-helices which slowly deuterate.

A comparison of other regions of the infrared spectrum for dehydrated and ^2H_2O-suspended photoreceptor membrane (cf. Figs. 1A, B and 2A, B) which

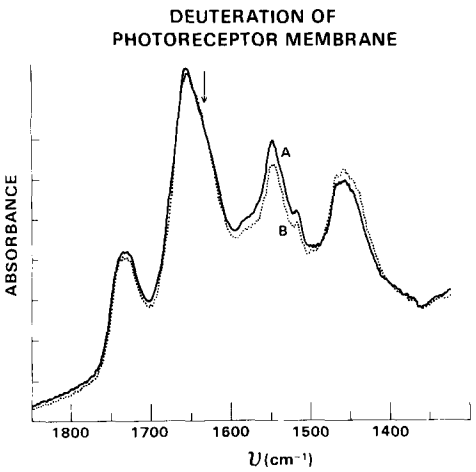


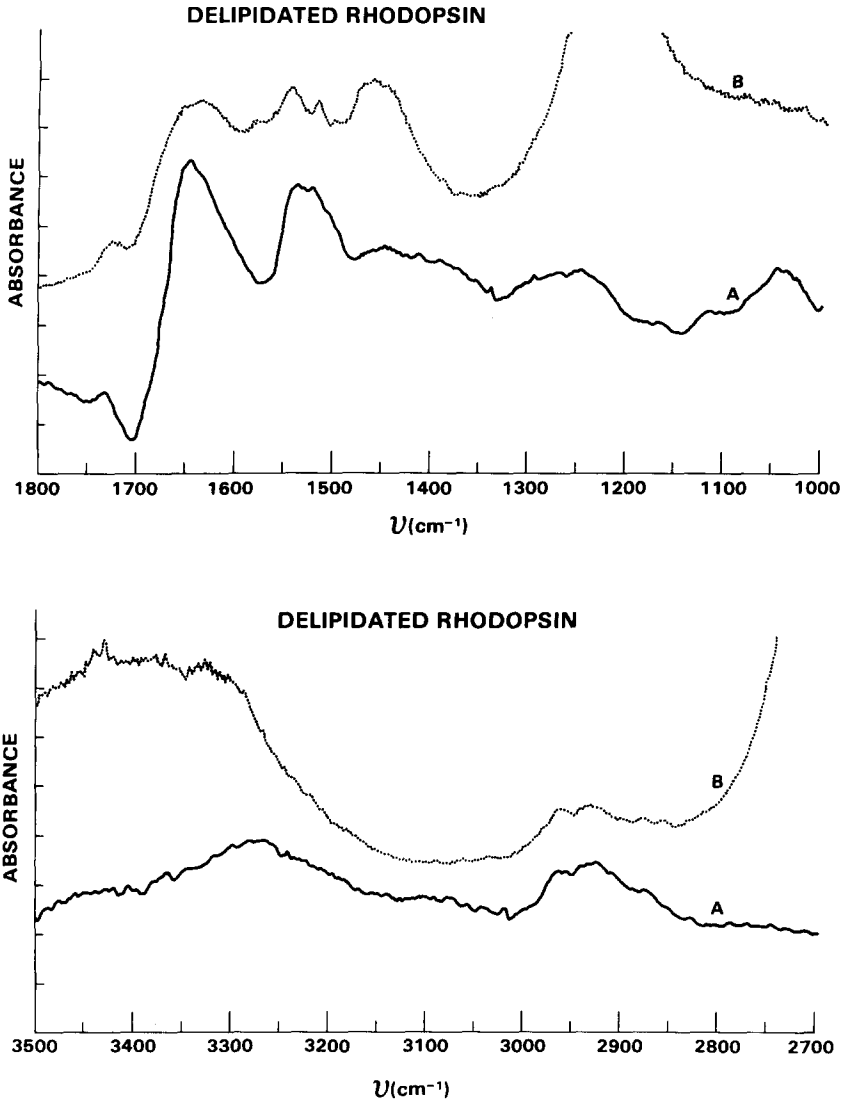
Fig. 4. Infrared absorption spectrum of photoreceptor membrane suspended in ^2H_2O. (A) spectrum recorded 6 min after ^2H_2O was added to sample (solid line); (B) spectrum recorded after 31 min of deuteration (dotted line).

should not be effected by deuteration reveals only small differences. For example, the 1740 cm^{-1} ester carbonyl stretching vibration is broader in ^2H_2O. This is likely to be due to solvating effects which the ^2H_2O exerts on the phospholipid head groups. The absence of other differences indicates that no major conformational change occurs when the unbleached photoreceptor membrane is dried, in agreement with other spectroscopic criteria [24,25].

Delipidated rhodopsin and the effects of dehydration

Fully delipidated rhodopsin was dehydrated using the isopotential spin-dry technique and the infrared spectrum recorded (Figs. 5A and 6A). The spectrum is noisy since the delipidated rhodopsin does not orient but forms a highly scattering powder. While the amide I and II peaks are still dominant, the amide II/amide I ratio is lower, probably due to lack of orientation. In addition, the ester carbonyl stretch is reduced in intensity relative to the native photoreceptor membrane due to the removal of lipids. The remaining absorption at 1735 cm^{-1} is due to carboxylate ($-COO^-$) groups of the Glu and Asp residues of rhodopsin. Other peaks absent or reduced in intensity due to delipidation are the $-POO^-$ symmetric stretch vibration (1095 cm^{-1}) and the symmetric CH stretch vibration (2855 cm^{-1}).

Evidence of conformation differences between delipidated rhodopsin and native rhodopsin can be surmised from a shift in the frequency of the amide II peak to 1535 cm^{-1} relative to 1545 cm^{-1} in the native photoreceptor membrane. In addition the amide I appears at lower frequency and is somewhat broader. These differences would suggest a change towards more disordered conformation. In contrast to native photoreceptor membrane, addition of ^2H_2O to dried delipidated rhodopsin leads to an apparent shift of the amide II to 1545 cm^{-1} (Fig. 5B). It is not clear, however, if this shift is real since an appreciable amount of delipidated rhodopsin is deuterated after only 20 min as



Figs. 5 and 6. Infrared spectra of delipidated rhodopsin on AgCl window. (A) dehydrated sample (solid line); (B) sample suspended in ^2H_2O for 20 min (dotted line).

determined by the marked reduction of the amide II intensity (the tyrosine vibration at 1515 cm^{-1} now appears only slightly smaller than the amide II vibration). Hence the 1545 cm^{-1} peak may represent residual amide II intensity which is hidden by the more intense amide II peak at lower frequency in the spectrum of dried delipidated rhodopsin (cf. Fig. 5A). The rapid deuteration of delipidated rhodopsin in contrast to the slower deuteration of rhodopsin in native photoreceptor membrane (cf. Fig. 4) indicates a much greater accessibility of the amide hydrogens for exchange. This may be due to increased accesibility of the rhodopsin amide groups to ^2H_2O either because of a con-

formational change or removal of lipids which might normally shield the protein amides. A similar increase in deuteration has been observed in detergent micelles subsequent to bleaching [11].

Reconstituted rhodopsin membrane

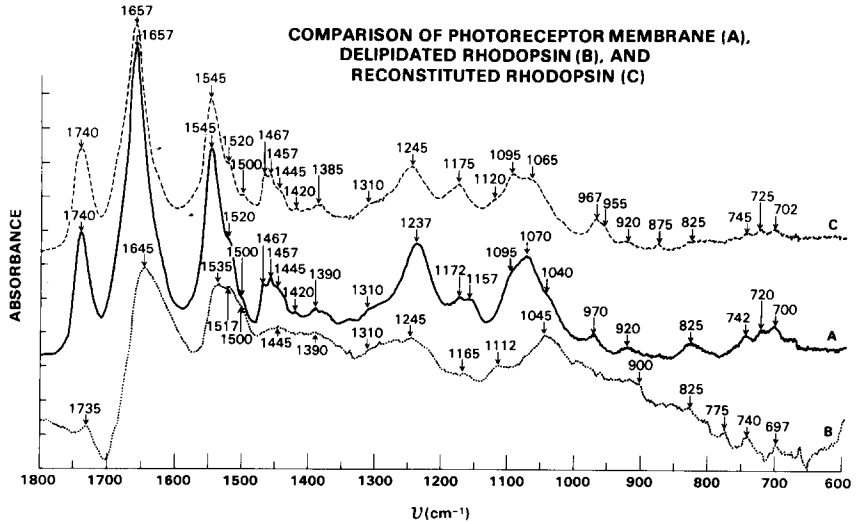
As described in the Methods and Procedures section, delipidated rhodopsin was reconstituted with dioleyl-phosphatidylcholine. The infrared spectrum of these dehydrated membranes is shown in Figs. 7C and 8C. While the delipidated rhodopsin spectrum differs significantly from the native photoreceptor membrane, particularly at the amide I and II peaks, the amide peaks of native and reconstituted rhodopsin appear almost identical. In fact, only peaks associated with lipids such at 1467 cm^{-1}, 1095 cm^{-1} and 3010 cm^{-1} exhibit change indicating that reconstituted rhodopsin is conformationally equivalent to native rhodopsin. Hence any changes occurring due to delipidation must be completely reversible. This strongly reinforces biochemical parameters such a regenerability, and SH availability which also suggests reversibility.

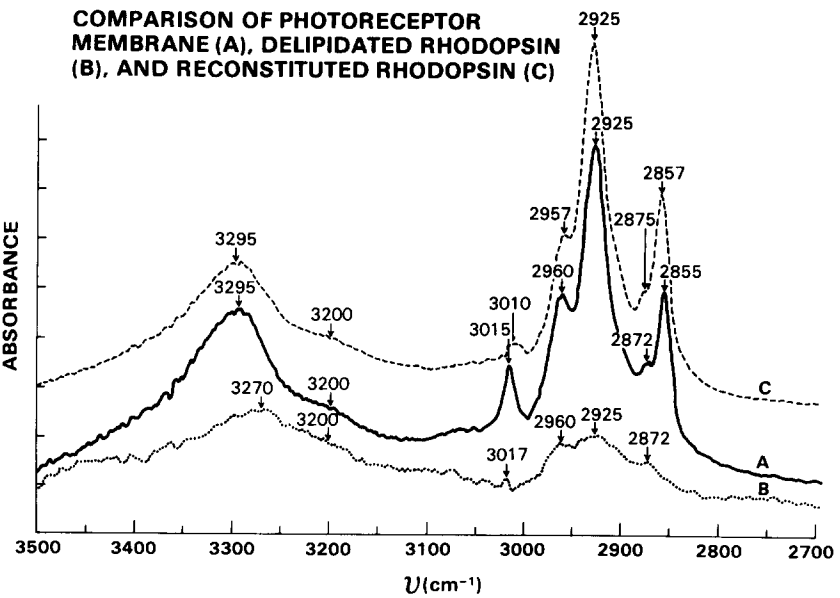
Comparison of infrared spectra from photoreceptor membrane, delipidated rhodopsin and reconstituted rhodopsin membranes

Figs. 7A, B and C and 8A, B and C show the infrared spectra of dried photoreceptor membrane, delipidated rhodopsin and reconstituted rhodopsin membranes. Comparison of these spectra enables separation of protein and lipid vibrations as well as providing an indication of possible conformation differences which might exist between protein and lipid components in the different samples.

Protein. We find evidence for some conformational change when rhodopsin is removed from the membrane. In contrast, the rhodopsin conformation is similar in native photoreceptor and reconstituted membranes. As discussed previously, this evidence is based on a comparison of the major peptide vibrations. In particular, in both the native and reconstituted membrane, the amide A, I and II vibrations of the peptide groups are observed at 3295, 1657 and 1545 cm^{-1}, respectively. In contrast, dried delipidated rhodopsin displays amide A, I and II frequencies at 3270, 1645 and 1535 cm^{-1}, respectively. This could indicate secondary structural change and is more characteristic of disordered or even β-structure. As noted a conformational change towards greater disorder may account for the increased deuteration rate of delipidated rhodopsin.

It is possible by comparing the spectra in Figs. 7A, B and C and 8A, B and C to unambiguously assign peaks to either protein or lipid vibrations. For example, the small shoulders at 1520 cm^{-1} and 1500 cm^{-1} in the photoreceptor membrane spectrum are clearly due to rhodopsin and are assigned to the Tyr ring mode [26] and the Lys NH deformation stretch, respectively. Other prominent protein vibrations include the amide B mode (3200 cm^{-1}), the amide III mode (1245 cm^{-1}) and a peak of unknown origin at 1045 cm^{-1} (this may be due to a C-N stretch mode). It is interesting to note that the amide III peak is entirely hidden in the native infrared spectrum of photoreceptor membrane by the intense P=O stretch mode at 1237 cm^{-1}. The same is true of the Asp, and Glu ester C=O vibrations appearing at 1735 cm^{-1} in the spectrum of





Figs. 7 and 8. Infrared absorption spectra of (A) photoreceptor membrane, (B) delipidated rhodopsin and (C) reconstituted rhodopsin; all prepared using the isopotential spin-dry method on AgCl cell.

delipidated rhodopsin. The spectrum of delipidated rhodopsin is somewhat noisy below 1000 cm^{-1} and hence the peak frequency is uncertain. However, by comparing several spectra of different samples, protein peaks near 920, 825 and 740 cm^{-1} are found, which agrees with peaks in the spectrum of the native and reconstituted membranes.

Lipids. Differences in the 2700—3100 cm^{-1} region of spectra 8A, B and C reflect largely the differences in the fatty acids and protein residues such as

valine and leucine of the different samples. For example, delipidation is expected to lower the CH content of a membrane. In agreement, the CH peaks intensity relative to the amide A (NH stretch) intensity at 3295 cm^{-1}, is lowest in the spectrum of delipidated rhodopsin. Delipidated rhodopsin also displays several other distinct differences from the native and reconstituted membranes. In particular, there is a relative increase in dominance of the 2960 cm^{-1} and 2872 cm^{-1} vibrations, and a disappearance of the 2855 cm^{-1} peak. These differences can be understood if one considers the increased proportion in the delipidated rhodopsin, compared to the native and reconstituted membranes, of CH from the terminal methyl groups (CH_3). Since the 2960 cm^{-1} peak is assigned to terminal methyl groups, we expect this vibration to be more prevalent in delipidated rhodopsin. In contrast the 2855 cm^{-1} peak is due to CH_2 groups and is only seen in the native and reconstituted membrane. Finally the 2872 cm^{-1} peak is again due to vibrations of the terminal methyl groups and its presence as a shoulder in the spectra of native and reconstituted membranes is largely due to protein contributions. The 3015 cm^{-1} peak in the spectrum of native photoreceptor membrane is assigned to CH stretching in double-bonded carbons. Since photoreceptor membrane lipids are highly unsaturated, with almost 50% of the lipid-acyl chains containing six double bonds [27] relative to dioleyl-phosphatidylcholine which contains one double bond per acyl chain, this peak appears more intense in native photoreceptor membrane (cf. Fig. 8A).

Other group assignments are indicated in Table I. In particular, the 1740 cm^{-1} peak is due predominantly to ester carbonyl stretching, but its presence in delipidated rhodopsin is most likely due to the carbonyl groups in Glu and Asp residues. The strong 1237 cm^{-1} vibration due to PO_2^- appears to cover a weaker protein vibration found in delipidated rhodopsin.

Conclusions and Perspectives

Infrared absorption measurements on photoreceptor membrane indicate that rhodopsin contains extensive alpha-helical structure but little or no β-structure. This is in substantial agreement with other biophysical studies such as Raman light scattering [9] and circular dichroism [28,29]. In addition, the apparent change in the amide II/amide I ratio towards 1 in highly oriented samples, indicates that the alpha-helices are predominantly oriented perpendicular to the membrane plane as found in bacteriorhodopsin, and indicated by diamagnetism of rod outer segments [30]. The presence of at least 10% random structure can be deduced from the rapid shifts in the amide I peak which occurs within 5 min of initial exposure of the membranes to ^2H_2O. However, since subsequent deuteration does not lead to an appreciable change in the amide I peak, it is likely that a high percentage of the rhodopsin structure is alpha-helical in agreement with a previous infrared study [10], but contrary to a reports based on tritiation exchange kinetics [31,32].

We also find from our data that rhodopsin undergoes some structural change when lipids are removed completely. This conformational change might be related to the inability of delipidated rhodopsin to bleach beyond the Meta I intermediate [33,34,35]. In contrast, we find no evidence for conformational

differences of rhodopsin in the reconstituted and native membrane. This is further evidence that any conformation changes which might be caused by delipidation are reversed upon reconstitution. It is therefore likely that lipids are essential for maintaining the native rhodopsin structure. We can also conclude from our studies that dehydration does not lead to any major changes in the rhodopsin structure. This agrees with spectroscopic criteria [4,25]. Further evidence that photoreceptor membranes are not irreversibly altered by dehydration is found from chemical and photochemical regeneration of dried oriented layers of reconstituted photoreceptor membrane [25,26]. This is an indication that the lipid environment of rhodopsin even in the dehydrated state protects it from any major structural rearrangement in contrast to soluble proteins which are often denatured upon drying. A similar conclusion has been reached based on infrared measurements in the case of dehydrated purple membrane (Rothschild, K.J., DeGrip, W.J. and Sanches, R., unpublished data). This does not of course exclude the possibility that small conformation changes occur upon dehydration. In fact, such changes have recently been measured using Fourier transform infrared difference spectroscopy and will be discussed in detail in a future paper.

In conclusion, we find the comparison of infrared spectra from delipidated and reconstituted membrane protein to be an essential step in using infrared spectroscopy as an effective tool for studying membrane structure. We have been able with this method to identify both lipid and protein peaks associated with specific groups. It will now be interesting to study the effects of pH, ionic composition, lipid composition, temperature and other factors on specific membrane groups. The demonstration that extremely low noise infrared spectra can be obtained in less than 1 min permits the study of the relatively slow changes, such as the transition to Meta II, while earlier photolytic intermediates are accessible by lowering the temperature or blocking the photolytic cycle.

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