*Biochimica et Biophysica Acta*, 679 (1982) 95–100
Elsevier Biomedical Press



BBA 41012

# LIGHT-DEPENDENT CONFORMATIONAL CHANGE AT RHODOPSIN'S CYTOPLASMIC SURFACE DETECTED BY INCREASED SUSCEPTIBILITY TO PROTEOLYSIS

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(Received April 8th, 1981)
(Revised manuscript received July 31st, 1981)

*Key words: Rhodopsin; Light-receptor protein; Disk membrane; Proteolysis; Vision; (Bovine rod cell outer segment)*

**Rhodopsin in bovine photoreceptor disk membranes was subjected to limited proteolysis by thermolysin, removing twelve amino acids from rhodopsin's carboxyl terminus. (1) The rate of proteolysis is significantly faster with rhodopsin following exposure to light than with unbleached rhodopsin, provided that the incubation conditions (pH, temperature) favor the formation of metarhodopsin II. (2) If the disk membranes are illuminated under conditions in which metarhodopsin I is the predominant photoproduct (pH 8.5, 0°C), no increase in the rate of proteolysis is observed compared to unilluminated membranes. (3) The light-induced increase in the rate of proteolysis is transient: it slowly decays in the dark to the original rate found for unbleached rhodopsin. The enhanced susceptibility to proteolysis appears to measure a conformational change at rhodopsin's cytoplasmic surface which is first exhibited at the metarhodopsin II stage. This and possibly other light-dependent changes may allow rhodopsin to mediate its signal as a light-receptor protein by binding to and activating certain rod cell enzymes.**

## Introduction

Rhodopsin is the predominant intrinsic membrane protein in disk membranes of rod cell outer segments [1]. Absorption of light by rhodopsin leads to isomerization of its chromophore, 11-*cis*-retinal, followed by a series of events which includes (a) activation of a GTPase [2] and a phosphodiesterase [3], (b) phosphorylation of rhodopsin by a kinase and ATP [4], (c) binding of the GTPase, kinase and another protein to disk membranes [5,6]. Such enzyme activation and binding of proteins to disk membranes are expected to occur at the cytoplasmic surface of rhodopsin and imply that rhodopsin's surface conformation should be different in the bleached and unbleached states. However, evidence for such a surface conformational change has to date been indirect and inferential. We report here direct evidence for such a light-induced conformational change at rhodopsin's surface monitored as an increased susceptibility to proteolytic cleavage following illumination.

In disk membranes of native sidedness, rhodopsin is oriented with its amino terminus located at the inside surface of the disks [7] and its carboxyl terminus exposed at the membrane's external (cytoplasmic) surface [8]. Limited proteolysis of disks using thermolysin cleaves rhodopsin at two positions in its carboxyl terminus, viz., -Thr-Val$\overset{\downarrow 12'}{}$-Ser-Lys-Thr-Glu-Thr-Ser-Gln-Val$\overset{\downarrow 4'}{}$-Ala-Pro-Ala$\overset{1'}{}$ [8]. In this

Abbreviations: SDS, sodium dodecyl sulfate; des(1'-12')-rhodopsin, rhodopsin in which 12 amino acids from the carboxyl terminus have been removed (the primed numbers indicate the position of amino acids starting from the carboxyl terminus).

study we show that proteolysis occurs at a faster rate with bleached rhodopsin than with unbleached rhodopsin, and that this light-induced increase in susceptibility is transient. We present evidence that this increased susceptibility is first exhibited at the meta-rhodopsin II stage.

## Methods

Osmotically intact disk membranes were prepared from freshly purified bovine rod cell outer segments [6] and were separated from broken disks and other material by flotation on Ficoll [9]. Suspensions of freshly prepared disks, bleached or unbleached, were treated in the dark with thermolysin (Calbiochem). Thermolysin stock solution, prepared according to the method of Matsubara [10], was added either to unbleached disks, or to freshly bleached disks (addition about 1 min after illumination), or to disks which had been bleached and then allowed to stand in the dark for 50–100 min. Disks were illuminated using orange light ($\lambda > 540$ nm) either for 3 min (Figs. 1 and 2, Table I) or for 1 min (Table II); about 80% of the rhodopsin was bleached after 1 min. The same temperature was used during illumination of the disks as during their subsequent digestion with thermolysin: either 20°C using a thermostatically controlled water bath (Figs. 1 and 2, Table I), or 0°C using an ice-water bath (Table II). A heat filter (Schott KG 2) was sometimes inserted between the lamp and the samples; the digestion rate of illuminated samples was the same with and without the heat filter. The final concentrations in the incubation mixture were: 1 mg rhodopsin/ml, 2 mM $CaCl_2$, and 10 mM buffer. The buffer was Tris-acetate, pH 7.2 and 8.5, or acetic acid-sodium acetate, pH 5.1, as specified in each case. The weight ratio of rhodopsin to thermolysin was between 500 and 1000 in the experiments shown in Table I and the figures.

At measured times following the addition of thermolysin, 50- or 100-$\mu$l aliquots of the digestion mixture were quenched with 100 or 200 $\mu$l, respectively, of 5 mM EDTA. The samples were then solubilized in SDS and subjected to SDS-polyacrylamide gel electrophoresis [11]. The gels were stained with Coomassie brilliant blue and destained under carefully controlled conditions [12] to allow quantitative determination of the polypeptides present by measuring peak areas of densitograms.

## Results

In the early events of digestion by thermolysin, rhodopsin is converted to des(1′-12′)rhodopsin, and the peptides $\overset{12'}{\text{Val}}$-Ser-Lys-Thr-Glu-Thr-Ser-$\overset{5'}{\text{Gln}}$ and $\overset{4'}{\text{Val}}$-Ala-Pro-$\overset{1'}{\text{Ala}}$ are released into the supernatant [8]. When the digestion mixtures are analyzed by the electrophoretic procedure of Laemmli [11] (Fig. 1), it becomes apparent that a previously undetected product is transiently present. This product is resolved visually on polyacrylamide gels (not shown) and can

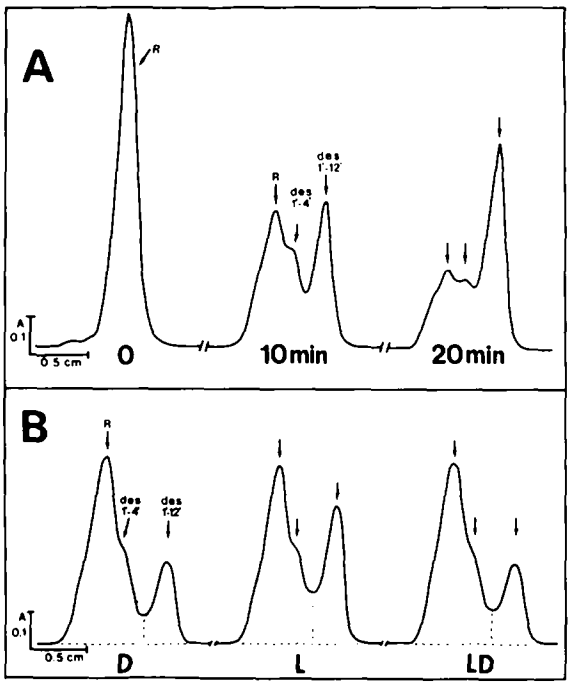


Fig. 1. Densitograms of SDS-polyacrylamide gels [11] showing the separation of rhodopsin and its digestion products. Rhodopsin (R) is converted to des(1′-4′)rhodopsin and des-(1′-12′)rhodopsin (the stained bands on the gels represent opsin, the apoprotein of rhodopsin). Ordinate, $A_{580\,nm}$; abscissa, gel length. Digestion was at 20°C and pH 7.2. Two different thermolysin stock solutions of slightly different activity were used in the two series A and B. A, digestion of unbleached disks for 0, 10 and 20 min. B, three samples were digested for 7 min each: D, unbleached disks; L, freshly bleached disks; LD, bleached disks allowed to stand at 20°C in the dark 52 min prior to addition of thermolysin. The dashed lines indicate baselines and lines separating the peaks as drawn for quantitative evaluation of the densitograms.

only be des(1′-4′)rhodopsin; rhodopsin from which the C-terminal tetrapeptide has been removed. At the thermolysin concentrations used in this study, the digestion does not significantly proceed beyond the formation of des(1′-12′)-rhodopsin.

We have used this analytical system to determine whether rhodopsin which has been bleached behaves in the same manner toward proteolysis. We find that the same products are formed (Fig. 1B) but that for the same length of digestion time, the bleached sample (L) produces more des(1′-12′)rhodopsin than the unbleached sample (D). This effect of illumination is reversible: a sample which has been bleached but then allowed to stand in the dark for nearly an hour at 20°C (Fig. 1B, last trace) now forms the same amount of des(1′-12′)rhodopsin as the sample which had never been illuminated.

The early time course of digestion for all three types of illumination of rhodopsin is shown in Fig. 2. The freshly bleached rhodopsin (L) is clearly digested more rapidly than the unbleached rhodopsin (D),

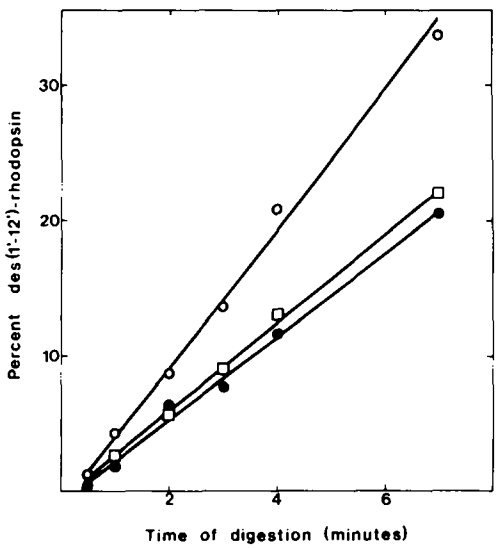


Fig. 2. Formation of des(1′-12′)rhodopsin as a function of time after addition of thermolysin. Digestion in all three cases was carried out in the dark, at 20°C and pH 7.2. (●——●) D, unbleached disks; (○——○) L, disks bleached immediately before, and (□——□) LD, disks bleached 52 min before addition of thermolysin. The ordinate represents the percentage of the area of the des(1′-12′)-rhodopsin peak compared to the total area of rhodopsin, des(1′-4′)- and des(1′-12′)rhodopsin in densitograms.

indicating that illumination has increased the accessibility of rhodopsin's carboxyl terminus to proteolysis. This light-induced increase in accessibility is, however, reversible: disks incubated for 52 min in the dark following illumination are digested at nearly the same rate as unbleached disks. Essentially the same results have been obtained in four time course experiments using four different disk preparations and thermolysin stock solutions *.

The difference in digestion rates between freshly bleached disks and unbleached disks seen in Fig. 2 is statistically significant ($P < 0.01$), as shown with an additional series of samples each treated individually and digested for fixed periods of time (Table I). During the first 2 min of reaction, rhodopsin in freshly bleached disks was digested 1.7-times faster than in unbleached disks. The light-induced increase in proteolysis rate was fully reversed within 95 min at 20°C in the dark (Table I).

The observed increase in proteolytic susceptibility appears to reflect a conformational change of rhodopsin due to illumination. The question arises: at which point in the decay sequence of rhodopsin's photoproducts does this conformational change occur? Which is the earliest photoproduct exhibiting increased susceptibility to proteolysis? Conditions have been described [13,14] which lead to a 'stable' equilibrium between bovine metarhodopsin I and metarhodopsin II and which can be manipulated in such a way that either metarhodopsin I (at high pH) or metarhodopsin II (at low pH) is the predominant photoproduct. We applied these conditions in proteolysis experiments; spectroscopic measurements indicated that, although the equilibrium was not quite stable even at 0°C, metarhodopsin I ($\lambda_{max}$ = 480 nm) was in fact the predominant photoproduct present at 0°C and pH 8.5, and metarhodopsin II ($\lambda_{max}$ = 380 nm) the predominant photoproduct at

* Normally, all of the digestion experiments were carried out in the dark, with or without previous bleaching of the disks, in order to have comparable digestion conditions for all of the samples. In a few additional experiments, digestion of bleached samples was carried out under continuous illumination with white light. The early time course of digestion in these cases (not shown) was essentially the same as for the freshly bleached samples which were then digested in the dark.

TABLE I

FORMATION OF DES(1'-12')RHODOPSIN AFTER 2 AND 4 min OF DIGESTION

Each sample was pipetted and incubated separately (20°C, pH 7.2). Unbleached (D) and bleached (L) samples were incubated alternately in order to exclude artifacts possibly produced by ageing of the disks or of the thermolysin stock solution. No indications of such ageing were, however, observed. Amounts of des(1'-12')rhodopsin are given in percent of total rhodopsin (see legend to Fig. 2), ±S.D. (and number of samples).

| Digestion time (min) | Treatments of disks before addition of thermolysin | | | Ratio L/D |
|---|---|---|---|---|
| | D | L | Light, then 95 min dark | |
| 2 | 11.5 ± 2.6 (5) | 19.2 ± 2.3 (4) | 11.9 ± 2.8 (5) | 1.7 |
| 4 | 25.0 ± 3.0 (5) | 35.0 ± 3.3 (5) | 22.1 ± 2.9 (5) | 1.4 |

0°C and pH 5.1. The results of digestion experiments performed under these conditions, with and without previous illumination of the disks, are shown in Table II. More thermolysin had to be added in order to compensate for the decrease in activity due to the low temperature and extremes of pH, but otherwise the digestion proceeded as shown in Figs. 1 and 2, with the same products being formed.

Illumination at pH 8.5 and 0°C clearly does not increase the rate of proteolysis as compared to the unbleached sample (Table II), whereas illumination at pH 5.1 and 0°C leads to the same increase in proteolytic rate as observed at 20°C (Tables I and II). This indicates that the conformation which is more susceptible to proteolysis is present only when the conditions (low pH) favor the formation of metarhodopsin II. The existence of various forms of metarhodopsin II has been suggested in the literature (e.g., Ref. 14); our data cannot distinguish which of them is the most susceptible substrate for thermolysin, nor can they exclude the possibility that photoproducts later than metarhodopsin II may still be in this more susceptible form. In any event, the data indicate that metarhodopsin II is the first photoproduct with increased susceptibility to proteolysis. Metarhodopsin I is no more susceptible than unbleached rhodopsin (Table II), and opsin has the same low susceptibility (Figs. 1B and 2, Table I).

The data shown in Table II are representative of five similar series of experiments using different ratios of rhodopsin to thermolysin leading to extents of proteolysis between 8 and 45% over a 2 min time period at 0°C. The light-induced increase in proteolytic susceptibility was always significant at pH 5.1 and 0°C, and was never observed to a significant extent at pH 8.5 and 0°C. The highest L/D ratio ever observed in a single experiment at pH 8.5 and 0°C was 1.15.

TABLE II

pH DEPENDENCE OF DES(1'-12')RHODOPSIN FORMATION

Samples were pipetted and incubated individually, alternating between unbleached (D) and bleached (L) samples, as described in Table I. The conditions of temperature and pH were the same during illumination and digestion of each series of samples. Digestion time was 2 min in all cases. Data are given ± S.D. (and number of samples). Meta, metarhodopsin.

| Conditions | Predominant photoproduct | Thermolysin/ rhodopsin mass ratio | % des(1'-12')rhodopsin formed following treatment of disks in | | Ratio L/D |
|---|---|---|---|---|---|
| | | | D | L | |
| 0°C, pH 8.5 | Meta I | 1 : 11 | 17.1 ± 1.4 (4) | 17.3 ± 0.9 (4) | 1.0 |
| 0°C, pH 5.1 | Meta II | 1 : 110 | 16.0 ± 1.4 (5) | 24.2 ± 1.4 (5) | 1.5 |
| 20°C, pH 6.5 | Meta II | 1 : 400 | 23.7 ± 2.0 (6) | 34.8 ± 2.0 (8) | 1.5 |

## Discussion

Our results demonstrate that light induces a transient conformational change at rhodopsin's cytoplasmic surface: proteolytic attack at rhodopsin's carboxyl terminus proceeds more rapidly with freshly bleached disks than with unbleached disks, provided metarhodopsin II has been formed in the sequence of photoproducts. This light-induced increase in the initial rate of digestion is slowly abolished with time following illumination, probably on a time scale of tens of minutes (unpublished observations).

It cannot be decided at the present time whether the primary site of light-enhanced cleavage is at the 12′- or at the 4′-position or at both positions. The final product, des(1′-12′)rhodopsin, is obviously produced more rapidly following illumination; but it is always accompanied by some des(1′-4′)rhodopsin, even at very early (not shown in Fig. 1) as well as at late digestion times, with and without previous illumination (Fig. 1). This demonstrates that at least a considerable part of the cleavage at the 4′-position must occur before cleavage at the 12′-position; but the poor resolution of des(1′-4′)rhodopsin on gels does not allow quantitative determination of its rate of formation and decay in light as compared to darkness.

The following arguments point out that the observed increase in the rate of proteolysis is not an artifact but is really due to the formation of metarhodopsin II. (i) Any direct activation of thermolysin by light can be excluded (see Methods). (ii) The samples did not significantly warm up during illumination; the insertion of an additional heat filter had no influence on the results. (iii) Illumination at pH 5.1 leads to an increased rate of proteolysis, whereas illumination at pH 8.5 under otherwise similar conditions does not significantly increase the rate (Table II). The major difference between these two conditions is only that at pH 5.1, metarhodopsin II is formed whereas at pH 8.5, it is not formed to an appreciable extent.

Different lines of published evidence have indicated that there are differences in the conformation of rhodopsin following bleaching, although none of these studies has localized the region of the protein in which the change occurs. Changes in the environment of a tryptophan residue upon bleaching have been monitored by circular dichroism [15] and by linear dichroism [16]. Some other amino acids change their environment in bleached rhodopsin as monitored by proton uptake or release [13,14,17] and by the appearance of a reactive sulfhydryl group [18].

Rhodopsin's carboxyl terminus contains serine and threonine residues which are phosphorylated by a specific protein kinase and ATP in a light-dependent reaction [4,19]; four of these serine and threonine residues are located in the 1′-12′ sequence of rhodopsin which is removed by limited proteolysis with thermolysin [19]. The ability of the kinase to phosphorylate rhodopsin's carboxyl terminus following bleaching, and the increased rate of proteolysis with thermolysin of that region, probably measure the same conformational change. It should be noted, however, that the stimulation by light is much greater for the phosphorylation reaction than for the proteolysis: about 50–100-times more phosphate is incorporated into bleached than into unbleached rhodopsin [6,20], whereas the proteolytic susceptibility is only increased by a factor of less than two. This is mainly due to the relatively high susceptibility to proteolysis even of unbleached rhodopsin. The kinase thus recognizes much more specifically the light-induced conformational change than does the protease. It has been shown that the kinase undergoes a transient light-induced binding to disk membranes which probably serves to regulate the activity of the phosphorylation reaction [6], whereas no such specific and regulatory interaction is to be expected for the protease.

## Acknowledgements

This work was supported by a grant from Deutsche Forschungsgemeinschaft, SFB 160 (to H.K.), NATO grant RG 076.80, NIH grant EY 2875 and a grant from Deutscher Akademischer Austauschdienst (to P.A.H.). We thank Dr. M. Chabre for stimulating discussions.

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