Biochimica et Biophysica Acta, 357 (1974) 151–158

BBA 46769

BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS XXVI. BINDING SITE AND MIGRATION OF RETINALDEHYDE DURING RHODOPSIN PHOTOLYSIS*

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(Received February 19th, 1974)

SUMMARY

The binding site of retinaldehyde during photolysis of rhodopsin was studied by probing the retinaldehyde binding site of native rhodopsin after illumination. This was carried out by $NaBH_4$ fixation of the chromophore during and at various time intervals after illumination, followed by treatment with 11-*cis*-retinaldehyde. With $NaBH_4$ present during illumination there is very little recombination with 11-*cis*-retinaldehyde. With increasing time intervals between illumination and addition of $NaBH_4$ the recombination activity increases. This increase follows first order kinetics with a half time of about 10 min at 25 °C. This coincides with the decay of metarhodopsin II at this temperature as confirmed by the time course of the appearance of free retinaldehyde.

Thus the recombination capacity of illuminated, reduced rhodopsin parallels the vacation of the metarhodopsin II-binding site. This indicates that in intact rhodopsin and in metarhodopsin II retinaldehyde is bound to the same ε-amino lysine group, while migration of the chromophore occurs during the decay of metarhodopsin II.

INTRODUCTION

During the photolysis of cattle rhodopsin, the chromophoric group retinaldehyde remains bound by means of an aldimine linkage to opsin, at least up to metarhodopsin II, in spite of its conversion from the 11-*cis* to the all-*trans* isomer [1]. The binding site of the chromophore in metarhodopsin II has been shown to be the ε-amino group of a lysine residue of opsin [2, 3]. More recently this has also been proven to be the case for intact rhodopsin [4–6]. This still leaves the possibility of a migration of the chromophore from one lysine group to another during the transition of rhodopsin to metarhodopsin II, since cattle rod outer segment mem-

* Presented in preliminary form at the Symposium on Biochemistry and Physiology of Visual Pigments, August 27–30, 1972, Bochum, W. Germany (ref. 21).

branes contain 16 lysine groups per mole of rhodopsin [6, 7], at least 11 of which are on the rhodopsin molecule itself [8, 9]. Since at physiological temperature metarhodopsin II is formed within milliseconds, while its decay takes several minutes [10, 11], visual excitation must be triggered during this transition (ref. 12, p. 373). Hence, it would be of great importance for our understanding of the mechanism of visual excitation to know more about the binding site of the chromophore during rhodopsin photolysis.

We have studied this question in two parallel ways: (1) by observing the availability of the retinaldehyde binding site of native rhodopsin after illumination by its reactivity towards 11-*cis*-retinaldehyde, and (2) by following the release of the (isomerized) chromophore after illumination. In the first approach it is assumed that no recombination with 11-*cis*-retinaldehyde under formation of new photopigment is possible, as long as the original binding site is occupied by the isomerized chromophore. $NaBH_4$ reduction is used to fix bound chromophore in position by reducing the aldimine bond to a secondary amine and to reduce free retinaldehyde to retinol. The various modes of reaction, which could occur, are depicted schematically in Fig. 1.

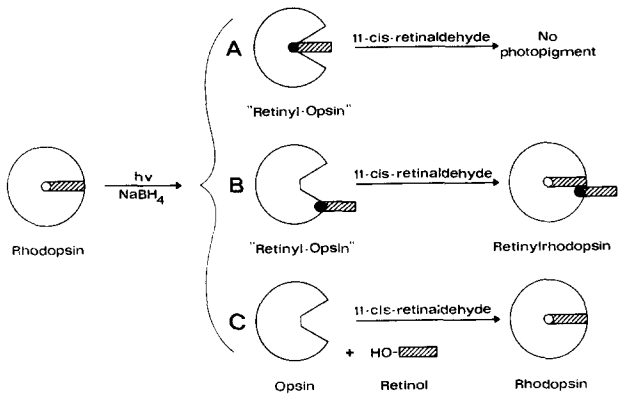

Fig. 1. Possible reaction schemes for $NaBH_4$ reduction of illuminated rhodopsin, followed by reaction with 11-*cis*-retinaldehyde (○, aldimine bond at original binding site, ●, reduced aldimine bond). Case A: no migration of chromophore, reaction with 11-*cis*-retinaldehyde cannot lead to formation of a photopigment. Case B: migration of chromophore followed by reduction of the aldimine bond, reaction with 11-*cis*-retinaldehyde should lead to formation of a new photopigment: "retinylrhodopsin". The binding of migrated retinaldehyde does not necessarily take place on the rhodopsin molecule itself, but may occur on amino groups of other membrane proteins or phospholipids as well. Case C: chromophore is first released and then reduced to retinol by $NaBH_4$, reaction with 11-*cis*-retinaldehyde should lead to formation of rhodopsin.

We find that the reactivity towards 11-*cis*-retinaldehyde in time parallels the vacation of the metarhodopsin II binding site, indicating that the binding of retinaldehyde in intact rhodopsin and in metarhodopsin II occurs at the same ε-amino lysine group, and that migration of the chromophore takes place during metarhodopsin II decay.

MATERIALS AND METHODS

Cattle rhodopsin is prepared by the enrichment procedure of de Grip et al. [13], which gives rod outer segment membrane preparations of high purity, containing no free opsin, no free retinaldehyde and virtually no retinol. Photolyzed rhodopsin is prepared by illumination with a 75-W tungsten lamp for 10 min at a distance of 15 cm behind orange and infrared filters (OG 370 and KG 1, thickness 3 mm each, Schott-Jena, Mainz, W. Germany). Opsin is prepared by addition of a 5-fold molar excess of NADPH to a 25 μM suspension of illuminated rhodopsin [13]. The resulting retinol and excess coenzyme are removed by washing with serum.

Aldimine links between photolyzed rhodopsin and retinaldehyde are stabilized by reduction with $NaBH_4$ to secondary amines. The reduction is carried out by adding 2 mg $NaBH_4$ (Fluka, Switzerland) at various time intervals after illumination to 1.0 ml of a 25 μM suspension of rhodopsin in 0.25 M phosphate buffer (pH 6.7). Similarly reduction of metarhodopsin II is effected by adding $NaBH_4$ prior to illumination. Free retinaldehyde, present at the time of reduction is reduced to retinol. This is, in contrast to retinylopsin, completely extractable with hexane after pretreatment with methanol [2]. Since retinyl-phospholipids are virtually absent in this extract, the percentage of free retinaldehyde, present at any moment after photolysis, can be calculated from the retinol absorbance of the hexane extract at 330 nm. The extraction is carried out in a nitrogen atmosphere. Retinaldehyde, both aldimine bound and free, is determined directly by the thiobarbituric acid method [14, 15]. The amount of aldimine bound retinaldehyde is measured directly by protonation followed by two-wavelengths spectrophotometry at 380 and 440 nm [6] after solubilization in Triton X-100 (final concentration 1 %). This method is applicable since all absorbance at these wavelength is due to either free retinaldehyde or retinylidene-aldimine, and the molar absorbances of each compound at both wavelengths are known.

The recombination capacity of (reduced) illuminated rhodopsin preparations is determined after removal of excess $NaBH_4$. The suspension is centrifuged, the pellet is washed once with 0.25 M phosphate buffer (pH 6.7) and the resulting pellet is taken up in 0.067 M phosphate buffer (pH 6.3). 1 ml of this suspension is added, under vigorous stirring, to a 5-fold molar excess of 11-*cis*-retinaldehyde in 50 μl of methanol. The control consists of 1.0 ml of a non-reduced sample treated in the same way. No denaturation of opsin is observed in the presence of this amount of methanol. After 3 h incubation in the dark, Triton X-100 (final concentration 1 %) and hydroxylamine (final concentration 0.05 M) are added to an aliquot of the suspension. The amount of regenerated rhodopsin is determined from the decrease in absorbance at 500 nm upon illumination and is expressed in percent of the amount of rhodopsin originally present in the preparation. This percentage is designated as the recombination capacity. When the regeneration is compared to that of an appropriate control preparation, the term "relative recombination capacity" is used. For convenience these figures include the relatively low percentages of visual pigment (5–10 %) remaining after photolysis, both in the presence and absence of $NaBH_4$ under the conditions used.

RESULTS

$NaBH_4$ reduction in this study has been carried out at pH 6.7 in order to prevent any damage to the recombination capacity of opsin. At this pH illumination of rhodopsin in the presence of $NaBH_4$ results in reduction of 90–95 % of the retinaldehyde originally present, while 5–10 % of the visual pigment survives these conditions, as shown by the absorption spectrum of the product. Analysis of the chloroform–methanol extract of the reaction product by thin-layer chromatography shows only trace amounts of retinol and no retinylphospholipids. Only the residue, remaining after extraction, displays fluorescence, indicating that the retinyl group must be covalently bound to opsin. Since reduction is complete within a few seconds, the product must represent reduced metarhodopsin II.

When the reduction is carried out at increasing time intervals after illumination, the amount of retinol found in the hexane–methanol extract increases, and a constant level is reached at reduction 60 min after illumination (Fig. 2, open circles). At this time only about 40 % of the retinaldehyde has been released, as confirmed by the two-wavelengths method after protonation (Fig. 2, closed circles). The remaining 60 % of the retinaldehyde remains bound covalently as an aldimine. It must largely be bound to protein amino groups at the time of reduction, since chloroform/methanol extraction of illuminated, reduced rhodopsin yields only minor amounts of retinylphospholipids.

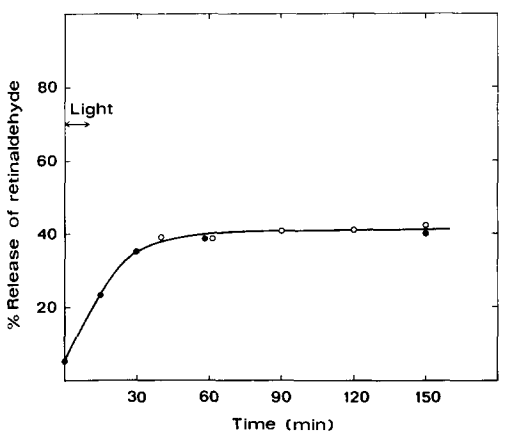

Fig. 2. Retinaldehyde release from rhodopsin after illumination as a function of the time elapsing after illumination. (○, determined by the protonation method. ●, determined by $NaBH_4$ reduction.)

The availability of the original binding site of retinaldehyde in illuminated, reduced rhodopsin preparations is studied by determining their recombination capacity. The recombination capacity of reduced metarhodopsin II, i.e. rhodopsin illuminated in the presence of $NaBH_4$, is very low, whereas in the absence of $NaBH_4$, photolyzed rhodopsin recombines with 11-*cis*-retinaldehyde to visual pigment for about 80 % (Table I). Control experiments with $NaBH_4$ treatment of both intact rhodopsin and opsin, show that the loss of recombination capacity of $NaBH_4$ reduced metarhodopsin II is not due to denaturing effects (Table I).

TABLE I

RECOMBINATION CAPACITY OF PHOTOLYZED RHODOPSIN PREPARATIONS, BEFORE AND AFTER VARIOUS TREATMENTS WITH $NaBH_4$

Data are expressed as percentage of the original rhodopsin content. n = number of independent determinations.

Preparation	Percent ±S.E.	n
Photolyzed rhodopsin	78±6	11
$NaBH_4$-reduced metarhodopsin II	5±1	11
$NaBH_4$-treated, washed, photolyzed rhodopsin	77±1	4
$NaBH_4$-treated, washed opsin	68±5	3

In subsequent experiments the time span between illumination and reduction has been systematically enlarged, and the recombination capacity of the preparation has been determined. A representative experiment is shown in Fig. 3 (upper curve). When the time interval between illumination and reduction is increased from 0 to 60 min, the relative recombination capacity rises from 5 to 90 % as compared to non-reduced controls, and remains constant thereafter. When illumination is carried out at 0 °C, and the suspension is kept at this temperature until reduction, the recombination capacity increases very little (Fig. 3, lower curve).

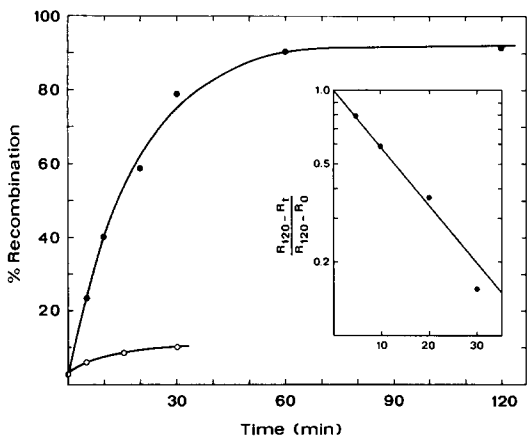

Fig. 3. Relative recombination capacity of reduced illuminated rhodopsin as a function of time interval between illumination and reduction. During the experiment the solution is kept either at 25 °C (●–●) or at 0 °C (○–○). The recombination reaction is carried out at room temperature. The recombination capacity of a non-reduced sample is taken as 100 %. Inset: semilogarithmic plot of $R_{120}-R_t/R_{120}-R_0$ vs time. R_0, R_t and R_{120} are the relative recombination capacities at 0, t and 120 min respectively.

The absorption spectrum of retinylrhodopsin, i.e. the pigment resulting upon treatment of illuminated, reduced rhodopsin with 11-*cis*-retinaldehyde, has been measured after removal of the excess retinaldehyde by repeated hexane extraction of the lyophilized preparation (Fig. 4, curve a). It displays maxima at 330 and 500 nm.

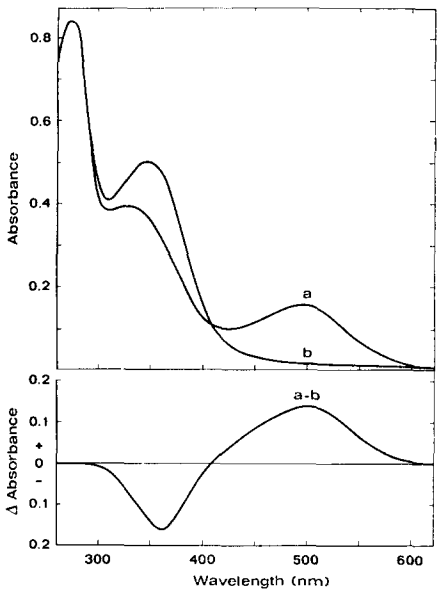

Fig. 4. Photolysis of retinylrhodopsin, prepared from photolyzed rhodopsin by reduction with $NaBH_4$ 1 h after illumination and subsequent treatment with 11-*cis*-retinaldehyde. The absorption spectra were measured after solubilization in 1 % Emulphogene, 0.05 M NH_2OH before (a) and after (b) illumination. The difference spectrum is depicted below by a−b. Curve a represents retinylrhodopsin, Curve b retinylopsin.

The 330 nm peak must be due to the retinyl group, resulting from reduction of the original chromophoric group, now bound as a secondary amine to another amino group. Illumination in the presence of NH_2OH causes a decrease in absorbance at 500 nm and an increase at 360 nm, much as is the case for rhodopsin (Fig. 4, curve b and the difference spectrum, a−b). In order to ensure that the molar amounts of retinylrhodopsin are correctly calculated, the molar absorbance of retinylrhodopsin at 500 nm has been determined. Comparison of the absorbance difference of hexane-extracted retinylrhodopsin at 500 nm before and after illumination with its retinaldehyde content on a molar basis (by the thiobarbituric acid method) gives a molar absorbance of 500 nm of 38 250$\pm$500 ($n = 3$), which is slightly lower than the value of 40 600 for normal rhodopsin [17]. This discrepancy is probably due to a small, residual amount of retinaldehyde, bound elsewhere than at the chromophoric site and difficult to remove by hexane extraction.

DISCUSSION

Our study was aimed at investigating whether migration of the chromophore occurs during the photolytic sequence of rhodopsin. In order to approach the condition of the native photoreceptor membrane as closely as possible we have worked with aqueous membrane suspensions in the absence of detergent. Another requirement for these experiments is the absence of free opsin, so that any photopigment formed upon incubation with 11-*cis*-retinaldehyde after illumination is indeed derived from

illuminated rhodopsin. In order to satisfy this condition, we have used rod outer segments enriched in rhodopsin content by treatment with 11-*cis*-retinaldehyde during isolation [13].

The degree of binding of retinaldehyde in photolyzed rhodopsin has been determined by reduction with $NaBH_4$. Reduction at increasing time intervals after illumination shows that in the absence of NADPH the chromophore is only partially released. Maximal release is 40 % after 60 min, while nearly 60 % of the all-*trans* retinaldehyde remains bound, mainly to protein amino groups (Fig. 2). It seems likely that the retinaldehyde, which is no longer covalently bound to amino groups, does not leave the photoreceptor membrane fragments in view of the lipophilic character of both the medium and retinaldehyde.

Probing the original chromophoric binding site by incubation with 11-*cis*-retinaldehyde appears possible (Table I). Control experiments show that $NaBH_4$ treatment of rhodopsin or retinaldehyde-free opsin does not seriously decrease the recombination capacity as compared to non-treated controls. However, metarhodopsin II, reduced momentarily during illumination, has almost completely lost this capacity. This strongly suggests that the chromophore binding site of rhodopsin remains occupied during the formation of metarhodopsin II. In other words, in metarhodopsin II the chromophore appears to be bound to the same ε-amino group as in native rhodopsin (see also Fig. 1A). It must be admitted that these experiments cannot exclude transiminization to a site so close to the original chromophore binding site that the latter would be shielded from the added 11-*cis*-retinaldehyde through steric hindrance. However, very recently de Grip in our laboratory has observed, that rhodopsin, in which all amino groups except the chromophore bearing group are completely blocked by amidination [7], shows a normal appearance of metarhodopsin II upon illumination. Since amidinated amino groups can no longer form aldimines, this confirms that the metarhodopsin II binding site is identical to the rhodopsin binding site. This conclusion is also supported by the studies of Waggoner and Stryer [18], who find a great similarity in the induced circular dichroism of rhodopsin, metarhodopsin I and metarhodopsin II.

Upon enlarging the time span between illumination and reduction, the recombination capacity gradually increases (Fig. 3). Under these conditions the original chromophoric binding site is apparently becoming available to 11-*cis*-retinaldehyde. The simplest explanation for these observations is that upon illumination the original binding site is vacated during the decay of metarhodopsin II (Fig. 1, B and C). The observed increase in recombination capacity fits a first order process with a half time of about 10 min at 25 °C (Fig. 3, inset) and is paralelled by the time course of the release of retinaldehyde from illuminated rhodopsin (Fig. 2). Metarhodopsin II decays with first order kinetics and a half time of about 7 min at 25 °C (refs 10 and 19). This slightly shorter half time for the metarhodopsin II decay can easily be explained by the different modes of illumination, flash photolysis [10, 19] vs. a 10-min illumination at much lower intensity in this study. The decay of metarhodopsin II at 0 °C takes many hours [20] and at this temperature indeed only a slight increase in recombination capacity is seen (Fig. 3, lower curve).

Under our conditions, apparently 40 % of the chromophore is released (or else bound in such a way that borohydride reduction and protonation results in retinol and free retinaldehyde, respectively), while 60 % migrates to another mem-

brane amino group (Fig. 1, C and B, respectively). This migration could represent the mechanism by which retinaldehyde migrates to other functionally important sites, e.g. the active center of retinoldehydrogenase or isomerase. The involvement of retinoldehydrogenase in chromophore migration will be the subject of a subsequent paper. The place of metarhodopsin III (pararhodopsin) in this chain of events is not yet clear, and is presently being investigated.

ACKNOWLEDGEMENTS

The excellent technical assistance of Mr G. L. M. van de Laar and Miss P. H. M. Geurts is gratefully acknowledged. Financial support was received from the Netherlands Organization for Basic Research (Z.W.O.) through the Netherlands Foundation for Chemical Research (S.O.N.).

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