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BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS

XL. SPECTRAL AND CHEMICAL ANALYSIS OF METARHODOPSIN III IN PHOTORECEPTOR MEMBRANE SUSPENSIONS

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Summary

The late photointermediates of rhodopsin photolysis have been analyzed spectrally and chemically in bovine rod outer segment membrane suspension at 25°C and pH 6.5. The decay of metarhodopsin II follows two spectrally distinct routes, resulting 40 min after illumination in a stable mixture of photoproducts with absorbance maxima around 380 and 452 nm, free retinal and metarhodopsin III, respectively. Chemical analysis shows that three different products are involved: free retinal (approx. 34%), protein-bound retinal (approx. 51%) and lipid-bound retinal (approx. 15%). The latter fraction consists of retinylidene-phosphatidylethanolamine exclusively.

Photolysis of membranes reconstituted with various phospholipids gives a qualitatively normal spectral picture, but the production of metarhodopsin III may vary with the phospholipid composition, i.e. with the percent of phosphatidylethanolamine present. Chemical analysis shows that with increasing phosphatidylethanolamine content of the membrane, the retinylidene phosphatidylethanolamine fraction increases proportionally at the expense of free retinal, while the fraction of protein-bound retinal remains unaffected.

The results indicate that under these conditions metarhodopsin III (defined as a long wavelength product of metahrodopsin II decay) is composed of two chemically distinct components: opsin-bound retinal and retinylidene phosphatidylethanolamine.

Introduction

In the usual photolysis schemes for vertebrate rhodopsin, metarhodopsin III appears as a long-wavelength product of the thermal decay of metarhodopsin II.

However, the published data on this photoproduct are rather confusing. This is clearly illustrated by the fact that at least four other names have been used by various authors for this intermediate: transient orange, 460—470 chromophore, metarhodopsin$_{465}$ and pararhodopsin (cf. Refs. 1 and 25). About its chemical identity relatively little was known until recently.

Earlier work in this laboratory has shown that during metarhodopsin II decay retinal leaves its original binding site and partly binds to other amino groups [2]. More recently we have noticed that photolysis of rhodopsin in isolated and washed bovine rod outer segment membranes leads to a stable mixture of photoproducts consisting of 65% metarhodopsin III and 35% free retinal. The same phenomenon was observed in membranes, reconstituted from pure rhodopsin and various phospholipids, but the ratio between metarhodopsin III and free retinal varied, depending on the phospholipids used [3].

This led us to investigate in more detail the formation and composition of the products of metarhodopsin II decay in native membranes and in reconstituted membranes of varying phospholipid composition.

Materials and Methods

Rhodopsin preparations

Bovine rod outer segment membranes were isolated according to the method of De Grip et al. [4], omitting the enrichment with 11—*cis* retinal. The membranes are twice washed with distilled water and resuspended in 0.1 M phosphate buffer (pH 6.5). Reconstituted lipid-rhodopsin membranes [3,5] were prepared with rhodopsin and purified by affinity chromatography together with dioleoyl phosphatidylcholine (Serdary Res. Lab., London, Ontario, Canada), egg phosphatidylethanolamine or bovine spinal cord phosphatidylserine (both from Lipid Products, South Nutfield, England). The phospholipid to rhodopsin ratio is always 66 : 1, and the reconstituted membranes are resuspended in 0.1 M phosphate buffer (pH 6.5).

Spectral measurements

Spectral measurements of the various rhodopsin suspensions (10—30 μM) are performed at pH 6.5 and 25°C in a Rapid T3 spectrophotometer (Howaldtswerke, Deutsche Werft, Kiel, F.R.G.). This double-beam instrument is particularly suited for measurement of turbid solutions since the thermostated cuvettes are placed immediately in front of an integrating sphere-photomultiplier system. The spectra are recorded from long to short wavelength at a scanning speed of 4 or 6 nm/s. Illumination is performed with a Rollei Strobofix flasher equipped with a 3 mm thick OG_{530} filter (Schott-Jena, Mainz, F.R.G.) or for 30 s with a 300 W tungsten source at a distance of 25 cm behind a 3 mm OG_{530} filter, both methods giving quite similar results. Spectra are recorded before illumination and at various time intervals thereafter. The actual time interval after illumination at each wavelength is calculated from the scanning speed, knowing the wavelength at which recording has been started, and the time elapsing between illumination and beginning of recording.

After recording of the spectral transitions, hydroxylamine (final concentration 50 mM) is added to the suspension, which in 10 min converts all photo-

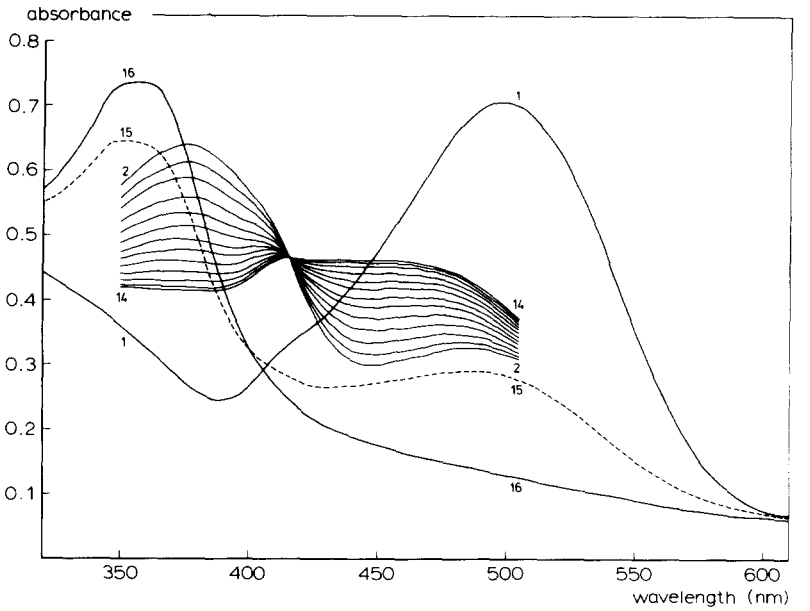

Fig. 1. Decay of metarhodopsin II after flash-photolysis of a bovine rod outer segment membrane suspension. Reaction takes place in 0.1 M phosphate buffer (pH 6.5) at 25° C. The rhodopsin spectrum (1) is obtained before illumination. The recordings of spectra 2—14 are started from 505 nm at the following times (in min after the flash): 0.48; 1.64; 2.81; 4.06; 5.39; 6.73; 8.23; 9.81; 11.56; 13.81; 16.64; 19.52; 21.06. Subsequently, hydroxylamine is added and 10 min later spectrum 15 is recorded, yielding a spectrum of residual visual pigment and retinylidene oxime (λ_{max} = 365 nm). A second light exposure photolyses all pigment, yielding spectrum 16.

products completely to retinylidene-oxime. Another spectral recording is then made, which gives the residual visual pigment (spectrum 15 in Fig. 1). A subsequent 5 min exposure of the suspension to filtered light (OG_{530} filter) completely photolyzes the residual pigment and again a spectrum is recorded (spectrum 16, Fig. 1).

In analogy to observations of Bridges with frog rod outer segments [6], photolysis of bovine outer segment membranes causes, under our conditions, the photoregeneration of some isorhodopsin, as follows from the λ_{max} of the residual visual pigment (difference spectrum of recordings 15 and 16 of Fig. 1). This fact does not interfere with the calculations described in the next paragraph.

Photoproduct analysis

The analysis of the distribution of all-*trans* retinal over the three pools of photoproducts (free, protein-bound, lipid-bound) is schematically presented in Fig. 2 and involves four procedures.

(1) Total all-*trans* retinal is calculated from the amount of rhodopsin photolyzed, which is determined from the difference in absorbance at 500 nm between the spectra 1 and 15 of Fig. 1 [4].

(2) Aldimine-bound and free all-*trans* retinal are determined by two-wavelength spectrophotometry after acidification [7]. The A_{440}/A_{380} is measured after adding 100 µl 2 M HCl to 1 ml of the (illuminated) rhodopsin suspension,

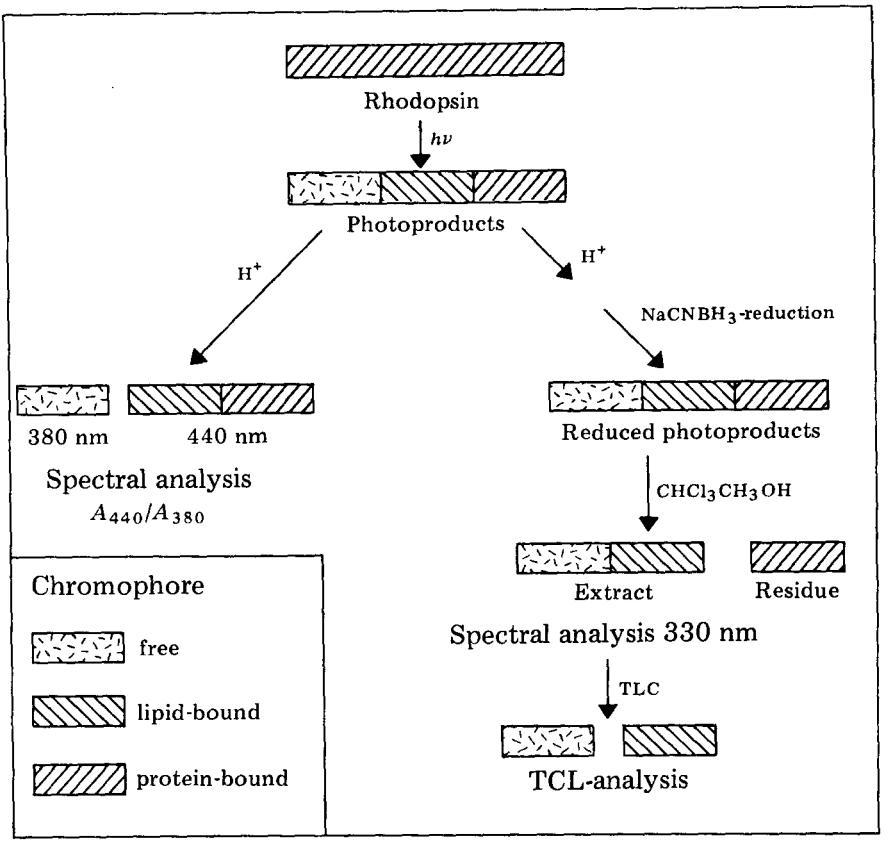

Fig. 2. Schematic presentation of photoproduct analysis. The composition of photoproducts is analyzed either by two-wavelength spectrophotometry after acidification (free chromophore vs. protonated aldimine) or by reduction and subsequent spectral analysis at 330 nm of the chloroform/methanol extractable chromophore (free + lipid-bound vs. protein-bound), sometimes followed by phosphorus analysis after chromatographic separation (lipid-bound). Corrections for non-photolyzed rhodopsin are not shown in this scheme. See also Materials and Methods.

followed by 100 μl Triton X-100 (10%, w/v) to obtain a clear solution. Under these conditions the absorbance maximum of free retinal remains at 380 nm, while all aldimines of retinal are converted to their stable protonated form with an absorbance maximum at 440 nm. Control experiments have shown that after the initial acidification no additional hydrolysis, formation or transminisation of aldimine occurs (cf. Ref. 8). The nomogram of the A_{440}/A_{380} ratio as a function of the ratio between protonated aldimines and free retinal is identical for such widely different aldimines of octylamine [7] and phosphatidylethanolamine.

(3) Chromophore extractable into lipid solvents after acidification and reduction with cyanoborohydride yields by spectral analysis at 330 nm, the sum of free and lipid-bound all-*trans* retinal, as well as, by subtraction, protein-bound all-*trans* retinal. Under the conditions used, free retinal is reduced to retinol and aldimines to retinyl-amines [9].

To 4 ml of the (illuminated) rhodopsin preparation 0.4 ml 2 M HCl is added

under vigorous agitation and 4 ml of the suspension is centrifuged for 30 min at 4°C (5000 rev./min, Sorvall RC2B, rotor SS34). After removal of 3.0 ml of the clear supernatant, 3.8 ml of chloroform/methanol (1 : 2, v/v), containing 10 mM of the antioxidant butylated hydroxytoluene, is added to the residue. All material, including the protein, dissolves in the homogeneous solution (4.7 ml). Cyanoborohydride ($NaCNBH_3$, 5—10 mg) is added to this clear solution, and instantaneous reduction of all free retinal to retinol and of protonated aldimines to retinyl-amines is observed. From the 330 nm absorbance of the clear solution of the reduced compounds the total chromophore concentration is calculated. Then 1.2 ml chloroform and 1.2 ml 0.1 M KCl are added to the solution. The mixture is shaken 10 min in sealed tubes under a nitrogen atmosphere. It is then centrifuged for 10 min at room temperature (5000 rev./min, Sorvall RC2B, rotor SS34). About 1.5 ml of the organic lower phase (2.35 ml total) is removed to measure the 330 nm absorbance. The proteins are assembled in a thin layer at the boundary of the two phases. The upper phase is invariably free of compounds absorbing between 300 and 600 nm. All manipulations are carried out in darkness. The antioxidant is added to prevent oxidation of retinol or retinyl compounds.

The fraction of the chromophore which can be extracted by this procedure is calculated from the absorbance of the clear, homogeneous solution before extraction and that of the lower layer after separation of the phases, and the volume ratio between both solutions. Retinol and retinyl-amines have the same molar absorption (ϵ = 51 000) and absorbance maximum (λ_{max} = 330 nm).

(4) Finally, thin-layer chromatography of the reduced lipid extract, followed by phosphorus determination of the fluorescent spots, gives the amount of lipid-bound all-*trans* retinal and allows identification of the lipid-bound retinal.

Quantitative thin-layer chromatography is carried out on 0.3 mm thick purified silica gel H (Merck), mixed with 4% alkaline magnesium silicate [9] with chloroform/methanol/acetic acid (90 : 15 : 6, v/v/v) as eluent. Retinol and retinyl-amines are detected under ultraviolet light. Retinol migrates near the front. Retinyl phosphatidylethanolamine (R_F = 0.75) is completely separated from the normal phospholipids ($R_F < 0.45$), so that it can be determined by phosphorus determination after scraping off from the plate and acid destruction [10]. Retinyl phosphatidylserine (R_F = 0.50) is virtually absent in our preparations. Reference compounds are prepared by combining retinal and the amine in 0.1 M KCl (pH 10), followed by acidification, $NaCNBH_3$ reduction and extraction, as described above.

Corrections for non-photolyzed visual pigment (protein-bound 11-*cis* and 9-*cis* retinal) are always made. Errors due to the presence of 11-*cis* and 9-*cis* chromophore in the acidified mixtures (methods 2 and 3) deriving from residual pigment are eliminated by a 10 min illumination of the acidified (Triton X-100 solubilized) preparation with white light (300 W tungsten source at a distance of 40 cm).

The calculation of the photoproduct composition requires only three of the four assay methods described (either 1, 2 and 3, or 1, 3 and 4). Both approaches give the same result. Control experiments with native rhodopsin and with model compounds (all-*trans* retinal, 11-*cis* retinal and their aldimines) confirm the reliability of the methods employed. Quantitative recovery requires careful

manipulation in dim red light and rigorous exclusion of chromophore oxidation by the use of an antioxidant and/or a nitrogen atmosphere.

Results

Spectral analysis in native membranes

A typical example of the spectral transition observed in illuminated suspensions of rod outer segment membranes at 25°C and pH 6.5 is shown in Fig. 1. Within the time-scale used, rhodopsin (Fig. 1, spectrum 1) is immediately converted by illumination to a component with maximal absorbance at about 380 nm (Fig. 1, spectrum 2; Fig. 3A), which is called metarhodopsin II [13]. Subsequently a gradual decrease of the 380 nm absorbance is observed, concomitantly with the appearance of a component (metarhodopsin III) absorbing maximally at about 452 nm (Fig. 1, spectra 2 to 14; Fig. 3B). After 40 min the spectrum remains stable for at least 2 h. There is a sharp isosbestic point at 418 nm.

The spectral transition is analyzed by applying the formula:

$$\ln[(A_{t_\infty} - A_{t_i})/(A_{t_\infty} - A_{t_1})] = -k(t_i - t_1)$$

The absorbance difference at infinite time $(A_{t_\infty} - A_{t_i})$ is obtained by the least-squares method. For every analysis the absorbance differences at 6 or more time intervals are used, covering over 80% completion of the transition. In all nine cases tested the correlation coefficient obtained by the least-squares method invariably exceeds 0.999, indicating that the transition obeyes first-order kinetics.

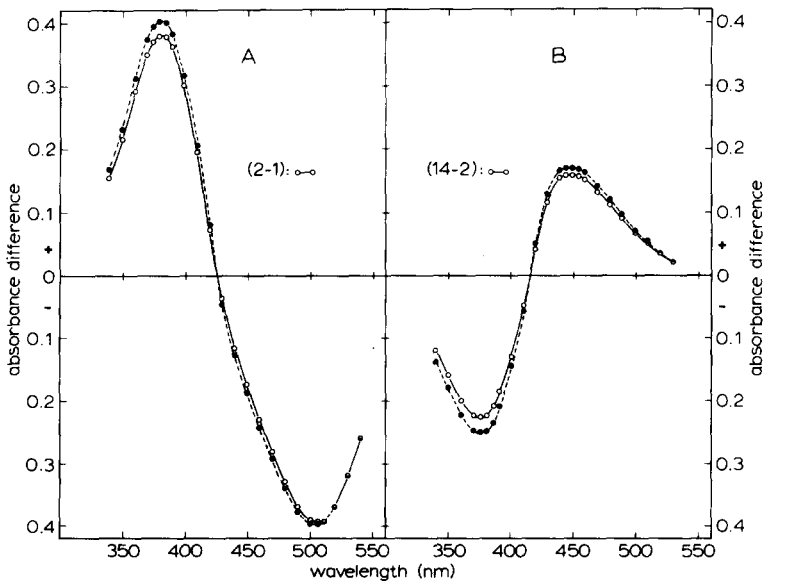

Fig. 3. Difference spectra obtained from the data of Fig. 1. The solid curves in A and B represent the differences in absorbance between spectra 2 and 1 and between spectra 14 and 2, respectively. The dashed curves represent the same difference spectra, but are corrected for the time delay in the recording of spectrum 2.

TABLE I

KINETIC AND SPECTRAL CONSTANTS OF METARHODOPSIN II DECAY

Native bovine rod outer segment membrane suspensions are illuminated in 0.1 M phosphate buffer (pH 6.5) at 25°C. Average results of 9 experiments with S.D.

Apparent λ_{max}	metarhodopsin II	380 ± 1 nm
Apparent λ_{max}	metarhodopsin III	452 ± 1 nm
Isosbestic point		418 ± 2 nm
$(A_\infty - A_0)_{380}/(A_\infty - A_0)_{452}$		1.49 ± 0.09
Rate of absorbance decrease at 380 nm		0.12 ± 0.02 min^{-1}
Rate of absorbance increase at 452 nm		0.12 ± 0.03 min^{-1}
Half-time of absorbance decrease at 380 nm		5.8 ± 1.1 min
Half-time of absorbance increase at 452 nm		5.9 ± 1.1 min

The kinetic constants of the rate of the absorbance decrease at 380 nm and increase at 452 nm (Table I) are identical within the experimental error. This is not unexpected, since the occurrence of an isosbestic point indicates that only two spectrally distinct compounds are involved in the transition.

The spectrum at the real zero time of the transition (t_0) can be calculated using the kinetic constant k. Since illumination itself takes time, the midpoint of the time of illumination is the best approximation of t_0 [12]. The calculated extrapolation to this zero-time gives, at any wavelength, the total difference in absorbance at time zero and at infinite time ($A_{t_\infty} - A_{t_0}$). In this way the ratio between the total absorbance difference at 380 nm and 452 nm, expressed by $(A_{t_\infty} - A_{t_0})_{380}/(A_{t_\infty} - A_{t_0})_{452}$, is calculated to be about 1.5.

Difference spectra can be corrected by a similar calculation. Since the scanning of a spectrum takes about 1 min, the difference spectra obtained from actual recordings are deformed by the time delay between the measurements at different wavelengths. This effect is eliminated when the kinetic constant is used to recalculate the recorded spectra to spectra that would have been found if the scanning time was reduced to zero. Difference spectra corrected in this way are shown in Fig. 3 (dashed curves), taking spectra 1 and 14 of Fig. 1 as time-invariant. The effects on the absorbance difference are obvious, but the position of the absorbance maxima is hardly influenced.

Figs. 1 and 3 indicate that only 60—65% of the initial absorbance gain at 380 nm after illumination is lost during the transition to metarhodopsin III. Calculation shows that approx. 35% of the total initial absorbance gain at 380 nm (at 'zero' time of illumination) persists after completion of the transition, 40 min after illumination. This fraction largely represents free retinal, as will be shown in the next paragraph.

Photoproduct analysis in native membranes

The relative concentrations of free, protein-bound and lipid-bound retinal at various times after illumination of the membrane suspension have been determined as described under Materials and Methods (cf. Fig. 2). The formation of all three compounds exhibits first-order kinetics with similar time courses as found for the spectral transition. In metarhodopsin II all retinal is bound to the protein, since no lipid-extractable photoproducts are found when illumination is immediately followed by acidification and reduction. Product analysis, after

TABLE II

DISTRIBUTION OF RETINAL OVER DIFFERENT POOLS AFTER COMPLETION OF METARHODOPSIN II DECAY

Results are expressed as percent of total photoproducts. Three experiments for native rod outer segment membranes with S.D. and single experiments in all other cases. Phospholipid/rhodopsin molar ratio is 66 in all preparations.

Metarhodopsin III preparation	Retinal (%)		
	free	lipid-bound	protein-bound
Native rod outer segment membranes	34 ± 4	15 ± 2	51 ± 5
Reconstituted rod outer segment membranes	33	14	53
Lipid free rhodopsin reconstituted with:			
Dioleoyl phospatidylcholine	44	0	56
Egg phosphatidylethanolamine	9	34	57
Bovine phosphatidylserine	47	0	53

equilibrium has been reached (40 min after illumination), shows that free retinal represents about 34% of all photolysed rhodopsin, while the protein-bound and lipid-bound retinylidene-imines account for 51 and 15%, respectively (Table II).

Thin-layer chromatography of the lipid extract, obtained after acidification and $NaCNBH_3$ reduction, more than 40 min after illumination, shows that retinol, retinyl phosphatidylethanolamine and phospholipids are the major compounds. This confirms that retinal is a substantial product of metarhodopsin II decay. Since only a trace of retinyl phosphatidylserine is present, this means that the lipid-bound retinal is almost exclusively retinylidene phosphatidylethanolamine. Quantitative determination of retinyl phosphatidylethanolamine by phosphorus analysis indicates that this compound constitutes 18 ± 3% of the final photoproducts, in agreement with the spectrally obtained data of Table II.

Spectral analysis in reconstituted membranes

When rod outer segment membranes are solubilized in 100 mM dodecyltrimethylammonium bromide at 4°C and the detergent is subsequently removed by dialysis, membranes are obtained, reconstituted with their own original lipids. The metarhodopsin II decay and the appearance of metarhodopsin III in this preparation is similar to that found in native rod outer segment membranes, except that the rate of transition is somewhat increased, maximally by a factor of 1.5.

When pure, delipidated rhodopsin is reconstituted with dioleoyl phosphatidylcholine, egg phosphatidylethanolamine or phosphatidylserine from bovine spinal cord, illumination of these reconstituted membranes also leads to the normal appearance of metarhodopsin II, which decays to metarhodopsin III. The isosbestic point remains at 418 nm and the absorbance maxima of metarhodopsin II and III are unchanged. The ratio between the absorbance decrease at 380 nm and the increase at 452 nm, following the appearance of metarhodopsin II, remains approximately 1.5 in all reconstituted preparations. The transition still exhibits first-order kinetics, but the rate is increased, maximally

by a factor two as compared to that of the native rod outer segment membrane suspensions.

However, the nature of the polar head groups of the phospholipids used for reconstitution does influence the ratio of metarhodopsin III to free retinal formed. With phosphatidylcholine and phosphatidylserine relatively less metarhodopsin III seems to be formed, while relatively more metarhodopsin III is observed when phosphatidylethanolamine is the only phospholipid present. The effects are in the order of 20—30%. Since the different scattering properties of the various reconstituted preparations hamper accurate calculations, photoproduct analysis was carried out to study this phenomenon quantitatively.

Photoproduct analysis in reconstituted membranes

Membranes reconstituted with their own lipids (40% phosphatidylethanolamine), show upon illumination a distribution of retinal over the three pools, which is almost identical to that in native membranes under the same conditions: 33% free, 14% lipid-bound and 53% protein-bound retinal (Table II). In membranes reconstituted of rhodopsin and dioleoyl phosphatidylcholine or phosphatidylserine, i.e. in the absence of phosphatidylethanolamine, additional free retinal (44—47%) is formed at the expense of the lipid-bound fraction, which is absent. When pure phosphatidylethanolamine is used, more retinylidene phosphatidylethanolamine (34%) and less free retinal (9%) are formed, as compared to native membranes. In all reconstituted membranes, the fraction of retinal that remains bound to the protein (53—57%) after completion of metarhodopsin II decay is virtually unaffected by the membrane composition. The retinylidene phosphatidylethanolamine fraction thus increases about proportionally to the phosphatidylethanolamine percentage of the lipid fraction.

Discussion

Suspensions of bovine rod outer segment membranes show upon illumination the usual sequence of photointermediates [13]. On the time scale used, metarhodopsin II is instantly formed and decays with a half-time of about 6 min at 25°C. When the decay of metarhodopsin II is completed, 40 min after illumination, a stable mixture of 65—70% metarhodopsin III and 30—35% free retinal is observed. It should be noted that in this paper the term 'metarhodopsin III' is used in a purely operational sense: a long-wavelength decay product of metarhodopsin II.

Bovine metarhodopsin III, obtained in suspension, shows an apparent maximal absorbance at 452 nm. The use of difference spectra does not allow the exact localization of the absorbance maximum. Whereas rhodopsin and metarhodopsin II have approximately the same molar absorbance (Fig. 3A), formation of metarhodopsin III from metarhodopsin II is consistently accompanied by a relative decrease in absorbance (Fig. 3B); $\Delta A_{380}/\Delta A_{452} \approx 1.5$). Taking into consideration the spectral contributions of retinal and metarhodopsin II at 452 nm, we estimate the molar absorbance of metarhodopsin III to be about 80—90% of that of rhodopsin.

The decay of metarhodopsin II to metarhodopsin III leads to three products: free, protein-bound and lipid-bound retinal. The spectral transition is character-

ized by a well-defined isosbestic point at 418 nm, an absorbance peak near 452 nm and a residual absorbance at 380 nm. This suggests that one of the two peaks must represent two of the products. Free retinal is known to have its absorbance maximum near 380 nm. Since the residual absorbance at 380 nm after completion of the transition is about 35% of that of metarhodopsin II and our chemical analysis shows that 35% of metarhodopsin II goes to opsin and free retinal, it appears that the residual absorbance at 380 nm can be assigned to free retinal.

Since protein-bound retinal accounts for over 50% of the final decay products, it must be responsible for the major part of the 452 nm peak. Lipid-bound retinal would then seem to fall in the 452 nm peak as well, since about 65% of metarhodopsin II decays to metarhodopsin III, while after completion of the decay of metarhodopsin II 65% of the retinal is bound to either protein or lipid, i.e. phosphatidylethanolamine. This is supported by the experiments with reconstituted membranes, where the percent of phosphatidylethanolamine in the membrane is positively correlated with the relative amount of metarhodopsin II decaying to metarhodopsin III. This also agrees with the corresponding photoproduct analyses (Table II) and leads to the conclusion that, under our conditions, the spectrally defined species metarhodopsin III is composed of two components: retinylidene phosphatidylethanolamine and protein-bound retinal.

The protein in protein-bound retinal must be opsin, since metarhodopsin III from membranes reconstituted from pure rhodopsin and phospholipids contains protein-bound retinal to the same extent as that derived from native membranes.

Our data indicate that the decay products of metarhodopsin II are formed in parallel, or else very rapid consecutive reactions must be involved. Certainly the rate of metarhodopsin II decay and the rate of metarhodopsin III formation agree closely (Table I). The time resolution of our analytical techniques is not good enough to make more definitive statements about the decay routes of metarhodopsin II. Whether or not our findings about three decay products of metarhodopsin II should be related to reports that rhodopsin and its earlier photoproducts, also in the absence of detergent, occur in two or more isochromic forms [11,12], is presently unclear. However, our data do not indicate any heterogeneity in the kinetics of the metarhodopsin II decay.

Our observations on metarhodopsin III differ rather drastically from those reported by other authors [13—21]. First, we find a much more stable metarhodopsin III. This can, however, be easily explained. In the intact retina [14—20] the decay of metarhodopsin II is accelerated by the enzymic reduction of retinal to retinol in the presence of endogenous NADPH. In digitonin solutions [13,18,21] the possibilities for hydrolysis are greatly increased, since the product retinal will readily dissolve in the aqueous detergent solution. In accordance with this, we observe that 'stable' metarhodopsin III in rod outer segment membrane suspension decays upon addition of NADPH or a detergent like Triton X-100. Secondly, all difference spectra, observed in intact retina of rat [16], frog [14,17—19] and man [20], indicate an absorbance maximum for metarhodopsin III at 470 ± 5 nm. In digitonin solution metarhodopsin III of cattle [13,21] and frog [18] are reported to have their maximum absorbance

at 465 nm, whereas our difference spectra indicate a maximum at 450–455 nm. Finally, with respect to the molar absorbance, most authors seem to agree on nearly equal values for metarhodopsin II and III, whereas we find a distinctly lower value for metarhodopsin III.

Rather than trying to formulate speculative explanations for the last two discrepancies, we prefer to emphasize the variability in properties of metarhodopsin III, as opposed to those of metarhodopsin II and the earlier photointermediates. While metarhodopsin II always has an absorbance maximum at 380 nm and a molar absorbance close to that of rhodopsin, metarhodopsin III exhibits a varying absorbance maximum and/or molar absorbance. While in our experiments with reconstituted membranes containing rhodopsin metarhodopsin II does not seem to be affected, the formation and composition of metarhodopsin III clearly depend on the phospholipid composition. These findings suggest that up to the formation of metarhodopsin II, the photolytic process is largely determined by conditions residing within the rhodopsin molecule, which impose a strict specificity on this reaction sequence. Once the chromophore is exposed and transiminizes or detaches [2,22,23], however, the environment of the rhodopsin molecule becomes important and the specificity of the decay of metarhodopsin II becomes dependent on the external conditions.

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