IDENTIFICATION OF THE ACTIVE SITE POLYPEPTIDE IN LABELED PHOTORECEPTOR MEMBRANES DIGESTED WITH PAPAIN

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1. Introduction

It is now generally recognized that an understanding of the mechanism through which photopic stimulation is translated into a visual impulse requires a knowledge of the organization of rhodopsin in the disc membrane of rod outer segments (ROS). Attempts directed towards this end include the work of Trayhurn et al. [1,2] who observed that rhodopsin in disc membranes was digested by papain into a functionally active membrane bound fragment, termed rhodopsin-core, which was assigned a mol.wt of 24 800 based on sodium dodecyl sulphate (SDS)–acrylamide gel electrophoresis. It was inferred that the retinal binding site resided in the rhodopsin-core and that in its formation about one-third of the polypeptide chain had been removed from rhodopsin (mol.wt 36 000). Using ROS in which rhodopsin is specifically labelled with tritium at the active site we have now critically examined the location of the retinal binding site in ROS digested with papain. Our results show that papain cleaves rhodopsin in disc membranes into fragments held as a non-covalent complex in which a polypeptide of mol.wt 15 500 contains the retinal binding site. From the evidence presented we have concluded that papain digestion of rhodopsin in disc membranes does not produce a true rhodopsin-core, but a multiple-cleaved species hereafter referred to as cleaved-rhodopsin.

2. Experimental

2.1. *Preparation of ROS-containing tritium at the active site of rhodopsin*

ROS were prepared from bovine retinae as previously described [3]. 11-*cis*-[15-^{3}H]retinal was synthesized and purified as described [3,4] and had a specific radioactivity of 10.9 Ci/mol except where stated otherwise. ROS were homogenised at 3–4 A_{500} units/ml in 0.067 M potassium phosphate buffer, pH 7.0, bleached, and regenerated by adding a 2-fold molar excess of 11-*cis*-[15-^{3}H]retinal in Tween-80 [3]. After incubation in the dark at 37°C for 1 h the ROS were successively washed and centrifuged (100 000 × *g* for 30 min) with water, 2% (w/v) hydroxylamine, 1% (v/v) formaldehyde and water. This procedure ensured that any excess tritiated retinal was converted into the corresponding oxime which could not form random Schiff-bases with the protein.

2.2. *Column chromatography of tritiated rhodopsin*

ROS regenerated from 11-*cis*[15-^{3}H]retinal were solubilized in 2% (w/v) Ammonyx LO (Venture Chemicals, Reading, Berks., England) containing 0.01 M imidazole, pH 7, as described [5]. 5–10 A_{500} units of the resulting rhodopsin were applied under dim red-light to a 10 × 1 cm column containing freshly prepared calcium phosphate [6]. After washing with 25 ml of the above buffer to remove any unbound radioactivity the radioactive rhodopsin was eluted with a 60 ml gradient from 5–300 mM potassium phosphate, pH 7, containing 2% (w/v) Ammonyx LO.

2.3. *Treatment of tritiated ROS with papain*

ROS regenerated with 11-*cis*[15-^{3}H]retinal were digested with papain using the incubation conditions exactly as described [2]. The digestion was terminated by the addition of iodoacetamide to a concentration of 10 mM, the ROS collected by centrifugation at 190 000 × *g* for 15 min and washed 2× by recentri-

fugation. No loss in 500 nm absorbance occurred during proteolysis.

2.4. *Polyacrylamide gel electrophoresis*

Tritiated ROS and those incubated with papain were suspended in water (2 A_{500} units/ml) and irradiated for 1 min in the presence of $NaBH_4$ (1.5 mg/A_{500} unit) to fix the label to the protein as a stable *N*-retinyl derivative [4]. Non-fixed tritiated retinal was removed by methanol washing. 70–80% fixation of tritium to the protein was obtained. The protein was then solubilized in 5% (w/v) SDS, 50 mM sodium carbonate in the absence or presence of 10% (v/v) 2-mercaptoethanol as indicated and 100–150 μg electrophoresed on gels containing 12.5% (w/v) acrylamide, 0.416% (w/v) bis-acrylamide and 6 M urea [7]. The gels were stained and destained by the method of Weber and Osborn [8] and scanned at 265 nm in a Joece Loebl densitometer.

2.5. *Radioactivity measurement*

Aqueous samples (0.2 ml) and methanol samples (1 ml) were counted in 15 ml NE260 (Nuclear Enterprises, Sighthill, Edinburgh, Scotland). Polyacrylamide gels containing radioactive bands were sliced into 2 mm discs. Each disc was analysed for radioactivity using a Packard Tri Carb sample oxidizer. All vials were then counted in a Phillips scintillation counter. The recovery of the radioactivity originally applied to the gels was greater than 90%.

3. Results

To test the radiochemical purity of the ROS regenerated from 11-*cis*-[15-^{3}H]retinal they were analysed by two different methods. Firstly, a sample was solubilized in Ammonyx LO and purified by column chromatography on calcium phosphate (fig.1). The only radioactive protein that eluted was rhodopsin and the appearance of radioactivity exactly followed the 500 nm absorbance peak. The stoichiometry of the incorporation of the tritiated retinal into rhodopsin, calculated from the 500 nm absorbance peak using ϵ_{500} = 40 600 [9], was 1.06 mol/mol rhodopsin which suggests that the label was specifically located at the active site. The spectrum of rhodopsin purified from

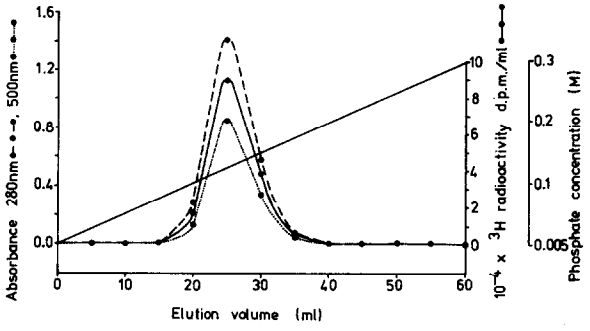

Fig.1. Chromatography of solubilized ROS regenerated from 11-*cis*-[15-^{3}H]retinal on calcium phosphate. 7.1 A_{500} units were purified as described in the Experimental section. Fractions of 5 ml were collected. 6.8 A_{500} units of labelled rhodopsin of A_{280}/A_{500} ratio = 1.7 and containing 1.06 mol tritiated retinal/mol were recovered. The specific radioactivity of the 11-*cis*-[15-^{3}H]retinal used in the initial regeneration in this experiment was 1.82 Ci/mol.

tritiated ROS was similar to that of native rhodopsin and had an A_{280}/A_{500} ratio = 1.7 (fig.2).

Secondly, the labelled ROS were fixed with $NaBH_4$ (see Experimental), solubilized in SDS in the absence (fig.3a) or presence (fig.3b) of 2-mercaptoethanol and analysed by acrylamide gel electrophoresis. In both cases most of the radioactivity on the gel was present in the 36 000 mol.wt region which corresponds to the position of bovine opsin. In the absence of

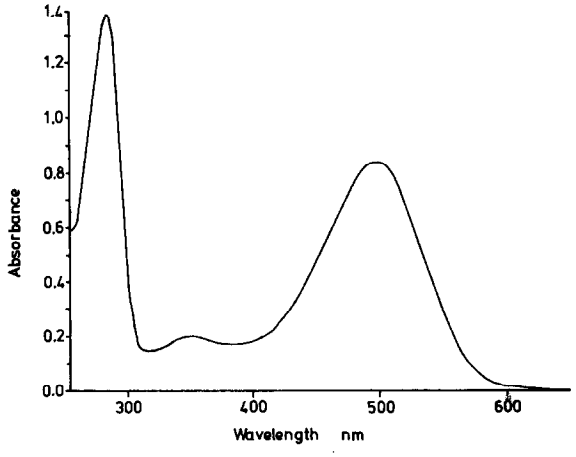

Fig.2. Spectrum of tritiated rhodopsin purified by column chromatography as in fig.1.

2-mercaptoethanol some of the radioactivity on the gel (fig.3a) was also found in species of higher mol.wts which we believe are polymers of rhodopsin formed by air oxidation. The minor radioactive peak near the dye-front may be attributed to a small amount of unbound tritiated retinol not removed by methanol washing. These results when taken in conjunction with the column data establish that our ROS regenerated from 11-*cis*-[15-^{3}H]retinal contain the label exclusively in the active site of rhodopsin and not bound to protein via random Schiff-base linkages.

The specifically labelled ROS were incubated with papain and then fixed with $NaBH_4$. Solubilization in SDS in the absence of 2-mercaptoethanol followed by electrophoresis gave three bands A, B and C (fig.4a). The apparent mol.wts of fragments A, B and C were estimated as 23 000, 15 500 and 6000, respectively. In view of the uncertain behaviour of intrinsic membrane bound proteins, in general, and of glycoproteins, in particular, during SDS-acrylamide gel electrophoresis [10] the mol.wts assigned to the three fragments A, B and C may not represent their true

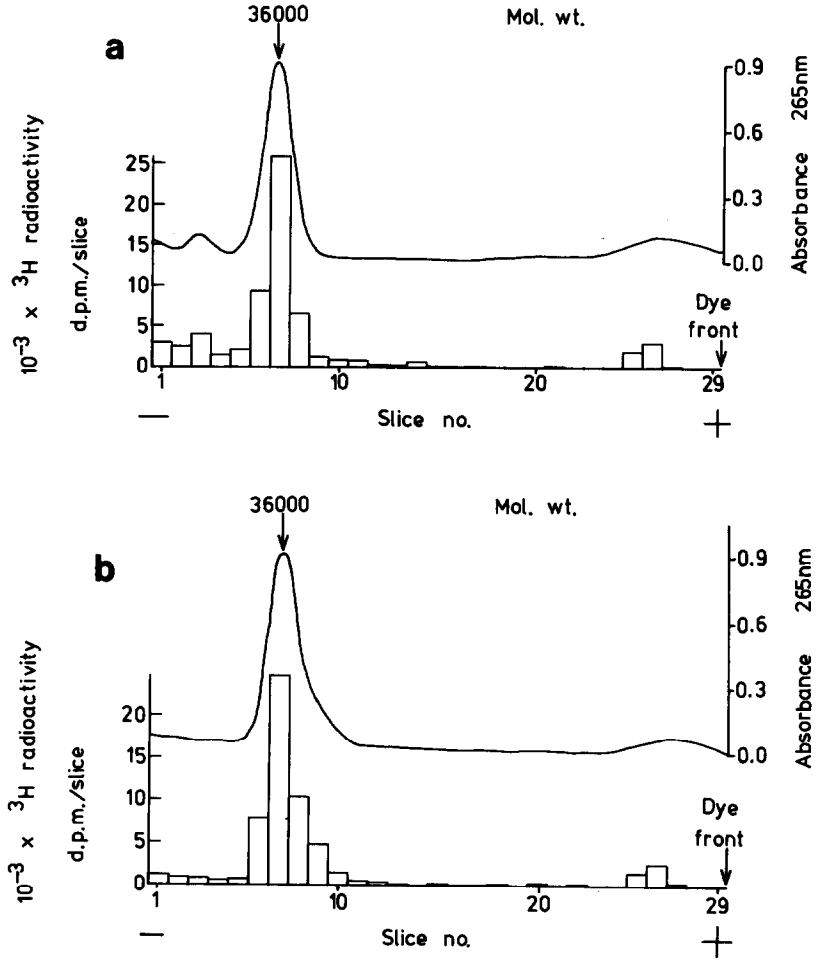

Fig.3. Polyacrylamide gel electrophoresis of ROS containing tritiated *N*-retinyl opsin. ROS were regenerated from 11-*cis*-[15-^{3}H]-retinal (spec. radioact. = 10.9 Ci/mol) $NaBH_4$-fixed and methanol-washed as described in the Experimental section. Protein (135 μg) containing tritium, 60 000 dpm, was then solubilized in SDS in the absence (a) or presence (b) of 2-mercaptoethanol and electrophoresed. The histograms show the radioactivity in 2 mm slices and the continuous lines the A_{265} scans. Of the radioactivity originally applied 96% and 93.4% was recovered on the gels in (a) and (b) respectively.

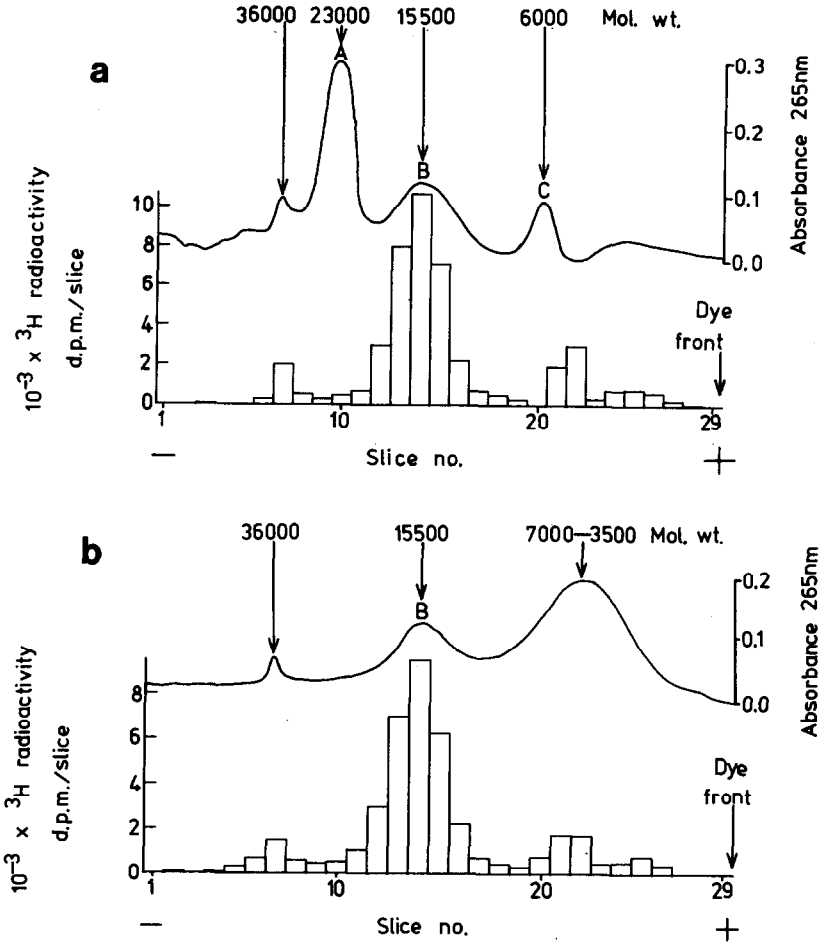

Fig.4. Polyacrylamide gel electrophoresis of papain treated ROS containing the tritiated *N*-retinyl opsin derivative. A sample of the labelled ROS as analyzed in fig.3 was incubated with papain, $NaBH_4$-fixed and methanol-washed as described in the Experimental section. Protein (100 μg) containing tritium, 45 000 dpm, was then solubilized in SDS in the absence (a) or presence (b) of 2-mercaptoethanol and electrophoresed. Of the radioactivity originally applied, 97% and 92% was recovered on the gels in (a) and (b) respectively. A small amount of undigested *N*-retinyl opsin is present on both gels.

values. Similar electrophoretic patterns were observed for papain treated ROS when the $NaBH_4$ fixation-step was omitted except that in this case fragment B was somewhat less pronounced. Fragment A, which corresponded to the rhodopsin-core described by Trayhurn et al. [2] contained less than 5% of the total radioactivity on the gel; 72.5% of the radioactivity coincided with fragment B showing that it contained the retinal binding site in papain treated ROS. Under the conditions of reductive electrophoresis (in the presence of 2-mercaptoethanol) fragment A was replaced by a broad-band in the 7000–3500 mol.wt region (fig.4b). Fragment A must, therefore, consist of several polypeptide chains linked by disulphide bonds. Fragment B was unaffected by 2-mercaptoethanol and still contained most of the radioactivity. Thus the apparent mol.wt of the retinal-binding polypeptide in papain-cleaved rhodopsin is 15 500.

We have considered that $NaBH_4$-fixation of ROS containing cleaved-rhodopsin may, in addition to reducing the active site retinyl–opsin linkage, break a disulphide bond in fragment A (apparent mol.wt

23 000) to produce the retinal-binding polypeptide of apparent mol.wt 15 500 (fragment B). Although this possibility cannot be completely excluded it is rendered unlikely by our repeated failures to show radioactivity in the 23 000 mol.wt region of the gel (fig.4a).

4. Discussion

Pober and Stryer [11] proposed that the visual pigment isolated from thermolysin treated ROS consists of a retinal binding polypeptide of apparent mol.wt 18 000 and another single chain polypeptide of apparent mol.wt 30 000. They also concluded that one specific region of rhodopsin is highly susceptible to enzymic digestion. The present work using a quantitative radioactive approach shows that proteolysis of ROS by papain appears to result in the formation of three species, a retinal-binding polypeptide of apparent mol.wt 15 500 (fragment B), a multi-chain species of apparent mol.wt. 23 000 (fragment A) and a low mol. wt polypeptide (fragment C). Since these species are dissociated from extensively washed papain-treated ROS by SDS alone this indicates that they are held within a complex by non-covalent interactions. Assuming that papain can only attack those parts of rhodopsin that are normally exposed to the aqueous environment, the presence of several polypeptides in papain-cleaved rhodopsin shows that many 'bulge' regions on the rhodopsin molecule must extend outside the membrane.

In conclusion digestion of ROS by papain produces a cleaved complex in which the constituent polypeptides are held together by both non-covalent interactions and by disulphide-linkages. Most interestingly, the conformation and environment of the retinal-binding polypeptide (apparent mol.wt 15 500) is maintained in the multiple-cleaved complex sufficiently for the characteristic spectral and regeneration properties [1,2] of rhodopsin to be preserved.

Acknowledgements

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