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BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS

XXXVIII. EFFECTS OF LATERAL AGGREGATION ON RHODOPSIN IN PHOSPHOLIPASE C-TREATED PHOTORECEPTOR MEMBRANES

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Summary

Treatment of bovine rod outer segments with phospholipase C leads to largely lipid-depleted membranous structures. Under these conditions rhodopsin remains spectrally intact, but its thermal stability and regeneration capacity are decreased, whereas upon illumination the metarhodopsin I to II transition is blocked. These observations can be explained on the basis of the previously demonstrated lateral aggregation of rhodopsin molecules which, on the one hand leads to a (partial) shielding of these molecules and, on the other hand, might impose constraints on the flexibility of the molecule to undergo light-induced conformational changes.

Upon reconstitution of these lipid-depleted preparations with amphipathic lipids by means of a detergent dialysis procedure, the aggregates are apparently rearranged to lipid bilayer structures with complete recovery of the original rhodopsin properties. Under our conditions the nature of the polar head groups and the fatty acids is not critical in this respect. Simple addition of amphipathic lipids, without the use of detergent, restores the rhodopsin properties only in the case of rod outer segment lipids and of didecanoylphosphatidylcholine, and even then only occasionally.

These results are discussed in the light of the strong analogy in properties between phospholipase C-treated rod outer segment membranes and lipid- and detergent-free rhodopsin obtained by affinity chromatography. It is concluded that rhodopsin must be in a freely dispersed state in order to function properly. Apparently, a non-specific lipid bilayer fulfills this condition for the regeneration capacity, whereas normal photolytic behaviour requires, in addition, a minimal membrane fluidity according to the observations of other investigators. Presumably, the uniquely high phospholipid unsaturation of rod outer segment membranes is important for another, as yet unassessed, function of rhodopsin or the photoreceptor membrane.

Introduction

Previously we have shown that phospholipase C treatment of bovine photoreceptor membranes leads to extensive delipidation [1], while membranous structures are still retained [2]. Rhodopsin remains spectrally completely intact and its molecules are apparently organized as lateral aggregates with relatively few phospholipids left in the membranous structures. Therefore, the microenvironment of the rhodopsin molecules in these preparations is considerably altered from that in the native membrane.

In view of reports [3,4] on the influence of the microenvironment on the properties of rhodopsin, it seemed of interest to study various properties of rhodopsin in phospholipase C-treated rod outer segment membranes. This technique is especially attractive since enzymatic hydrolysis represents a very mild way to reduce the amount of amphipathic lipids in membranes in a graded way. All other methods for removing lipids from outer segment membranes require solubilization in detergent with the inherent danger of detergent-induced artifacts, even after the detergent has been removed.

Rhodopsin in phospholipase C-treated membranes behaves very similarly to completely lipid- (and detergent-) free rhodopsin with regard to thermal stability, photolytic sequence and regeneration capacity. In combination with the evidence from electronmicroscopic observations and membrane reconstitution experiments, the results strongly suggest that rhodopsin must be in a freely dispersed state in order to function properly.

Materials and Methods

Materials

Concanavalin A Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden), α-methylmannopyranoside from Calbiochem (San Diego, U.S.A.). Dodecyltrimethylammonium bromide (DTAB; Eastman, Rochester, U.S.A.) was recrystallized from ethylacetate/ethanol before use. Dioleoyl- and didecanoyl-phosphatidylcholine and oleoylglycerides were obtained from Serdary Research Laboratories (London, Canada). Monogalactosyldiglyceride from *Bifidobacterium Bifidum* was a gift of Dr. J.H. Veerkamp. Most other phospholipids were purchased from Lipid Products (South Nutfield, England). Phosphatidic acid was obtained from pure egg phosphatidylcholine by phospholipase D treatment. The purity of the phospholipids is checked by thin layer chromatography.

Phospholipase C (EC 3.1.4.3) was purified by the method of Otnaess et al. [5], and is free of proteolytic activity, where 11-*cis* retinaldehyde was isolated essentially according to Brown and Wald [6]. All other chemicals are of the highest purity available.

Isolation of rod outer segment membranes

Cattle rod outer segment membranes are isolated according to de Grip et al. [7]. After two washings with water, the prearation shows an A_{278}/A_{500} ratio of 2.3—3.0 and an A_{400}/A_{500} ratio of 0.20—0.25. It contains 60—70 mol of phospholipid per mol rhodopsin.

Phospholipase C treatment

Rod outer segment membrane suspensions (2 mg/ml) in 0.2 M Tris-maleate (pH 7.0) are incubated at room temperature for 1 h with 0.25 units of phospholipase C (*Bacillus cereus*) per nmol of rhodopsin. About 90% of the membrane phospholipids are hydrolyzed to diglycerides and phosphate esters during this incubation, as measured by phosphate determinations in the supernatants. Residual phospholipid is mainly phosphatidylserine. Rhodopsin remains spectrally completely intact. Intermediate degrees of phospholipid hydrolysis are obtained by shorter incubation periods, after which the enzyme activity is inhibited by addition of 10 mM EDTA and cooling to 0°C. After incubation the suspensions are washed and resuspended in 0.1 M phosphate buffer (pH 6.5).

Isolation of lipid- and detergent-free rhodopsin

Cattle rod outer segment membranes are solubilized in 100 mM dodecyltrimethylammonium bromide and subjected to affinity chromatography over a concanavalin A-Sepharose 4B column, with elution by means of α-methylmannose, as described by van Breugel et al. [8]. The resulting rhodopsin solutions show an A_{278}/A_{500} ratio of 1.6—1.8 and an A_{400}/A_{500} ratio of 0.18—0.23. Removal of detergent by dialysis (see next paragraph) yields rhodopsin, which appears to be pure upon polyacrylamide gel electrophoresis in sodium dodecyl sulfate and contains less than 0.3 mol phosphorus per mol of rhodopsin. The recoveries are in the order of 80%.

Preparation of reconstituted lipid-rhodopsin membranes

Preparation of reconstituted lipid-rhodopsin membranes, both from phospholipase C-treated membranes and from lipid- and detergent-free rhodopsin is performed in 300 mM dodecyltrimethyl ammoniumbromide, followed by exhaustive dialysis against *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid buffer, in the presence of 1 mM dithioerythritol and 1 mM EDTA, according to Hong and Hubbell [3]. The lipid/rhodopsin molar ratio is in all experiments about 100 : 1. Under these circumstances rhodopsin is incorporated into a lipid bilayer, as is apparent from freeze-fracture electronmicroscopic observations (see also ref. 9).

Attempts to recombine either phospholipase C-treated membranes or pure rhodopsin with lipids in the absence of detergent are carried out in 0.1 M phosphate buffer (pH 6.5) with and without 20 mM $MgCl_2$ by adding a suspension of the rhodopsin preparation to a dry lipid film in a test tube. The mixture is vigorously shaken (15 min at room temperature) and sonicated at 0°C (3 × 3 s) to obtain homogeneous suspensions, which are left at room temperature for at least 1 h before measuring their characteristics.

Rhodopsin determination

Rhodopsin is determined by measuring the decrease in 500 nm absorbance upon illumination by a 300 W tungsten lamp for 10 min at a distance of 30 cm behind 3 mm thick OG_{475} and infrared KG_1 filters (Schott-Jena, Mainz, G.F.R.) in the presence of 50 mM NH_2OH. Absorbance measurements are either made in suspension in a Rapid T3 spectrophotometer (Howaldtswerke-Deutsche

Werft, Kiel, G.F.R.), or after solubilization in Triton X-100 (final concentration 1% in a Zeiss PMQ II spectrophotometer.

Absorbance spectra

Absorbance spectra of suspensions or solutions are recorded from 600 to 320 nm on the Rapid T3 double-beam spectrophotometer with a scanning speed of 4 or 6 nm/s. This instrument permits the use of suspensions, since the thermostated cuvette is placed immediately in front of an integrating sphere-multiplier system. The recordings have not been corrected for residual scattering. The metarhodopsin II to metarhodopsin III transition is measured after illumination with a Rollei Strobofix E60 flash lamp through an OG_{530} filter. Illumination with a tungsten source for 30 s through an OG_{530} filter gives the same results.

Determination of thermal stability

The thermal stability of the absorbance spectrum of rhodopsin is measured by following the 500 nm absorbance at various temperatures and is expressed as the temperature at which 50% of rhodopsin is spectrally denatured in 10 min. Samples are introduced in thermostated, preheated tubes, and aliquots are taken at five or more time intervals and frozen immediately. The 500 nm absorbance is measured at 10° C after addition of Triton X-100 and buffered NH_2OH to final concentrations of 1% and 50 mM, respectively. The absorbance decay at 500 nm consistently follows first order kinetics.

Determination of regeneration capacity

The rhodopsin preparation is illuminated, as specified under Rhodopsin determination. It is then incubated in darkness at room temperature with a 5-fold molar excess of 11-*cis* retinaldehyde for 1.5 h in 0.1 M phosphate buffer (pH 6.5), containing 24% glycerol in order to improve the homogeneity of the suspensions. Incubation for longer periods of time never resulted in additional rhodopsin formation. Rhodopsin determinations are carried out before (A) and after (B) photolysis and after incubation with 11-*cis* retinaldehyde (C). The regeneration capacity is expressed as $(C - B)/(A - B) \times 100\%$.

A complication might arise with phospholipase C-treated membranes and lipid- and detergent-free rhodopsin, where photolysis results in a rather stable 480 nm pigment (see Results). However, it appears that regeneration with 11-*cis* retinaldehyde can occur from this photointermediate. If NH_2OH is added during photolysis, the 480 nm intermediate is rapidly decomposed to retinylidene oxime (λ_{max} = 360 nm) and excess NH_2OH can be removed by washing and centrifugation. The regeneration capacity of this sample is the same as found with the standard procedure without the use of NH_2OH.

Results

Absorbance spectrum

Determination of difference spectra before and after illumination in the presence of 50 mM hydroxylamine invariably shows an unchanged absorbance maximum at 498 nm in all rhodopsin preparations used in this study: native photoreceptor membranes, phospholipase C-treated membranes, lipid- and detergent-free rhodopsin and reconsituted lipid-rhodopsin membranes.

TABLE I

THERMAL STABILITY OF THE 500 nm ABSORBANCE OF RHODOPSIN PREPARATIONS

Results are expressed as the temperature at which 50% loss of absorbance occurs in 10 min in 0.1 M phosphate (pH 6.5).

Preparation	$T_{50\%}$ (°C)
Rod outer segment membranes	70.1
Phospholipase C-treated rod outer segment membranes	58.9
Lipid- and detergent-free rhodopsin	52.8
Egg phosphatidylcholine-rhodopsin reconstituted membranes *	70.1

* Molar ratio phosphatidylcholine : rhodopsin = 66 : 1.

Thermal stability

Treatment of rod outer segment membranes with phospholipase C (90% phospholipid hydrolysis) decreases the thermal stability of rhodopsin (Table I). Complete removal of all lipids by affinity chromatography and detergent dialysis, yielding lipid- and detergent-free rhodopsin, causes an even greater decrease in thermal stability. A complete recovery of the thermal stability is observed, when reconstitution with egg phosphatidylcholine is performed. The thermal stability of rhodopsin in native rod outer segment membranes is found to be 70.1° C, which agrees fairly well with the value of 72.6° C reported by Hubbard [10] for bovine rod outer segments.

Photolytic behaviour

The change in the absorbance spectra upon illumination is shown in Fig. 1 for three rhodopsin preparations: A, native rod outer segment membranes; B, phospholipase C-treated membranes and C, lipid-rhodopsin recombinant membranes. Curve 1 in each case represents the situation before illumination, curves 2, 3 and 4 have been recorded 0.5—1 min, 7 min (for C, 4 min), and 30 min (for C, 15 min) after illumination, respectively. Then NH_2OH is added in a final concentration of 50 mM in order to obtain complete conversion of all photo-intermediates to retinylidene oxime (λ_{max} = 360 nm), and curve 5 is recorded. In the bottom part of Fig. 1 the difference spectra for curves 2 and 1 and for curves 4 and 2 are plotted. The amount of rhodopsin photolyzed during the first illumination is calculated by tracing the spectrum after a second complete illumination (curve not shown).

In the case of rhodopsin in native rod outer segment membranes (Fig. 1A) the first recording after photolysis is dominated by the disappearance of rhodopsin (λ_{max} = 498 nm) and the appearance of metarhodopsin II (λ_{max} = 380 nm). Between 1 and 30 min after photolysis the dominant transition is from metarhodopsin II to metarhodopsin III (λ_{max} = 455 nm). The half time of the metarhodopsin II decay, which shows first-order kinetics, is 5.5 min at 25° C and pH 6.5 (Van Breugel, to be published). Formation of retinol is not observed, since the appropriate coenzyme (NADPH) for retinol dehydrogenase is absent.

A completely different picture is obtained with membranes treated exhaustively with phospholipase C (Fig. 1B). In the first minute after illumination a photo-intermediate with λ_{max} of about 480 nm appears, which slowly decom-

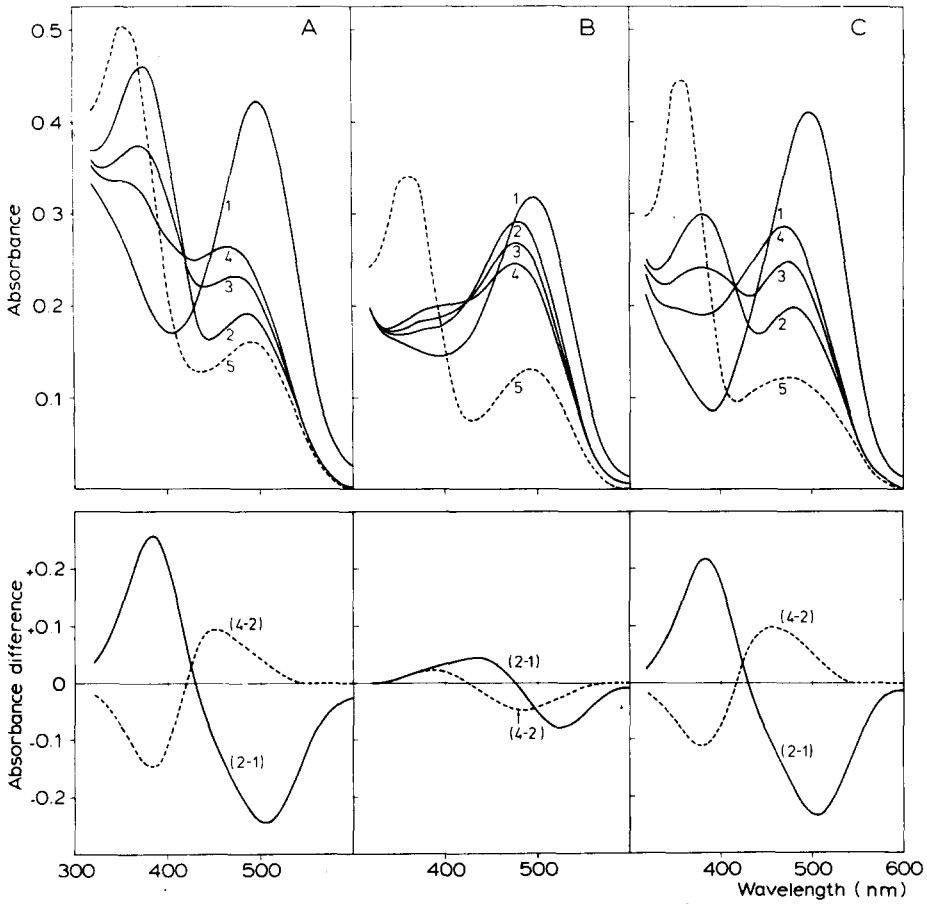

Fig. 1. Absorption spectra of various rhodopsin preparations at 25°C in 0.1 M phosphate, pH 6.5. Illumination during 30 s with a tungsten source through an OG_{530} filter to cut off wavelengths below 480 nm. A. Rod outer segment membrane suspension. B. Suspension of rod outer segment membranes exhaustively treated with phospholipase C (90% phospholipid hydrolysis). C. Suspension of rhodopsin reconstituted with egg phospholipids (70% phosphatidylcholine; 15% phosphatidylethanolamino and 15% phosphatidylserine. Molar ratio rhodopsin:phospholipid, 1:100). Upper figures: Curve 1 is taken before illumination. The curves 2, 3 and 4 are scanned starting at about 1 min, 7 min (for C, 4 min) and 30 min (for C, 15 min) after illumination started, respectively. Curve 5 is in the presence of 50 mM hydroxylamine added after illumination. Lower figures are difference spectra obtained by subtracting curves as indicated.

poses to a species with λ_{max} = 380 nm. The half time of the latter transition is greater than 5 min at 25°C (pH 6.5). Exactly the same behaviour is shown by suspensions of lipid- and detergent-free rhodopsin, obtained by affinity chromatography. The photoproduct with λ_{max} = 380 nm is probably free retinaldehyde, since there is no bathochromic shift upon acidification as would be expected for an aldimine of retinaldehyde. The absorbance gain at 380 nm is substantially less than the loss at 480 nm. Since there is an isobestic point at 425 nm, this cannot be due to trivial loss of chromophore, e.g. by oxidation. In addition, the chromophore absorbance is regained at 360 nm by treatment with hydroxylamine (Fig. 1B, curve 5). Below 40% hydrolysis of membrane

TABLE II

RESTORATION OF THE APPEARANCE AND SUBSEQUENT DECAY OF METARHODOPSIN II AT 25°C IN 0.1 M PHOSPHATE (pH 6.5)

+, Restoration; —, no restoration; ±, restoration occasionally observed; n.d., not determined.

	Reconstitution (detergent dialysis procedure)		Simple addition of lipids	
	Phospholipase C-treated membranes	Lipid- and detergent-free rhodopsin	Phospholipase C-treated membranes	Lipid- and detergent-free rhodopsin
Egg phosphatidylcholine	+	+	—	—
Dioleoylphosphatidylcholine	+	+	—	—
Didecanoylphosphatidylcholine	+	+	±	±
Egg phosphatidylethanolamine	+	+	—	—
Bovine phosphatidylserine *	+	+	—	—
Phospholipid mixtures **	+	+	n.d.	—
Phosphatidic acid	n.d.	+	n.d.	—
Monogalactosyldiglyceride	n.d.	+	n.d.	—
Rod outer segment lipids	+	+	±	—
1-Monooleoylglyceride	—	—	—	—
1,2-Dioleoylglyceride	—	—	—	—
Trioleoylglyceride	—	—	—	—

* From spinal cord.

** Mixtures of egg phosphatidylcholine and bovine phosphatidylserine (molar ratios 90 : 10 and 50 : 50) as well as of egg phosphatidylcholine, egg phosphatidylethanolamine and bovine phosphatidylserine (molar ratios, 70 : 15 : 15 and 45 : 45 : 10).

phospholipid the normal photolysis pattern of Fig. 1A is seen. With increasing phospholipid hydrolysis it gradually developes to the pattern of Fig. 1B.

Addition of detergent to photolyzed suspensions of phospholipase C-treated membranes or of lipid- and detergent-free rhodopsin, containing substantial amounts of the 480 nm pigment, largely restores the appearance of later photointermediates as observed when native rod outer segments membranes are photolyzed in the same detergent.

Reassembly of rhodopsin and lipids by means of the detergent dialysis procedure restores the original photolysis pattern (Fig. 1C). This is true both when phospholipase C-treated membranes or lipid- and detergent-free rhodopsin are used. The effect is observed with a variety of amphipathic lipids, as listed in Table II. Attempts to reconstitute with mono-, di- or tri-oleoylglycerides are unsuccessful in restoring the original photolytic behaviour.

Whenever metarhodopsin II is formed in reconstituted preparations, its decay consistently obeys first-order kinetics. The half-time of the decay (pH 6.5, 25°C), is generally decreased, maximally by a factor of two, as compared to native rod outer segment membranes, but the usual decay products (metarhodopsin III and retinaldehyde) appear. However, the relative amounts of these products do, to some extent, depend on the nature of the polar headgroups of the lipids used in the reconstitution (Van Breugel, to be published).

Attempts to restore the photolytic sequence by simply adding the lipids to suspensions of either phospholipase C-treated membranes, or lipid- and detergent-free rhodopsin, are generally unsucessful. Only in the case of didecanoyl-

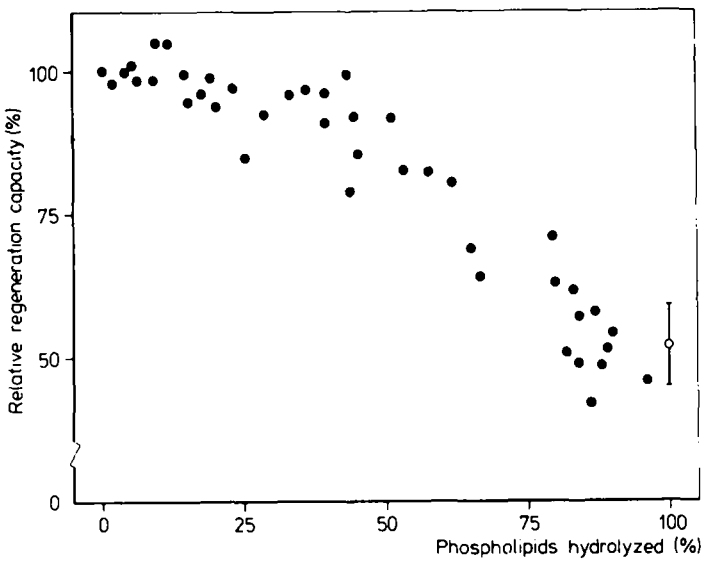

Fig. 2. Relative regeneration capacity of rod outer segment membrane preparations treated with phospholipase C as a function of the percentage phospholipid hydrolysis. The results of phospholipase C treatment of a number of preparations are combined in this figure. The regeneration capacity is expressed as percentage of the regeneration capacity of the corresponding untreated preparations, which exhibit an average capacity of 92% (see Table III). The open circle represents the regeneration capacity of lipid- and detergent-free rhodopsin suspensions (bar, ± S.D., $n = 6$).

phosphatidylcholine and lipids extracted from rod outer segments, a restoration of the photolytic sequence is observed, but not consistently (Table II).

Regeneration capacity

Treatment of rod outer segment membranes with phospholipase C, initially hardly affects the regeneration capacity, but beyond 40% phospholipid hydrolysis, a gradual decrease is observed (Fig. 2). Ultimately, after about 90% hydrolysis a regeneration capacity of about 50% is found (Table III). Lipid- and

TABLE III

REGENERATION CAPACITY OF RHODOPSIN PREPARATIONS

Preparation	% Regeneration (±S.D.)	Number of determinations	Molar ratio lipid/rhodopsin
Suspensions			
Rod outer segment membranes	92 ± 2	7	≈65
Phospholipase C-treated rod outer segment membranes (85—90% hydrosysis)	47 ± 6	5	<9
Lipid- and detergent-free rhodopsin	48 ± 7	4	<0.3
Reconstituted lipid-rhodopsin membranes *	85 ± 3	21	60—165
Solutions in 100 mM dodecyltrimethylammoniumbromide			
All rhodopsin preparations	0		variable

* See Table II and text.

detergent-free rhodopsin also shows a lowered regeneration capacity of about 50% (Table III, see also ref. 3).

Reconstitution of lipid-rhodopsin membranes both from phospholipase C-treated membranes and lipid-free rhodopsin by means of the detergent dialysis procedure restores the regeneration capacity nearly completely (Table III). All amphipathic lipids, listed in Table II, show this result. Only didecanoylcholine gives a slightly lower value (average 72%, $n = 2$). Simple addition of lipids does not restore the regeneration capacity, except in those cases (Table II), where the original photolytic sequence is restored.

Discussion

Effects of phospholipase C treatment

Phospholipase C treatment of bovine photoreceptor membranes results in extensive hydrolysis of phospholipids to water-soluble phosphate esters and diglycerides [1]. As shown in the previous paper [2], the diglycerides are extruded from the membrane as lipid droplets, and thus phospholipase C treatment results in effective removal of phospholipids from the membrane. With only about 7 phospholipid molecules per molecule rhodopsin remaining (90% phospholipid hydrolysis), membranous structures still persist. This suggests that large parts of these membranous structures consist of two-dimensional aggregates of rhodopsin. The present results show that phospholipase C treatment of photoreceptor membranes results in a decrease of the thermal stability of rhodopsin, in an inhibition of the dark transitions following the appearance of metarhodopsin I and also in a reduction of the regeneration capacity. These effects are most pronounced when about 90% of the phospholipids are hydrolyzed and are then about equal to those occurring in rhodopsin completely delipidated by affinity chromatography.

The decrease in thermal stability of rhodopsin in extensively phospholipase C-treated rod outer segment membranes suggests a changed condition of the rhodopsin molecule in the aggregates. While this does not present conclusions about the nature of the effect, the changes in the other parameters lead to further insight.

Extensive phospholipid hydrolysis also effects the photolytic process. A rather long lived 480 nm intermediate appears, which decays to opsin and free retinaldehyde without intermittent formation of metarhodopsin II. The absorption maximum of this intermediate suggests that it is metarhodopsin I, which normally at this temperature decays in milliseconds to metarhodopsin II. Previously, it has been found that in the absence of water [15] and after treatment of photoreceptor membranes with the cross-linking reagent glutaraldehyde (unpublished observation) photolysis stops at a 480 nm intermediate. In the case of lipid-depleted preparations the normal photolytic sequence can be restored by addition of detergent or reconstitution with phospholipids, while in the case of dry preparations addition of water allows the photolytic process to go to rapid completion. Since the metarhodopsin I to II transition is generally believed to be accompanied by substantial conformational changes [11], it seems reasonable to assume that this transition is blocked under conditions which interfere with the flexibility of rhodopsin molecules. This seems to be the case

in reversible fashion upon lateral aggregation due to deplication and upon dehydration, and in irreversible fashion upon cross-linking with glutaraldehyde.

The lowering of the regeneration capacity by about half can also be explained on the basis of rhodopsin aggregation. Aggregation would make part of the opsin molecules inaccessible to 11-*cis* retinaldehyde, due to shielding of up to about half of the chromophore binding sites by adjacent opsin molecules. An alternative interpretation, the rhodopsin molecule is damaged by lipid removal, is unlikely since regeneration is an all or none process (i.e., a molecule is either regenerated or not), and thus a 50% loss of regeneration would imply that only half of the pigment molecules would be damaged. The situation giving rise to the lowered regeneration capacity is rather static, since incubation with 11-*cis* retinaldehyde for extended time periods does not cause additional regeneration. Therefore, the lateral and rotational movements of the protein molecules appear to be restricted to such an extent that an inaccessible opsin molecule remains in this condition. This interpretation would imply that the removal of amphipathic lipids has no direct impact on the conformation of each individual protein molecule, as judged by the regeneration capacity.

The regeneration capacity gradually decreases upon increasing phospholipid hydrolysis (Fig. 2), while concomitantly the number of molecules that no longer show the normal photolytic pattern increases. In our view this means that the number of aggregated molecules increases at the expense of freely dispersed molecules when phospholipid hydrolysis is proceeding.

Comparison with lipid- and detergent-free rhodopsin

The properties of phospholipase C-treated photoreceptor membranes, in which about 90% of the phospholipids are hydrolyzed, are almost identical to those of lipid- and detergent-free rhodopsin. This shows that the latter preparation has not suffered damage due to the use of detergent during its preparation, at least with respect to the properties presently studied. The blocking of the metarhodopsin I decay in lipid- and detergent-free rhodopsin has previously been reported by Applebury et al. [4], while a decreased regeneration capacity in this preparation has been noted by Hong and Hubbell [3]. The analogy between enzymatically and chromatographically delipidated rhodopsin preparations suggests that the anomalies in these preparations have the same basis, i.e., molecular aggregation resulting in shielding and rigidity.

Membrane reconstitution

Attempts to restore the damage caused by delipidation are most successful when amphipathic lipids are added together with detergent, followed by the removal of the detergent by dialysis. This procedure does not work with mono-, di- or triglycerides. Mere addition of amphipathic lipids to the delipidated rhodopsin preparations generally does not lead to recovery of the normal rhodopsin behaviour either (cf. ref. 4). Obviously, with the exception of digitonin [3], only lipids capable of forming bilayers [9] have the potency to restore and maintain the proper functioning of rhodopsin. The nature of the polar head groups is not essential, since even galactolipids and phosphatidic acid are effective.

The reason that proper reconstitution of rhodopsin into lipid bilayers and

recovery of its properties are most easily achieved by the intermediate application of detergent may be that detergents can easily break up the aggregates. Simple addition of amphipathic lipids, generally, cannot do so: only addition of rod outer segment membrane lipids or didecanoylphosphatidylcholine is occasionally successful in restoring the original properties of rhodopsin in phospholipase C-treated membranes, but the former do not achieve this in completely delipidated rhodopsin. Apparently, the residual phospholipids may in this case guide the added phospholipids in between the aggregated rhodopsin molecules. Didecanoylphosphatidylcholine can occasionally restore the properties of rhodopsin when added to lipid- and detergent-free preparations. This phospholipid has been described as a lytic agent [12] and thus might be able to break up aggregates. These observations strengthen our hypothesis that the changes in the properties of rhodopsin are caused by mere aggregation without major structural reorganization in the protein itself, and can be reversed by disaggregation.

Effects of lipid unsaturation

Since all the lipids used in our reconstitution experiments are much more saturated than the lipids of native rod outer segment membranes, it would seem that none of the presently known properties of rhodopsin require the high degree of phospholipid unsaturation of these membranes (see ref. 13). Chen and Hubbell [9] have shown that the regeneration capacity of rhodopsin is even preserved upon reconstitution with completely saturated phosphatidylcholine. However, O'Brien et al. [14] have recently shown that reconstitution of rhodopsin with phosphatidylcholine containing completely saturated, medium chain-length fatty acids offers a substantially decreased metarhodopsin I decay rate. In agreement with our own unpublished observations, the same applies to slightly unsaturated (less than one double bond per molecule) egg phosphatidylcholine at a somewhat lower temperature (18°C).

In conclusion, these experiments support the idea that rhodopsin does require a proper microenvironment with a minimal bilayer fluidity in order to allow the appreciable (superficial?) conformational changes during the metarhodopsin I and II transition. Both a relatively rigid phospholipid bilayer and lateral aggregation might 'freeze' the conformational state of the molecule. Since the presence of two double bonds per molecule phospholipid seems to be sufficient above 18°C, the need for the uniquely high phospholipid unsaturation of the photoreceptor membrane remains unexplained, at least in warm-blooded animals. Presumably it is important for other, as yet unassessed functions of rhodopsin or the membrane. Most likely these functions would be involved with the still little understood events linking rhodopsin photolysis to photoreceptor cell excitation.

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