Chromophore Transfer from Lipid to Protein in Bovine Rhodopsin*

Raymond L. Kimbel, Jr., Raymond P. Poincelot, and E. W. Abrahamson

ABSTRACT: In native vertebrate (bovine) rhodopsin we have demonstrated that the retinylidene chromophore is bound to the phospholipid, phosphatidylethanolamine. Previous studies in two other laboratories indicate that in illuminated rhodopsin the chromophore binding site is an ϵ-amino group of a lysyl unit in the backbone protein. This suggests that the chromophore is transferred from the lipid to the protein in one of the early intermediate reactions. We have shown the chromophore is attached to the lipid at the metarhodopsin$_{478}$ I stage but is found on the protein at metarhodopsin$_{380}$ II. The transfer, therefore, occurs in the reaction, metarhodopsin$_{478}$ I $\rightarrow$ metarhodopsin$_{380}$ II, a process known to occur *in vitro* and *in vivo* in times on the order of 100 μsec. This suggests the lipid–protein chromophore transfer is a key molecular event in the generation of an electrical neural impulse in the visual receptors.

The preceding paper (Poincelot *et al.*, 1970) has established that in bovine rhodopsin the chromophore is bound *via* a Schiff base linkage to phosphatidylethanolamine, a major phospholipid found in rod outer segments (Poincelot and Zull, 1969). Two different laboratories have shown the chromophore can be reductively affixed to the ϵ-amino group of lysine at the metarhodopsin$_{380}$ II stage (Bownds, 1967; Akhtar *et al.*, 1968). However, the affixation at this intermediate stage was carried out at pH 6.0 and 8.0, which in aqueous systems favors imine exchange in model retinylidene Schiff base systems (Morton and Pitt, 1955). There is some reason to doubt, therefore, that this particular amino group is the actual binding site of the chromophore at metarhodopsin$_{380}$ II.

In an earlier note (Poincelot *et al.*, 1969) we examined the intermediates metarhodopsin$_{478}$ I and metarhodopsin$_{380}$ II under conditions which normally preclude imine exchange or Schiff base hydrolysis in solution. We found the chromophore linked to phosphatidylethanolamine at the metarhodopsin$_{478}$ I stage, but at the metarhodopsin$_{380}$ II stage it was attached *via* the ϵ-amino group to a lysyl unit in the backbone protein. Chromophore transfer from a protein binding site to a lipid binding site had been suggested as occurring at some stage in the photolysis cycle of rhodopsin (Bonting and Bangham, 1967). Our results bear out the notion of a chromophore transfer but clearly establish the transfer as occurring from the *lipid* to the *protein*, and specifically in the intermediate process, metarhodopsin$_{478}$ I $\rightarrow$ metarhodopsin$_{380}$ II (Poincelot *et al.*, 1969). This paper details the experimental results which led us to this conclusion.[1]

Experimental Procedures

Native bovine rod outer segments and rhodopsin in CTAB micelles (extracted with 2% CTAB in 0.1 M (pH 4.5) citric acid–phosphate buffer; McIlvaine, 1921) were isolated and purified as described previously (Poincelot *et al.*, 1970). Other germane experimental aspects also described in the preceding paper will not be repeated here.

Preparation of Metarhodopsin$_{478}$ I. Metarhodopsin$_{478}$ I was obtained by illumination of thin layers of nondelipidated, lyophilized rod outer segments or CTAB micelles of rhodopsin (maintained at 24°) with a 150-W tungsten flood lamp at a distance of 30 cm for 15 min. A resulting color change from red-pink to orange was observed.

Preparation of Metarhodopsin$_{380}$ II. Metarhodopsin$_{380}$ II was prepared in two ways. In the first we took 0.7 g of metarhodopsin$_{478}$ I as lyophilized CTAB micelles and wetted it with 10.0 ml of 0.1 M pH 4.5 citric acid–phosphate buffer (McIlvaine, 1921) at 4°. The slurry upon stirring immediately turned yellow-orange and was promptly lyophilized. For metarhodopsin$_{380}$ II as rod outer segments (0.2 g), a somewhat longer time (10 min) was required for the yellow color of metarhodopsin$_{380}$ II to develop. Reflectance spectra showed that under these conditions metarhodopsin$_{380}$ II had not decomposed to later photoproducts in the photolysis cycle, while difference spectra confirmed the presence of metarhodopsin$_{380}$ II. In the second method 10.0 ml of 0.1 M pH 4.5 McIlvaine's buffer was added at 4° to lyophilized native rod outer segments (0.2 g) or CTAB micelles of rhodopsin (0.7 g). These were illuminated with stirring (4°) under the same light source used for metarhodopsin$_{478}$ I. After a few minutes when the CTAB micelles of rhodopsin had turned from pink to yellow-orange, the mixture was rapidly lyophilized and stored at $-20°$ under N_2. For the rod outer segments a period of 15 min was required to effect the same color change.

Spectra of Photolytic Intermediates in Rod Outer Segments and CTAB Micelles of Rhodopsin. All spectra were taken on a Cary 14 R double-beam recording spectrophotometer with two matched 1-cm light-path cells, which were thermally jacketed and thermostatically controlled ($\pm 0.5°$). A diffuse reflectance

* From Case Western Reserve University, Department of Chemistry, Cleveland, Ohio 44106. *Received November 18, 1969.* This research was supported by National Eye Institute Grant EYs 00209 and EY 00471, U. S. Public Health Service.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CTAB, cetyltrimethylammonium bromide, *N*-RPE, *N*-retinylidenephosphatidylethanolamine (*N*-R_tPE-*trans* form); *N*-RH_2PE, *N*-retinylphosphatidylethanolamine; and PE, phosphatidylethanolamine.

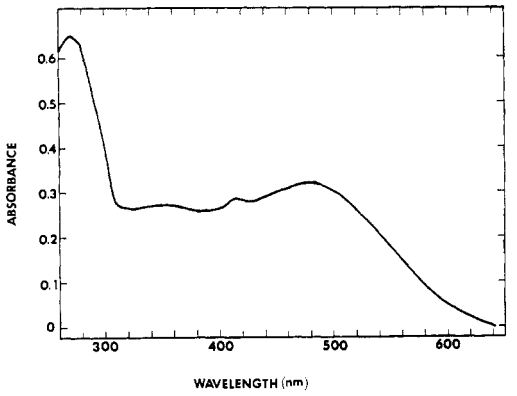

FIGURE 1: Powder spectrum of lyophilized metarhodopsin$_{478}$ I rod outer segments.

accessory was used for spectra of lyophilized material. The latter approach, though satisfactory for native rhodopsin and metarhodopsin$_{478}$ I, failed to give meaningful spectra for metarhodopsin$_{380}$ II as only a broad increase in absorbance was observed in the 350–400-nm region. This difficulty was resolved through the use of solution difference spectra, where the change in extinction in going from metarhodopsin$_{478}$ I to metarhodopsin$_{380}$ II was revealed by subtracting the curve of the former from the latter. For this separate solutions containing equal amounts of metarhodopsin$_{478}$ I rod outer segments and metarhodopsin$_{380}$ II rod outer segments were prepared in 2:1 glycerine–McIlvaine's buffer (0.1 M, pH 4.5) at $-40°$ and were sonicated below $-20°$ until clear solutions resulted. The spectrum of metarhodopsin$_{478}$ I rod outer segments was measured at $-20°$ while that at metarhodopsin$_{380}$ II rod outer segments was taken at 3°. Light-scattering problems were minimized by using the opal glass technique (Shibata *et al.*, 1954). The CTAB powder of rhodopsin was treated in the same way except that sonication and opal glass technique were not necessary.

Reductions. All reductions, with the exception of one, were carried out as described previously (Poincelot *et al.*, 1970). Metarhodopsin$_{380}$ II, both as rod outer segments or CTAB micelles (as derived from 50 retinas), can be directly reduced with $NaBH_4$. To the dry CTAB micelles, 10 ml (4°) of distilled water (buffered at pH 4.5 due to buffer salts in lyophilized metarhodopsin$_{380}$ II) was added, followed by the addition of 50 mg of $NaBH_4$ with stirring. The reduction was complete within a few minutes, yielding a colorless solution. With metarhodopsin$_{380}$ II rod outer segments, 50 mg of $NaBH_4$ was added directly, followed by the addition of 15 ml of 2% CTAB in 0.1 M pH 4.5 McIlvaine's buffer with stirring (4°). Stirring was continued for 1 hr to ensure complete extraction of the *N*-retinylopsin from the rod outer segments. Both of the above CTAB solutions were centrifuged at 12,000*g* for 15 min with the supernatant being filtered through a Millipore syringe (pore size 0.22 μ). This was followed by overnight dialysis against distilled water to remove extraneous ions, whereupon the dialysate was alkaline hydrolyzed (2 N NaOH) under vacuum at 100° for 8 hr. Afterward the hydrolysates were extracted three times with 20-ml portions of $CHCl_3$ and this extract was stored under N_2 at $-20°$.

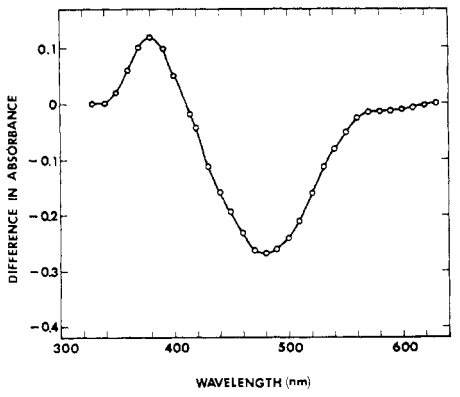

FIGURE 2: Difference spectrum between metarhodopsin$_{478}$ I and metarhodopsin$_{380}$ II in the rod outer segments.

Results

Dry native bovine rhodopsin as detergent micelles, when illuminated near room temperature, proceeds in the photolytic cycle only to the metarhodopsin$_{478}$ I stage as water is required for this intermediate to form metarhodopsin$_{380}$ II (Wald *et al.*, 1950). An ultraviolet–visible powder spectrum of the dry, illuminated bovine rhodopsin had a λ_{max} at 478 nm characteristic of metarhodopsin$_{478}$ I. Treatment of native rod outer segments in like manner also produced a peak at 478 nm (Figure 1).

Extracting lyophilized, nondelipidated metarhodopsin$_{478}$ I as rod outer segments or CTAB micelles with methanol or 0.1–$10^{-3.5}$ M HCl in methanol produced spectral results identical with those obtained when the native forms were extracted (Poincelot *et al.*, 1970). Absorption maxima were observed in methanol at 275 and 365 nm and at 275, 397, and 445 nm in acidified methanol. Chromophore determination (Poincelot *et al.*, 1970) showed about 90% to be present in the extracts. Reduction of the extract with $NaBH_4$ resulted in the disappearance of the 365-, 397-, and 445-nm peaks and the appearance of a new peak at 325 nm. Identification of the extracted and reduced materials by means of thin-layer and gas chromatographic methods identical with those employed for the native state (Poincelot *et al.*, 1970) showed it to be *N*-RH_2PE after $NaBH_4$ reduction and *N*-retinylethanolamine after alkaline hydrolysis.

For identification of metarhodopsin$_{380}$ II, we used the technique of difference spectra to follow the change in extinction between metarhodopsin$_{478}$ I and metarhodopsin$_{380}$ II. By subtracting the spectral curve of metarhodopsin$_{478}$ I from that of metarhodopsin$_{380}$ II, we obtained a difference spectrum (Figure 2) with a maximum at 380 nm and a minimum at 478 nm. Results were similar for rod outer segments and rhodopsin micelles.

When lyophilized metarhodopsin$_{380}$ II (rod outer segments or rhodopsin) was extracted with methanol, the amount of Schiff base (365 nm) removed as *N*-RPE accounted for less than 10% of the chromophore. Similarly, essentially the same amount of chromolipid in the protonated form (445 nm) was extracted when acidified methanol was used. Moreover, when metarhodopsin$_{380}$ II was reduced with $NaBH_4$ at pH 4.5 in the rod outer segments or rhodopsin micelles, the resulting solution upon extraction with CTAB followed by centrifugation and filtration showed absorption maxima at 278 and

TABLE I: Chloroform-Soluble Components in *N*-Retinylopsin Hydrolysates.

Sample	Fluorescent R_F (×100) Values	
	Solvent	
	I[a]	II[b]
$CHCl_3$ extract of hydrolysate		
N-Retinylopsin (rod outer segments)	35, 97	10, 92
N-Retinylopsin (rhodopsin)	34, 97	10, 93
Retinol	97	94
N-Retinyllysine	36	10

[a] Methyl ethyl ketone–pyridine–H_2O–acetic acid (70:15:15:2, v/v). [b] $CHCl_3$–CH_3OH–28% NH_4OH (70:30:4, v/v).

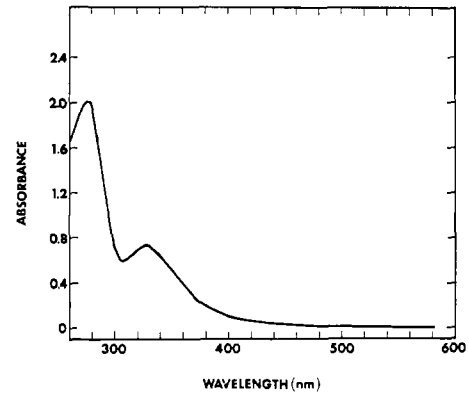

FIGURE 3: *N*-Retinylopsin as produced by $NaBH_4$ reduction (pH 4.5) of metarhodopsin$_{380}$ II.

325 nm (Figure 3). From the absorbance at 325 nm, 82% of the chromophore was estimated to be present in the form of *N*-retinylopsin.

After dialysis, alkaline hydrolysis, and chloroform extraction of the *N*-retinylopsin the results in Table I were found by thin-layer chromatography.

Discussion

Identification of the chromophore binding site at metarhodopsin$_{478}$ I follows the same line of reasoning as that for the native state (Poincelot *et al.*, 1970). On the basis of the evidence presented, the chromophore remains linked to phosphatidylethanolamine during the early stages of the photolytic cycle, *i.e.*, from native rhodopsin through metarhodopsin$_{478}$ I.

At metarhodopsin$_{380}$ II we found that methanol or acidic methanol removed less than 10% of the chromophore as *N*-RPE. This is what one would expect if small amounts of rhodopsin and isorhodopsin and perhaps some metarhodopsin$_{478}$ I in equilibrium with metarhodopsin$_{380}$ II were also present. The $NaBH_4$-reduced metarhodopsin$_{380}$ II (rod outer segments and CTAB micelles) yielded 82% *N*-retinylopsin (Figure 3) which was found as *N*-retinyllysine after alkaline hydrolysis.

At the metarhodopsin$_{380}$ II stage the chromophore is exposed to its solution environment as is attested to by its reactivity toward $NaBH_4$ and other reagents (Bownds, 1967; Wald and Hubbard, 1960; Wald and Brown, 1953–1954). The aqueous acidic conditions (pH 4.5) during the metarhodopsin$_{380}$ II reductive affixation of the chromophore assured that once the binding site was exposed, it underwent reduction immediately without competitive imine exchange (Morton and Pitt, 1955). There is no question, then, that at metarhodopsin$_{380}$ II the chromophore is bound to a lysyl unit in the backbone protein. The relative stability of native and metarhodopsin$_{478}$ I toward $NaBH_4$ reduction suggests that in these forms the chromophore binding site is largely protected from the solution environment. The action of acid methanol undoubtedly denatures the lipoprotein structure of metarhodopsin$_{478}$ I so that the chromolipid *N*-RPE can be extracted quantitatively. To argue that phosphatidylethanolamine is not the binding site in metarhodopsin$_{478}$ I or native rhodopsin is to postulate that the chromophore is transferred to phosphatidylethanolamine quantitatively from some other binding site—presumably after the denaturation but before the acid condition is established about the transfer region. It is indeed hard to defend such an alternative hypothesis, particularly in view of the fact that this postulated transfer does not occur at all or occurs to a different binding site under identical extraction conditions at metarhodopsin$_{380}$ II. Furthermore, heat denaturation under aqueous acidic conditions with concomitant $NaBH_4$ reduction quantitatively affixes the chromophore to phosphatidylethanolamine in native rhodopsin (Poincelot *et al.*, 1970). This same experiment, because of its transient character in aqueous solution, cannot be done with metarhodopsin$_{478}$ I, but the similarity in behavior to native rhodopsin points clearly to the same physiological binding site in both.

Recent studies in our laboratory (Rapp *et al.*, 1969; unpublished data) indicate that the metarhodopsin$_{478}$ I → metarhodopsin$_{380}$ II reaction can be fitted to a Michaelis–Menten scheme of the form

$$N\text{-}R_t PE + \text{protein} \rightleftharpoons N\text{-}R_t \cdots PE \cdots \text{protein} \rightleftharpoons N\text{-}R_t\text{-protein} + PE$$

This is a process which occurs in times of the order of 100 μsec.

The spectral maxima of metarhodopsin$_{478}$ I and metarhodopsin$_{380}$ II indicate that the transfer of the chromophore is from a protonated binding site to a nonprotonated one. Metarhodopsin$_{478}$ I is protected from its solution environment, perhaps through hydrophobic encapsulation in the membrane, whereas the reactivity of the binding site at metarhodopsin$_{380}$ II suggests that it is in the hydrophilic surface of the membranes surrounding the flattened ellipsoidal sacs constituting the rod outer segments. The transfer process should result in a considerable change in the charge environment of the transfer region which could logically lead to a significant change in the transmembrane potential. Cone and Cobbs (1969) claim that the early receptor potential in the rat correlates kinetically with the metarhodopsin$_{478}$ I → metarhodopsin$_{380}$ II reaction. A somewhat similar correlation was found by Ward, Pak, and Ostroy (unpublished data, 1969) for the excised frog retina.

The early receptor potential, then, may reflect the electrical change accompanying the chromophore transfer.

In conclusion it is worth pointing out that the transfer reaction discussed here is apparently the only established example of a membrane process in which two major constituents are clearly involved, *i.e.*, the prosthetic chromophore is transferred from a specific *lipid* to a specific *protein site*. Should the early receptor potential be shown to be in the mainstream of physiological events, then the transfer of the prosthetic chromophore would appear to be the key molecular event in the generation of the (late) membrane receptor potential.

Acknowledgments

We thank Dr. J. Wiesenfeld and Professors M. Sundaralingam and M. Bodanszky for helpful discussions.

References

Akhtar, M., Blosse, P. T., and Dewhurst, P. B. (1968), *Biochem. J. 110*, 693.

Bonting, S. L., and Bangham, A. D. (1967), *Exptl. Eye Res. 6*, 400.

Bownds, D. (1967), *Nature 216*, 1178.

Cone, R. A., and Cobbs, W. H. (1969), *Nature 221*, 820.

McIlvaine, T. C. (1921), *J. Biol. Chem. 49*, 183.

Morton, R. A., and Pitt, G. A. J. (1955), *Biochem. J. 59*, 128.

Poincelot, R. P., Millar, P. G., Kimbel, R. L., and Abrahamson, E. W. (1969), *Nature 221*, 256.

Poincelot, R. P., Millar, P. G., Kimbel, R. L., and Abrahamson, E. W. (1970), *Biochemistry 9*, 1809.

Poincelot, R. P., and Zull, J. E. (1969), *Vision Res. 9*, 647.

Shibata, K., Benson, A. A., and Calvin, M. (1954), *Biochim. Biophys. Acta 15*, 461.

Wald, G., and Brown, P. K. (1953–1954), *J. Gen. Physiol. 37*, 189.

Wald, G., Durell, J., and St. George, R. C. C. (1950), *Science 111*, 179.

Wald, G., and Hubbard, R. (1960), *Enzymes 3*, 369.

Phospholipid Composition and Extractability of Bovine Rod Outer Segments and Rhodopsin Micelles*

Raymond P. Poincelot and E. W. Abrahamson

ABSTRACT: Bovine rod outer segments, after exhaustive phospholipid extraction with hexane or diethyl ether, liberate additional amounts of phospholipid upon photolysis. Examination of rod outer segments at various stages in the photolytic cycle was carried out to determine at what photolytic intermediate(s) this release occurred. In the rod outer segments the largest portion of the phospholipid was liberated in the photolytic process

$$\text{native rhodopsin} \xrightarrow{h\nu} \text{metarhodopsin}_{478}\ \text{I}$$

while in the subsequent thermal process

$$\text{metarhodopsin}_{478}\ \text{I} \longrightarrow \text{metarhodopsin}_{380}\ \text{II}$$

an additional but smaller amount of phospholipid was released. Examination of the released phospholipids showed that in the rod outer segments phosphatidylcholine was tightly bound, while phosphatidylethanolamine constituted the bulk of the loosely bound phospholipids. Rhodopsin isolated by detergent extraction (Emulphogene) from the bovine rod outer segments contained 31 moles of phospholipid/mole of rhodopsin. Separation of the rhodopsin from the detergent by ammonium sulfate fractionation and subsequent hexane extraction of the lyophilized material yielded a native rhodopsin, containing an eightfold molar excess of tightly bound phospholipid, largely phosphatidylethanolamine. These tightly bound phospholipids which apparently were directly and indirectly involved in the chromophoric binding site were subsequently released after photolysis.

Phospholipids have an active role in the chemistry of the visual process through their involvement as *N*-RPE[1] in the binding site and in the lipid to protein chromophore transfer (Poincelot *et al.*, 1970; Kimbel *et al.*, 1970). Previously, it had been suggested that lipids had some indirect function in the visual cycle as there was a release of an extra lipid increment upon bleaching delipidated rod outer segments in the presence of a nondenaturing solvent (Ishimoto and Wald, 1946; Krinsky, 1958). Attempts by Krinsky (1958) to do the same type of experiment with digitonin solutions

* From Case Western Reserve University, Department of Chemistry, Cleveland, Ohio 44106. *Received November 18, 1969*. This research was supported by National Eye Institute Grants EY 00209 and EY 00471, U. S. Public Health Service.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: *N*-RPE, *N*-retinylidenephosphatidylethanolamine; PE, phosphatidylethanolamine.