Localization of light-induced conformational changes in bovine rhodopsin

Chantal Pellicone, Gérard Nullans and Noëlle Virmaux

Centre de Neurochimie du CNRS – 5, Rue Blaise Pascal, 67084 Strasbourg Cédex, France

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Conformational changes in the extradiscal regions of rhodopsin induced by illumination were investigated by modifying the visual pigment by mild treatment with cyanogen bromide prior to and after light exposure. Light induced an increased yield of cleavage of the Met bond 253–254 and a new cleavage at the Met bond 155–156 of the rhodopsin polypeptide chain. These residues, located at the beginnings of the membrane-buried helices 6 and 4, respectively, were concluded to become extradiscally exposed upon illumination.

Conformational change *Rhodopsin* *Photoreceptor* *CNBr cleavage* *Extradiscal region* *Dynamic structure*

1. INTRODUCTION

The primary event in visual excitation is the photon capture by rhodopsin leading to the isomerization of the 11-*cis*-retinal molecule bound to the apoprotein moiety, opsin. The action of light on retinal induces a transient conformational change of the membrane chromophoric site giving rise to the spectral intermediates of rhodopsin. These changes must be transmitted to the extradiscal surface of rhodopsin which in turn triggers the cyclic nucleotide enzymatic cascade [1,2].

Among the different experiments that have monitored extradiscal conformational changes only two investigations have demonstrated the involvement of defined structural regions of rhodopsin: (i) the third extradiscal loop, has been suggested to be involved in the light-dependent binding of a peripheral membrane protein (G-protein) [3], i.e., the third extradiscal loop must undergo a conformational change after light exposure (bleaching) unmasking a region that interacts with the G-protein; (ii) another region, the 12 C-terminal AAs, has been shown to change its conformation after bleaching because of its increased accessibility to limited thermolysin proteolysis [4]. Here, we focused our investigations of conformational change on two extradiscal regions: the second and third extradiscal loops. In fact, in the topographic models of rhodopsin in the disc membrane [5–7], the second extradiscal loop is rather large and could also undergo a light-induced conformational change. From the recently elucidated sequence of rhodopsin [5] we noticed that this second loop contains a methionine residue more cytosolic than that present in the third extradiscal loop. As methionyl bonds can be modified and split by cyanogen bromide (CNBr) we selected these methionine residues as probes of light-induced conformational changes. We used CNBr in a non-conventional way that is original, both in the mild conditions employed and in the limited proteolysis obtained, to demonstrate the differing accessibility of extradiscal methionines in unbleached and bleached rhodopsin.

Abbreviations: G-protein, GTP-binding protein or transducin; ROS, rod outer segments; PAS, periodic acid Schiff staining

2. MATERIALS AND METHODS

Rod outer segments (ROS) were purified from fresh bovine retinas as previously described [8].

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One fraction of a ROS preparation corresponding to 10 retinas was suspended in 10 ml of 66 mM phosphate buffer (pH 7.0) and divided into two equal portions. One portion was illuminated 1 min at 0°C under white light giving a 70% bleaching. Digestion of ROS was performed in darkness by adding 10 mg of CNBr representing a 50-fold excess per methionine residue. After 1 h at 20°C, reaction was stopped by immediate cooling at 0°C followed by a 100000 × *g* centrifugation. The purification of CNBr modified rhodopsin (Rh-B) and opsin (Ops-B) was as in [9] and also served to eliminate excess CNBr. Controls were rhodopsin (Rh) and opsin (Ops) purified from ROS and bleached ROS incubated in the same conditions without CNBr. Purified rhodopsins were suspended in 2.5 ml phosphate buffer for determination of absorption spectra. Protein concentrations were determined by the method of Lowry et al. [10] and suspensions adjusted to 1 mg rhodopsin/ml. Rh-B and Ops-B were reduced as suspensions in phosphate buffer with 100 mM dithioerythritol during 2 h at 20°C and then solubilized in 0.3% SDS during 1 h at 20°C. Reduced samples were analyzed by SDS electrophoresis in 12% polyacrylamide gels and stained for protein and carbohydrate contents [11]. For retinal detection Rh-B and Ops-B were reduced by sodium borohydride before dithioerythritol treatment followed by examination under UV light according to [12]. Isolation of Rh-B and Ops-B bands by semi-preparative SDS-polyacrylamide gel electrophoresis (SDS–PAGE) was performed as in [11]. For each band the AA N-terminals were identified by the dansylation procedure as in [11] and by the 4-*N,N*-dimethylaminoazobenzene-4′-isothiocyanate procedure and subsequent polyamide sheet chromatography [13].

3. RESULTS AND DISCUSSION

Cyanogen bromide cleavage of rhodopsin has been performed on the protein solubilized in formic acid to establish its primary structure [14–16]. Here CNBr was employed on rhodopsin-containing disc membranes and under mild conditions to preserve the native conformation of the protein. One of the best controls of rhodopsin conformation is its visible absorption spectrum (fig.1). Rh-B presents the characteristic 500 nm maximum

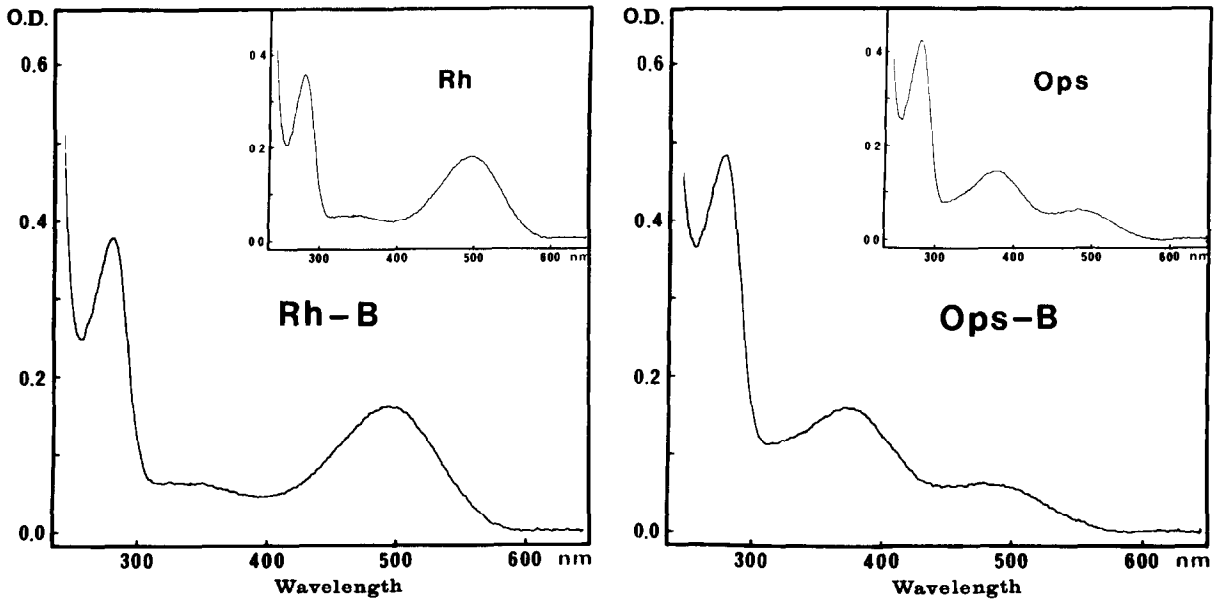

Fig.1. Absorption spectra of CNBr-modified rhodopsin (Rh-B) and CNBr-modified opsin (Ops-B). Insets: absorption spectra of native rhodopsin (Rh) and opsin (Ops). Samples containing 200 μg protein were dissolved in the detergent emulphogen.

of native rhodopsin and a very similar 280 nm/500 nm absorbance ratio. Ops-B gives the same spectral shape as opsin with two maxima: one at 500 nm corresponding to unbleached rhodopsin, the other at 367 nm due to all-*trans* retinal and Meta II rhodopsin. Comparing the 367 nm/500 nm absorbance ratios of Ops and Ops-B we noticed slightly more bleaching of Ops-B. If the spectral properties of CNBr-treated rhodopsins are conserved this means that the conformation of the retinal site is little or not at all affected and thus probably the same is true for other membrane domains of rhodopsin essential to stabilize the structure.

Electrophoresis patterns of Rh-B and Ops-B products are given in fig.2. The Rh-B gel shows 5 bands termed Rh-B1 (37 kDa), Rh-B2 (28 kDa), Rh-B3 (21 kDa), Rh-B4 (18 kDa) and Rh-B5 (14 kDa). After Rh-B1, Rh-B2 is the predominant band which means that the most accessible domain of rhodopsin to CNBr is the C-terminal region. The fragment sizes clearly indicate that they resulted from incomplete splitting of peptide bonds accessible to CNBr and/or are still in an aggregated state. Some of them are overlapping peptides from the N-terminus of rhodopsin as they contain the carbohydrate residues located in the N-terminal region of the protein: Rh-B1, Rh-B2 and Rh-B3. After the sodium borohydride reduction, Rh-B presents the typical fluorescence of the retinyl group. This fluorescence was recovered in each band except for Rh-B3. The Ops-B gel presents two critical features: (i) the yield of cleavage is dramatically increased as shown by the prevalence of the Rh-B3 and Rh-B5 fragments; (ii) one supplementary band, termed Rh-B'3 (19.5 kDa), containing the carbohydrate residues but not the retinal appears.

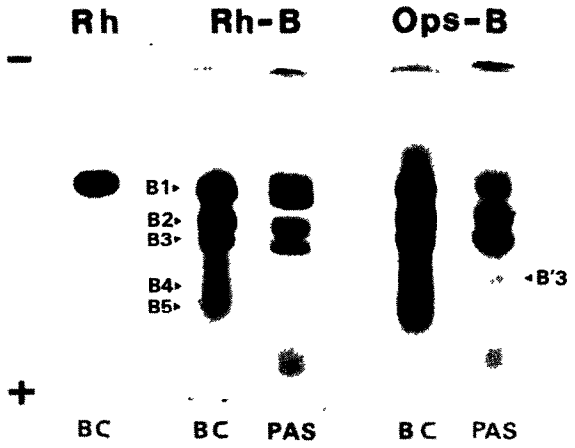

Fig.2. SDS–PAGE of rhodopsin vesicles prior to and after CNBr treatment of bleached and unbleached ROS. Samples were dissolved in 0.3% SDS. BC, Coomassie blue staining; PAS, carbohydrate staining.

Cyanogen bromide bands were further characterized after their isolation by N-terminal AA analysis. For Rh-B, two free N-terminal AAs were detected: valine and serine. The different characteristics of the Rh-B and Ops-B bands are summarized in table 1. From this table and according to the structure of rhodopsin [5] we have deduced the origin of each fragment of Rh-B. Rh-B1 presents all the characteristics of native rhodopsin and therefore it was concluded to be the non-digested visual pigment. Rh-B2 was shortened from the C-terminal end after helix 7 at Met 317 and Met 309 but the splittings in this region cannot be detectable by N-terminal A analysis because the cytosolic fragment(s) is (are) lost during the purification of Rh-B; the presence of the N-terminal serine indicates a cleavage of the Met 143–Ser 144 bond and that Rh-B2 must be a mixture of the fragments 1–308 (or 1–317) and 1–144 aggregated to 145–308 (or 145–317). These two last fragments are recovered in a non-aggregated form in Rh-B4 despite the negative PAS staining due to the poor yield of 1–144 peptide. The cleavage of the Met 143–Ser 144 bond known to be rather resistant to CNBr and located in the second

Table 1

Characteristics of Rh-B and Ops-B bands

Bands	Molecular mass (kDa)	Carbohydrate	Retinal	N-terminal
B1	37	+	+	none
B2	28	+	+	serine
B3	21	+	–	none
B'3	19.5	+	–	none
B4	18	–	+	serine, glycine[a]
B5	14	–	+	valine

[a] Only in Ops-B

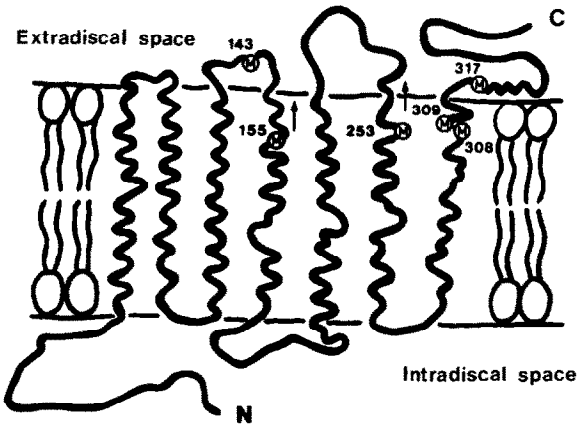

Fig.3. Localization of methionine residues modified by CNBr. The disposition of the rhodopsin polypeptide chain in photoreceptor membrane was taken from [5]. Arrows indicate possible shifts of helices 4 and 6 after illumination.

extradiscal loop can be explained by the fact that in our conditions an important excess of reagent is available to only the extradiscal methionine residues (fig.3). Rh-B3, derived from the N-terminal end and lacking the retinal site located in helix 7, comes from a cleavage of the methionyl bond 253–254 which produces its complementary fragment, Rh-B5. We found that Rh-B5 has a serine as N-terminal and contains the retinal site and therefore would correspond to the peptide 254–308 (or 254–317). The accessibility of the Met 253 which is considered to be located inside the membrane close to the third extradiscal loop [5,6] can be explained by the small size of the CNBr molecule.

In the case of Ops-B apart from valine and serine one supplementary N-terminal AA was detected: glycine (table 1). Therefore the main fragments obtained in Ops-B, Rh-B3 and Rh-B5 are the peptides 1–253 and 254–308 (or 254–317), respectively. The N-terminal glycine would originate from a cleavage of the methionyl-bond 155–156, located inside the membrane in darkness and neighbouring the second extradiscal loop (fig.3), producing the fragment Rh-B'3 (peptide 1–155). This cleavage also generates the cytosolic fragment 144–155 which is lost during the purification procedure and the glycine N-terminal fragment 156–308 (or 156–317) recovered in Rh-B4. This band also contains the preceding fragment 145–308 (or 145–317) found in Rh-B as retains an N-terminal serine.

In other words, the exposure of ROS to light has increased the cleavage yield at the methionyl bond 253–254 and has produced a supplementary cleavage close to the second extradiscal loop. Results of CNBr treatment of bleached ROS suggest that extradiscal loops of bleached rhodopsin become more accessible to the reagent. Apart from the C-terminal region, the third extradiscal loop changes its conformation so that a membrane residue (Met 253) was more dramatically accessible to CNBr. In the same way there is a light conformation change in the second extradiscal loop since a formerly membrane-buried residue (Met 155) is now accessible to CNBr.

Met 253 and Met 155 are residues located at the beginnings of helix 6 (connected to the third extradiscal loop) and of helix 4 (connected to the second extradiscal loop), respectively, which become more accessible to extradiscal reagents. This increased accessibility is perhaps due to a shift towards the extradiscal space of helices 6 and 4 induced by the structural change occurring in the retinal pocket upon illumination.

Thus CNBr treatment of ROS represents an alternative strategy to the limited proteolysis of rhodopsin, usually performed with proteolytic enzymes (review [17]) supplying an interesting probe to reveal light-induced conformational changes in rhodopsin.

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