STRUCTURAL STUDIES ON THE CHROMOPHORE ATTACHMENT SITE OF RHODOPSIN FOLLOWING BLEACHING

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Received 30 November 1981

1. Introduction

The capture of incident light by vertebrate rhodopsin is accompanied by isomerisation of the chromophore 11-*cis* retinal, and conformational changes in the polypeptide, opsin [1]. The resultant effect is the conversion of electromagnetic energy into a neural signal. Long after the effective primary event has occurred, opsin continues to progress through a series of spectrally defined structures [2,3]. During this process, the now all-*trans* chromophore migrates from the original binding site [4]. Following our identification of this primary attachment site for 11-*cis* retinal [5,6], we investigated the process of migration. These studies indicate that the chromophore remains attached to the same lysyl residue for at least 5 min after illumination and that subsequent migration occurs to the lipid milieu rather than onto any further lysyl side-chains.

2. Materials and methods

Dark-adapted, rod outer segment (ROS) discs were prepared from sheep retina as in [7]. The synthesis of 11-*cis* [15-^{3}H]retinal, the regeneration of rhodopsin and the digestion of the protein with *Staphylococcus aureus* V8 protease are described in [6].

In essence the experiment consisted of adding KBH_4 (3 mg/ml) to [^{3}H]retinal-regenerated intact and V8-cleaved protein in situ at various times after exposure of the system to light (0–5 min). This borohydride treatment will covalently fix the chromophore to any lysine with which it forms a Schiff base. The membranes were dissolved and subjected to SDS–urea polyacrylamide gel electrophoresis and 2 mm gel slices analysed for their radioactive content. The fragments generated by digestion of the protein with V8 protease were isolated from the washed membranes by gel filtration with Sephadex LH60 in formic acid (90%, v/v): acetic acid:chloroform: ethanol, 2:2:4:2 (v/v) [6]. Cleavage of the small V8 fragment (V8-S) was carried out in 70% formic acid for 24 h in the dark using 100-fold molar excess (with respect to methionines) of cyanogen bromide [6]. Further purification was carried out with Sephadex LH60 as above, only using formic acid (90%, v/v): ethanol as solvent.

Sephadex LH60 was obtained from Pharmacia Fine Chemicals, *S. aureus* V8 protease from Miles Biochemicals and KBH_4 from BDH Ltd.

Abbreviations: CNBr, cyanogen bromide; SDS, sodium dodecyl sulphate; PE, phosphatidyl ethanolamine

3. Results

Cleavage of the protein with V8 protease yielded the peptide fragments seen in fig.1. The pattern was identical whether reductive fixation of the chromophore had preceded or followed digestion of the protein. In all cases, radioactivity was associated with the smaller V8-S fragment and never with larger V8-L (fig.2). (A small 7 residue peptide was also released into the supernatant by protease treatment [5,8]. This peptide, which comes from the C-terminus of the protein, contained no lysines and no radioactivity.) The same profile of radioactivity was obtained when the borohydride was added 5 s before or at 30 s, 1 min, 2 min, 4 min and 5 min after the onset of bleaching. This indicated that no transfer of chromo-

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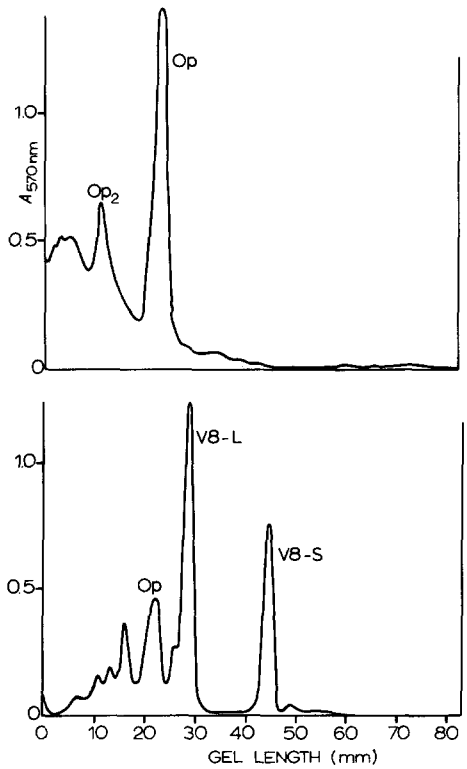

Fig.1. SDS–urea 10% polyacrylamide gel electrophoresis [12] of the intact and *S. aureus* V8 protease-cleaved ovine rhodopsin. Op denotes monomeric rhodopsin; $(Op)_2$, the dimeric form, and V8-L (27 000) and V8-S (12 000), the 2 principal cleavage products. The *Y*-axis indicates absorbance at 570 nm and the *X*-axis gel length. Protein loading was 20–25 μg/gel.

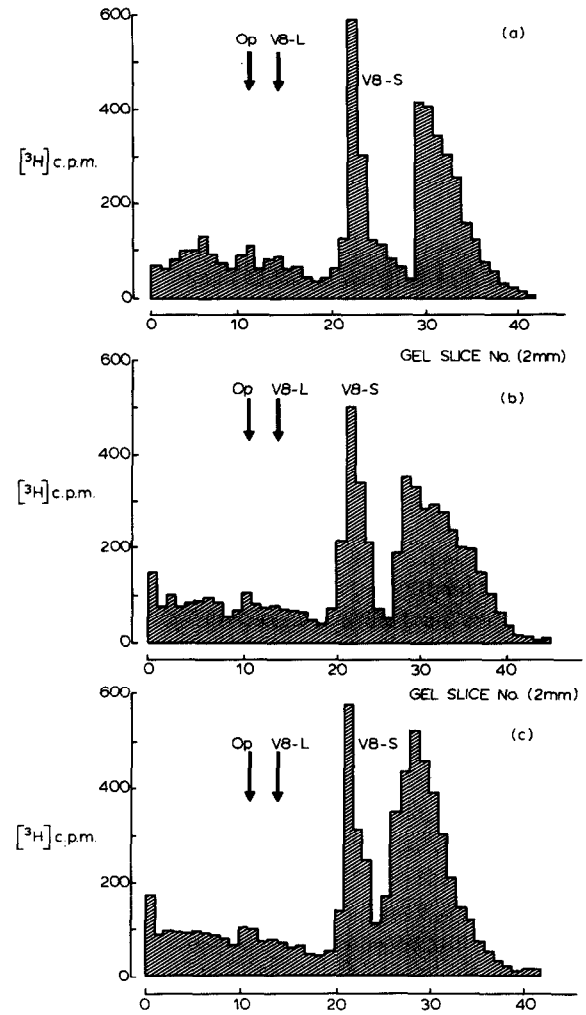

Fig.2. SDS–urea 10% polyacrylamide gel electrophoresis of [^{3}H]retinylopsin following treatment with KBH_4 and V8 protease. The 2 arrows denote the positions of intact monomeric opsin and the V8-L fragment, respectively. KBH_4 was added at (a) 0, (b) 2 min and (c) 4 min related to the onset of illumination.

phore to amino groups present on V8-L occurred during at least the first 5 min of bleaching of the intact protein in situ. At this time, ~90% rhodopsin had been bleached.

In order to examine more carefully the attachment sites on the [^{3}H]retinyl-protein, larger scale digestions with V8 protease were carried out and the V8-L and V8-S peptides isolated (fig.3). Again no radioactivity was associated with V8-L. Gel electrophoresis indicated that the radioactivity in the excluded fraction was due to uncleaved and aggregated opsin. Fractionation on Sephadex LH-60 of the peptide mixture resulting from cleavage of V8-S with cyanogen bromide, indicated that radioactivity was now to be found in the material designated CNBr 1 (fig.4). No chromophore was found associated with any of the other smaller peptides generated by cleavage with CNBr.

The small included peak of radioactivity (designated II) was found to represent a 20 residue fragment resulting from partial cleavage at a resistant Met–Thr bond [6,9]. This same result was obtained whether fixation occurred at 0,30s, 1 min, 2 min or 4 min after exposure to light. Since CNBr-1 contains only one lysine residue (fig.5), we may conclude that transiminization of retinal to another group on opsin does not occur.

The molar ratios of chromophore to peptide (as

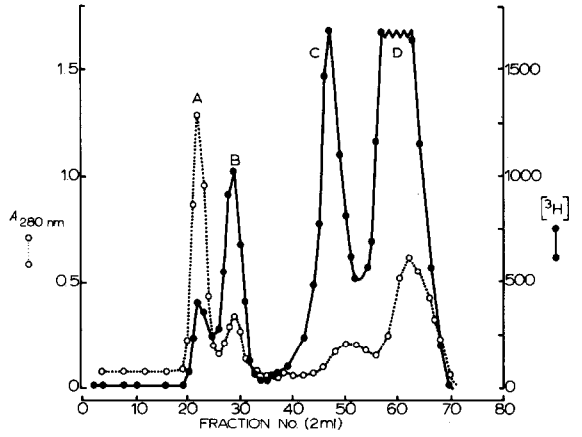

Fig.3. Separation of V8-L and V8-S. Reductively fixed and protease-cleaved opsin was solubilized from the membrane as described and the mixture fractionated using Sephadex LH-60 (1.6 cm × 65 cm) in formic acid (90%, v/v):acetic acid:chloroform:methanol (2:2:4:2, by vol.). The extinction was monitored at 280 nm (o——o) and 20 μl samples for the determination of radioactivity (•——•).

determined from amino acid analysis) were found to be 0.63 and 0.59 when fixation was performed at time $t = 0$ and $t = 4$ min. These values represent close to the maximum practicable yield since the preparation contained a proportion of opsin which varied according to the season (up to 30%) and which was

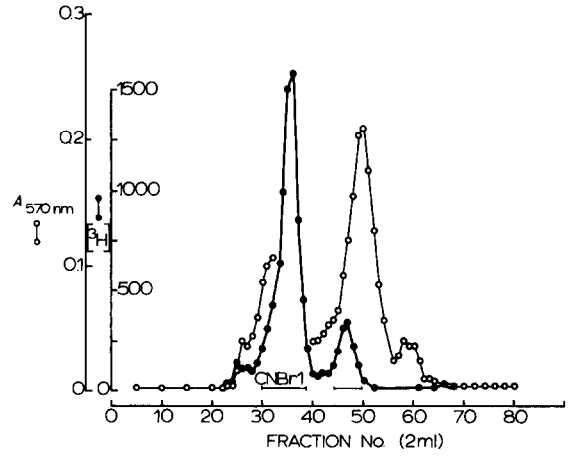

Fig.4. Separation of CNBr-peptides of V8-S. Purified V8-S was cleaved with a 100-fold molar excess of CNBr and the rotary evaporated material fractionated using Sephadex LH-60 (1.6 × 65 cm) equilibrated and eluted in formic acid (90%, v/v):ethanol (3:7, v/v); 400 μl was sampled for protein assay [13] (o——o) and 50 μl sampled for radioactivity (•——•).

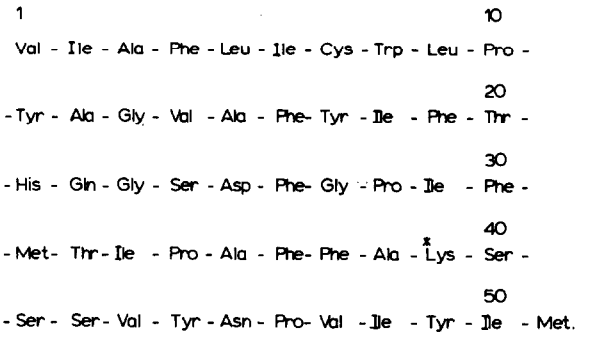

Fig.5. The primary structure of CNBrl: *denotes the attachment site of retinal.

not regenerable but did contribute to the protein estimations.

Two other radioactive peaks (fig.3) are seen in the profile of the separation of V8-L and V8-S. Neither of these contained peptide material, but both contain lipid, much more so in the case of peak D.

4. Discussion

These data indicate clearly that retinal associates with only a single lysyl residue in opsin. The molar ratios obtained are close to the theoretical maximum, indicating that the chromophore remains attached to this lysine for at least 5 min after exposure of the photocomplex to light, and hence long after the neural signal has been generated. Subsequent reductions in this value at longer time intervals probably represent dissociation of all-*trans* retinal from opsin prior to fixation. These periods of time represent stages in the photolytic cycle of rhodopsin corresponding to the decay of metarhodopsin II and events thereafter.

The identity of the immediate recipient, if any, for retinal has not been unambiguously confirmed. It is observed, however, that the excess chromophore becomes rapidly associated with the lipid fraction of the membrane and some of it forms, under the conditions of reductive fixation, a species which has a higher M_r than the average phospholipid. It may be relevant that where borohydride treatment is carried out on V8 protease-cleaved and subsequently delipidated and purified rhodopsin, fixation ratios (0.74) are slightly higher than the in situ fixed levels. Our results, therefore, are compatible with the interpre-

tation [4] obtained using an approach based on the liberation of the 11-*cis* retinal attachment site. Our preliminary analysis of the migration process indicates that in vitro the first receptor for the all-*trans* chromophore is a phospholipid molecule – probably PE since ethanolamine is recovered in the products obtained by hydrolysis of peak C (fig.4) with 6 M HCl, 18 h 110°C, [10,11].

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