Structural studies on membrane-bound bovine rhodopsin

Eric MULLEN and Muhammad AKHTAR

Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, U.K.

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Our previous work has shown that the treatment of bovine rhodopsin with the proteolytic enzyme papain gives rise to a cleaved, but fully functional, complex consisting of three fragments, H, M and L (heavy, medium and light), held together by strong non-covalent forces. By using some of the chemical and physical differences between the three fragments, a protocol for the preparative isolation of each fragment was devised. Purified M-fragment, which had been radiochemically labelled at the retinal-binding site was treated with CNBr and the mixture subjected to a multi-step separation to furnish a retinyl peptide. The sequence analysis of the latter showed that the retinal-binding lysine residue was located at position 296 from the *N*-terminal of rhodopsin (or residue 53 from the *C*-terminal). In order to ascertain the position of the cytoplasmic loop which exists between the M- and L-fragments, radiochemically labelled L-fragment was isolated from the cleaved complex. The purified L-fragment was shown to consist of two populations of peptides which were produced by the action of papain on the bonds between Lys-311 and Gln-312 and between Gln-312 and Phe-313.

Rhodopsin, the photosensitive pigment found in the rod outer segments of the retina, consists of a glycoprotein, opsin, covalently bound to 11-*cis*-retinal via a Schiff base linkage to the ϵ -amino group of a lysine residue (Akhtar *et al.*, 1965, 1967, 1968; Bownds, 1967). Light activation isomerizes the retinal chromophore from the 11-*cis* to an all-*trans* form and this initiates spectrally defined changes in the rhodopsin that lead to visual excitation. In order to describe these processes in molecular terms a knowledge of the structure of rhodopsin and its organization in the membrane is required.

A technique specifically aimed at pin-pointing regions of rhodopsin that protrude from the membrane into the aqueous environment utilizes the limited proteolysis of ROS with papain (Trayhurn *et al.*, 1974; Pober & Stryer, 1975; Sale *et al.*, 1977, 1978; Towner *et al.*, 1977; Albert & Litman, 1978). On the basis of such papain-cleavage experiments, Sale *et al.* (1977) proposed a model for rhodopsin that showed the molecule cleaved into three non-covalently linked fragments (heavy, medium and light). Continuing our studies on the structural analy-

sis of rhodopsin we now describe methods for the isolation of these three fragments of papain-cleaved rhodopsin, report on the location of the retinal-binding lysine residue and identify one region of the polypeptide chain between the M- and L-fragment which protrudes from the membrane into the interdiscal space. This work is based on a Ph.D. Thesis (Mullen, 1982), and some of the experiments herein have been described in a preliminary communication (Mullen & Akhtar, 1981).

Experimental and results

Materials

Cattle eyes were collected at an abattoir within 10 min of the animals' deaths. The retinas were removed immediately and stored in a light-proof flask. They were either processed on arrival at the laboratory or stored in the dark at 4°C for processing the following day. NaB³H₄, iodo[2-³H]-acetic acid and *N*-ethyl[2,3-¹⁴C]maleimide were obtained from The Radiochemical Centre, Amersham HP7 9LL, Bucks., U.K. Concanavalin A–Sephadex 4B, LH60–120 Sephadex and papain were from Sigma (London) Chemical Co., Kingston upon Thames KT2 7BH, Surrey, U.K. Ammonyx LO [an aq. 30% (w/v) solution of dodecyl-dimethylamine oxide/tetradecyldimethylamine oxide

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; ROS, rod outer segments; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulphate; h.v.p.e., high-voltage paper electrophoresis; Hse, homoserine; CmCys, carboxymethylcysteine.

(13:7, w/w)] was from Venture Chemicals, Reading, Berks., U.K. Vitamin A aldehyde (retinal) was from Fluorochem Ltd., Glossop SK13 9NU, Derbyshire, U.K. and CM32 cellulose and 3MM chromatography paper were from Whatman, Maidstone ME14 2LE, Kent, U.K. All other reagent-grade chemicals were from BDH Chemicals Ltd., Poole BH12 4NN, Dorset, U.K. Ethanol and distilled water for h.p.l.c. were further distilled in glass apparatus. Acetonitrile for the h.p.l.c. was obtained from Rathburn Ltd., Walkerburn, Peeblesshire, Scotland, U.K.

Preparation of ROS

Freshly collected bovine retinas (60–100) were vigorously shaken in 45% (w/v) sucrose (1 ml/retina) and then processed essentially as described by Sale *et al.* (1977). ROS, found at the 34/36% interface of a discontinuous sucrose gradient, were removed with a syringe, diluted fourfold with the buffer, collected by centrifugation at 40000g for 30 min and washed three times in water by suspension and centrifugation. This isolation procedure routinely yielded 19–29 nmol of rhodopsin per retina (measured from ϵ_{500} 40000 mol·litre⁻¹·cm⁻¹; Applebury *et al.*, 1974). The ROS had A_{280}/A_{500} ratios of 2.2–2.6.

Domain-specific labelling of papain-cleaved rhodopsin

Labelling with [³H]retinal. ROS containing rhodopsin (1.24 μ mol) were bleached and then regenerated with a 2-fold molar excess of 11-*cis*-[15-³H]retinal (prepared as described by Akhtar *et al.*, 1968; sp. radioactivity 5.6×10^6 d.p.m./ μ mol) as described by Sale *et al.* (1977). Regeneration, as measured by the reappearance of the A_{500} band of rhodopsin, was 100%.

The regenerated material was cleaved with papain under the conditions described by Trayhurn *et al.* (1974) as described by Sale *et al.* (1977). No loss of A_{500} occurred during proteolysis.

A portion of the papain-cleaved material (150 nmol) was suspended in water (75–100 nmol/ml) and irradiated in the presence of NaBH₄ (1.5 mg/25 nmol of rhodopsin) to fix the label to the protein as the stable *N*-retinyl derivative ([³H]-retinyl-opsin). The fixed material was collected by centrifugation. Non-fixed [³H]retinal was removed by washing the pellet twice in aq. 80% (v/v) methanol and once in water. A sample of papain-cleaved [³H]retinyl-opsin (25 nmol) was worked up as described by Mullen *et al.* (1981) to determine a stoichiometry of incorporation of 1.02 mol of retinal/mol of opsin.

Labelling with [¹⁴C]NEM. ROS were suspended in 200 mM-potassium phosphate buffer, pH 7.0 (50–100 nmol/ml), and incubated with a 20-fold molar

excess of *N*-ethyl[2,3-¹⁴C]maleimide ([¹⁴C]NEM, sp. radioactivity 1.7×10^6 d.p.m./ μ mol) for 18 h at room temperature with gentle shaking. The labelled rhodopsin in ROS was then collected by centrifugation and washed twice in 67 mM-potassium phosphate buffer, pH 7.0. The [¹⁴C]NEM-labelled rhodopsin (250 nmol) was cleaved with papain and washed. The stoichiometry of incorporation in a sample of the protein was calculated to be 2.0 mol of [¹⁴C]NEM/mol of rhodopsin (see also de Grip *et al.*, 1975).

Carboxymethylation and analysis of variously labelled cleaved-rhodopsin samples

Papain-cleaved [¹⁴C]NEM-labelled rhodopsin (52.6 nmol, 1.79×10^5 d.p.m.) was added to papain-cleaved [³H]retinyl-opsin (95.4 nmol, 5.3×10^5 d.p.m.) in 1 ml of water and the protein was carboxymethylated with iodoacetic acid and re-isolated as described by Mullen & Akhtar (1981).

A sample of the carboxymethylated material (4.72 nmol; 5.9×10^3 d.p.m. of ¹⁴C, 1.8×10^4 d.p.m. of ³H) was solubilized and analysed by SDS/15%-polyacrylamide-gel electrophoresis. The gel was stained for protein with Coomassie Blue. Three bands of apparent mol.wts. 23 000, 15 500 and 6000 were identified (Fig. 1a). The electrophoresis gel was sliced into 2 mm-thick discs, which were used for the determination of radioactivity. All of the ³H label originally associated with 11-*cis*-[15-³H]retinal co-migrated with the M-fragment (Fig. 1b), 56% (3.3×10^3 d.p.m.) of the ¹⁴C label was found in the H-fragment and 41% (2.4×10^3 d.p.m.) of ¹⁴C was found in the L-fragment (Fig. 1c).

Preparation of the three fragments of papain-cleaved rhodopsin

The preceding mixture (75 nmol, 9.35×10^4 d.p.m. of ¹⁴C, 2.85×10^5 d.p.m. of ³H) was dissolved in 5 mM-potassium phosphate buffer containing 3% (w/v) Ammonyx LO, pH 5.0 (2 ml) and applied to a column (0.5 cm $\times$ 4 cm) of CM-cellulose, previously equilibrated in the buffer. The column was washed with the buffer (50 ml). All of the ³H radioactivity (2.5×10^5 d.p.m.) and 54.7% of the ¹⁴C radioactivity (5.1×10^4 d.p.m.) was eluted from the column in seven fractions (1.5–10 ml) (Fig. 2a). This material corresponded to the M- and H-fragments. The material remaining on the column was eluted with 500 mM-potassium phosphate buffer containing 3% (w/v) Ammonyx LO, pH 7.0 (50 ml). The remainder of the ¹⁴C radioactivity (3.7×10^4 d.p.m.) was collected in seven fractions (1.5–10 ml) (Fig. 2b). When these fractions were pooled, dialysed against 2 litres of water in Medicell dialysis-Visking tubing size 1-8/32, freeze-dried and the protein washed with 80% (v/v) methanol and then water, 13 nmol (2.2×10^4 d.p.m.) of L-fragment were obtained.

Fig. 1. SDS/polyacrylamide-gel electrophoresis of carboxymethylated papain-cleaved [^{14}C]NEM-labelled opsin and [^3H]retinyl-opsin

Papain-cleaved [^{14}C]NEM-labelled rhodopsin was added to papain-cleaved [^3H]retinyl-opsin and the mixture carboxymethylated. A sample (5.9×10^3 d.p.m. of ^{14}C ; 1.8×10^4 d.p.m. of ^3H) was solubilized and subjected to SDS/polyacrylamide-gel electrophoresis. The gel was stained, scanned at 590 nm (a) and sliced into 2 mm-thick discs for the determination of ^3H radioactivity (b) and ^{14}C radioactivity (c). H, M and L refer to heavy, medium and light fragments of rhodopsin.

The material corresponding to the M- and H-fragments above (Fig. 2) was pooled, dialysed at room temperature, first against water (5 litre) and then against a solution of 50 mM-Tris/HCl containing 3% (w/v) Ammonyx LO, 1 mM- MnCl_2 , 1 mM- CaCl_2 and 1 mM- MgCl_2 , pH 7.0 (2 litres) for 1 h. The dialysis residue was applied to a column (1 cm $\times$ 4 cm) of concanavalin A-Sepharose 4B previously equilibrated with the buffer. Upon washing with the buffer, 68% of the original ^3H radioactivity (1.93×10^5 d.p.m.) and a negligible amount of ^{14}C radioactivity was eluted from the column (Fig. 3a). The fractions containing protein were pooled and worked up as described by Mullen & Akhtar (1981). A total of 26 nmol (1.48×10^5 d.p.m.) of M-fragment was obtained in this manner. The concanavalin A column was then washed with the buffer containing 250 mM-methyl glucoside (50 ml). The remainder of the ^{14}C radioactivity (3.99×10^4 d.p.m.) was eluted in 4.2–12.6 ml of the buffer (Fig. 3b). After work-up, 15 nmol

Fig. 2. CM-cellulose column chromatography of carboxymethylated papain-cleaved [^{14}C]NEM-labelled opsin and [^3H]retinyl-opsin

A mixture of papain-cleaved [^{14}C]NEM-labelled rhodopsin was added to papain-cleaved [^3H]retinyl-opsin and the mixture carboxymethylated. The mixture (9.35×10^4 d.p.m. of ^{14}C ; 2.85×10^5 d.p.m. of ^3H) was solubilized and applied to a column of CM-cellulose. The column was washed with 5 mM-potassium phosphate buffer, pH 5.0, containing detergent (a), and the remaining material was then eluted from the column with 500 mM-potassium phosphate buffer, pH 7.0, containing detergent (b). Portions of each (approx. 1.5 ml) fraction collected were analysed for ^{14}C ($\bullet$) and ^3H ($\blacktriangle$) radioactivity.

Fig. 3. Concanavalin A-Sepharose column chromatography of H- plus M-fragment

The fractions containing the H plus M-fragments, eluted from the CM-cellulose column (Fig. 2, first peak) were pooled and worked up as described in the text. The mixture (4.5×10^4 d.p.m. of ^{14}C ; 2.2×10^5 d.p.m. of ^3H) was then applied to a column of concanavalin A-Sepharose. The column was washed with a Tris buffer containing detergent (a), and then with the Tris/detergent containing α -methyl glucoside (b). Portions of each (approx. 1.5 ml) fraction collected were analysed for ^{14}C ($\bullet$) and ^3H ($\blacktriangle$) radioactivity.

Fig. 4. SDS/polyacrylamide-gel electrophoresis of [^{14}C]-NEM-labelled L-fragment from carboxymethylated papain-cleaved rhodopsin

A sample of L-fragment (5.2 nmol, 9.0×10^3 d.p.m.), isolated on a column of CM-cellulose, was subjected to SDS/polyacrylamide-gel electrophoresis as described in the text. The gel was stained, scanned at 590 nm (a) and sliced into 2 mm-thick discs for the determination of radioactivity (b).

Fig. 5. SDS/polyacrylamide-gel electrophoresis of [^3H]-retinyl-M-fragment (M) from carboxymethylated papain-cleaved rhodopsin

[^3H]Retinyl-M-fragment was isolated by chromatography on columns of CM-cellulose and concanavalin A-Sepharose. A sample of the purified material (3.3 nmol, 20×10^3 d.p.m.) was subjected to SDS/polyacrylamide-gel electrophoresis as described in the text. The gel was stained, scanned at 590 nm (a) and sliced into 2 mm-thick discs for the determination of radioactivity (b).

(2.6×10^4 d.p.m.) of H-fragment were recovered. [^{14}C]NEM-labelled L-fragment (5.2 nmol, 9.0×10^3 d.p.m.), [^3H]retinyl-M-fragment (3.3 nmol, 20×10^4 d.p.m.) and [^{14}C]NEM-labelled H-fragment (5.0 nmol, 8.6×10^3 d.p.m.) were each analysed by SDS/polyacrylamide-gel electrophoresis. Analysis of the gel of the L-fragment showed 69% (6.2×10^3 d.p.m.) of the applied radioactivity was associated with a single protein band of approx. mol.wt. 6000 (Figs. 4a and 4b). The major peak on the gel of the M-fragment, corresponding to an apparent mol.wt. of 15 500, was associated with 72% (1.4×10^3 d.p.m.) of the applied radioactivity (cf. Figs. 5a and 5b). Analysis of the gel of the H-fragment showed a single protein band of apparent mol.wt. 23 000. All of the applied radioactivity was found associated with this band (Figs. 6a and 6b).

Preparation of the active-site peptide

Isolation and purification. Rhodopsin in ROS (1 μmol) was bleached, regenerated with 11-*cis*-[$^{15}\text{-}^3\text{H}$]retinal (sp. radioactivity 5.0×10^6 d.p.m./mol), cleaved with papain, and the radioactive label fixed to the protein as described above. The protein

was then carboxymethylated and the stoichiometry of incorporation of the label calculated to be 1.03 mol of retinal/mol of papain-cleaved opsin.

[^3H]Retinyl-M-fragment (600 nmol, 3.0×10^6 d.p.m.) was then prepared from this material by chromatography on columns of CM-cellulose and concanavalin A-Sepharose as described previously. Upon analysis of a portion of the material by SDS/polyacrylamide-gel electrophoresis, all of the applied radioactivity was found associated with the major protein band corresponding to the [^3H]retinyl-M-fragment (Fig. 5).

The CNBr-cleaved fragments were subjected to a two-step separation involving h.v.p.e. and h.p.l.c. to yield 120 nmol (6.1×10^5 d.p.m.) of purified [^3H]retinyl-peptide. Not allowing for material taken for analysis at various stages in the purification, the overall yield of [^3H]retinyl-peptide was 12% from [^3H]rhodopsin and 20% from retinyl-M-fragment. The largest loss of material occurred at the elution of the retinyl-peptide from the paper in the electrophoresis step. A portion of the purified peptide

Fig. 6. SDS/polyacrylamide-gel electrophoresis of [^{14}C]NEM-labelled H-fragment (H) from carboxymethylated papain-cleaved rhodopsin

A sample of H-fragment (5.0 nmol, 8.6×10^3 d.p.m.) isolated on a column of concanavalin A–Sephadex was subjected to SDS/polyacrylamide-gel electrophoresis as described in the Experimental section. The gel was scanned at 590 nm (a) and sliced into 2 mm-thick discs for the determination of radioactivity (b).

(25 nmol, 1.2×10^5 d.p.m.) was hydrolysed overnight in the absence of oxygen with 6 M-HCl and analysed on a Rank–Hilger J80 automated amino acid analyser to yield the following amino acid composition: Asp_{0.9} Thr_{2.3} Ser_{1.4} Glu₀ Pro_{1.9} Gly_{0.2} Ala_{3.3} Val_{2.2} Met₀ Ile_{3.0} Leu_{0.1} Tyr_{0.8} Phe_{0.9} His₀ Lys_{0.7} Arg₀ Hse_{0.5}.

Sequence analysis. A further portion of the peptide (25 nmol, 1.2×10^5 d.p.m.) was taken for sequence analysis by the method of Chang *et al.* (1978) as described by Mullen *et al.* (1981). The result of the sequence analysis is shown in Table 1.

A portion of the peptide (44.5 nmol) was dissolved in formic acid (0.5 ml) and ozone was passed through the solution at a rate of approx. 0.6 mg of ozone/min for 10 min. Performic acid (1 ml), freshly prepared by adding H_2O_2 (0.5 ml) to 98–100% formic acid (9.5 ml) and leaving in a stoppered vessel for 2 h at room temperature, was added and the mixture was left for 30 min in an ice/water bath. The solution was then diluted with water (10 ml) and freeze-dried. A 20 nmol portion of this material was acid-hydrolysed and subjected to h.v.p.e. in pyridine/acetic acid/water (1:10:289, by vol.),

pH 3.5, for 2.5 h at 2 kV. Then electrophoretogram was revealed with ninhydrin and 1 cm strips cut for the determination of radioactivity. The only radioactivity detected on the paper was found in the region that corresponded to the position of authentic ϵ -N-carboxymethyl-lysine, 8 cm from the origin towards the anode (Mullen *et al.*, 1981).

Purification of labelled L-fragment for sequence analysis

Purification. The thiol groups on papain-cleaved rhodopsin (1.3 μmol) were carboxymethylated by reaction with iodo[2- ^3H]acetic acid (sp. radioactivity 1.96×10^6 c.p.m./ μmol) for 2 h and then an equal amount of non-radioactive iodoacetic acid for a further 2 h. The carboxymethylated material (470 nmol, 5.5×10^6 c.p.m.) was isolated by precipitation as described above and dissolved in 5 mM-potassium phosphate buffer containing 2% (w/v) dodecyltrimethylammonium chloride (prepared as described by Applebury *et al.*, 1974), pH 5.0 (4 ml), for chromatography on CM-cellulose as described above. The resulting L-fragment (2.5×10^6 c.p.m.) was chromatographed on a column (2 cm $\times$ 100 cm) of Sephadex LH-60 equilibrated with 90% (v/v) formic acid/ethanol (3:7, v/v). Approx. 70 ml of the eluant was discarded and fractions (2 ml) were then collected, analysed for protein with ninhydrin and samples were taken for the determination of radioactivity. The elution profile of the peptide from the column showed a single radioactive ninhydrin-positive peak, which was eluted between 40 and 60 ml. The fractions containing radioactivity, which contained purified L-fragment, were evaporated under vacuum. A portion of the L-fragment (4.0×10^4 c.p.m.) was analysed by SDS/polyacrylamide-gel electrophoresis to reveal a single protein band of apparent mol.wt. 6000 containing 75% of the applied radioactivity (3.0×10^4 c.p.m.).

L-peptide (1.2×10^4 c.p.m.) was then subjected to h.p.l.c. on a Waters μ -Bondapak C₁₈ column previously equilibrated with aq. 0.3% trifluoroacetic acid/ethanol 0.1% trifluoroacetic acid (3:2, v/v). The peptide was eluted from the column with a linear gradient generated from solvent A [trifluoroacetic acid/ethanol/water, 0.1:40:60 (by vol.)] and solvent B [trifluoroacetic acid/ethanol, 1:1000 (v/v)] over 30 min at a flow rate of 1.5 ml/min. Samples (100 μl) of each fraction (1.5 ml) were used for the determination of radioactivity. A single peptide which contained 83% of the applied radioactivity (1.0×10^4 c.p.m.) was eluted from the column at about 9 min. The remainder of the L-fragment (4.2×10^5 c.p.m.) was divided into two portions and each was run on the h.p.l.c. column. The eluted radioactive peaks were pooled (3.6×10^5 c.p.m.) and evaporated under vacuum.

Purified L-fragment (4.0×10^4 c.p.m.) was acid-

Table 1. Amino acid sequence of the active-site peptide isolated from the M-fragment of [^3H]retinyl-opsin

The peptide (25 nmol, 1.2×10^5 d.p.m.) was sequenced by the method of Chang *et al.* (1978). A portion of the coloured hydantoin derivative was used for the identification of the amino acid residue and the remainder for the determination of radioactivity. Abbreviation used: n.d., not determined.

Turn no.	Amino acid identified	Radioactivity from butyl acetate extract (d.p.m.)
1	Thr	245
2	Ile	120
3	Pro	170
4	Ala	200
5	Phe	205
6	Phe	185
7	Ala	185
8	n.d.	52518
9	Thr	3440
10	Ser	840
11	Ala	289
Radioactivity remaining in peptide after turn 11 (d.p.m.) ...		3444

hydrolysed overnight and the amino acid composition calculated relative to the amount of alanine ($=3.0$) detected in the hydrolysate: Asp_{3.4}Thr_{3.3}Ser_{2.5}Glu_{3.8}Pro_{2.4}Gly_{3.6}Ala_{3.0}Val_{2.4}Met_{1.0}Ile_{0.2}Leu_{2.5}Tyr₀His₀Lys_{1.2}CmCys_{1.7}.

Sequence analysis. A portion of purified L-fragment (2.9×10^4 c.p.m.) was treated with 0.2M-HCl for 2 h at room temperature, freeze-dried and then subjected to sequence analysis by the method of Chang *et al.* (1978). The results showed that two residues were detected at each cycle and radioactivity, corresponding to carboxymethylcysteine, was released at turns 4 and 5 in the ratio of 2:1 (Table 2).

Analysis by SDS/polyacrylamide-gel electrophoresis

Samples of variously labelled proteins were solubilized and applied to gels prepared by the method of Swank & Munkres (1971) containing 15% polyacrylamide, SDS, urea and *NN'*-diallyltartardiamide. Electrophoresis, protein analysis and determination of radioactivity were carried out as described by Mullen *et al.* (1979).

Discussion

Our previous work has shown that when membrane-bound rhodopsin is treated in the dark with papain under carefully controlled conditions, a multiply cleaved but fully functional complex is produced (Sale *et al.*, 1977). The cleaved complex, on non-reductive SDS/polyacrylamide-gel electro-

Table 2. Amino acid sequence of L-fragment from carbomethylated papain-cleaved rhodopsin

[^3H]Carboxymethyl-L-fragment (2.9×10^4 c.p.m.) was treated with 0.2M-HCl and then sequenced as described in the Experimental and results section. A portion of the coloured hydantoin derivative was used for the identification of the amino acid residues and the remainder was used for the determination of radioactivity. Abbreviation used: CmCys, carboxymethylcysteine.

Turn no.	Amino acid identified	Radioactivity from butyl acetate extract (c.p.m.)
1	Gln + Phe	135
2	Phe + Arg	142
3	Arg + Asn	182
4	Asn + CmCys	4076*
5	Met + Xaa†	2035*
6	Met + Val	95
7	Val + Thr	103
Radioactivity remaining in peptide after final turn of sequence (c.p.m.) ...		15921*

* The L-fragment contains three cysteine residues. Of the radioactivity originally associated with the peptide, 21% was removed at turns 4 and 5; 55% of the radioactivity remained with the peptide (see the text).

† CmCys was detected at turn 5 in insufficient yield to give a positive identification on the plate.

phoresis, shows three fragments (Fig. 1). Multiple-labelling techniques revealed that the fragments had originated from three discrete regions of the rhodopsin molecule. These species were designated H, M and L (heavy, medium and light) fragments and were assigned the following features.

(1) The L-fragment had an estimated mol.wt. of about 6000 and was shown to contain three to four phosphorylation sites (Sale *et al.*, 1978), a reactive thiol group (Sale *et al.*, 1977) and the C-terminal portion of rhodopsin (Hargrave *et al.*, 1980). It was hypothesized that the L-fragment represented one of the most hydrophilic regions of the rhodopsin molecule.

(2) The M-fragment with an approx. mol.wt. of 15500 had the retinal-binding site and was considered to reside between the L- and H-fragments.

(3) The H-fragment, heaviest of the three species, contained the *N*-terminus, the two carbohydrate chains, the disulphide bonds, a second reactive thiol group modified by *N*-ethylmaleimide and other thiol groups which become available for modification only under denaturing conditions. It is also worth mentioning that the H-fragment was cleaved between the disulphide loops, since reductive SDS/polyacrylamide-gel electrophoresis resulted in the

degradation of H-fragment into three to four species, ranging in molecular weight from 4000 to 6000.

Isolation of the three fragments of papain-cleaved rhodopsin

From the foregoing discussion it follows that, if the three fragments hitherto detected only by an electrophoretic technique could be separated on a preparative scale, they could provide information on the location of the retinal-binding site and other strategic domains in rhodopsin, and also be used for the determination of the regions of membrane-bound rhodopsin which protrude into the interdiscal phase. A rational approach for the separation of these fragments has now been designed and is based on the predicted hydrophilicity of the L-fragment and the presence of carbohydrate chains in the H-fragment.

The first step in the separation strategy required masking the thiol groups of cleaved-rhodopsin by *S*-carboxymethylation under the denaturing conditions in which the protein could be maintained in a soluble state. This was conveniently achieved by performing the alkylation process in 1% SDS at a protein concentration of 6 mg/ml. It is our experience that precipitation of rhodopsin or its modified derivatives from detergent solutions invariably results in an irreversible aggregation of protein thus rendering the material unsuitable for structural work. In the present study, successful use of SDS in protein-modification experiments depended on the observation that the carboxymethylated fragments from papain-cleaved rhodopsin could be precipitated in an unaggregated form and then freed from the detergent by the addition of methanol under carefully defined conditions. The methodology emerging from this study is both rapid and applicable to the processing of small amounts of the protein material.

Samples of papain-cleaved rhodopsin, which were radioactively labelled by domain-specific reagents, were subjected to *S*-carboxymethylation by the procedure above. The modified fragments were solubilized in a buffer containing detergent and applied to a column of CM-cellulose. The L-fragment was tightly bound to the column. The eluant containing the mixture of H + M-fragments, after appropriate work up, was applied to concanavalin-A-Sepharose. The carbohydrate-containing H-fragment was retained on the column and the M-fragment was recovered in the void volume. The removal of detergent from the latter fraction yielded the M-fragment (Fig. 5). The purified H-fragment was later eluted from the column of concanavalin A with a buffer containing 250 mM- α -methyl glucoside (Fig. 6). The L-fragment was eluted from the column of CM-cellulose with a high-ionic-strength high-pH buffer.

Isolation of the retinyl-peptide from M-fragment

In a recent publication (Mullen & Akhtar, 1981) a sample of ROS containing rhodopsin labelled at the active site with 11-*cis*-[15-³H]retinal was cleaved with papain and the complex processed to yield a mixture of M- plus L-fragments which was used to obtain a homogeneous retinyl-peptide with the sequence:

Thr-Ile-Pro-Ala-Phe-Phe-Ala-Lys-Thr-Ser-Ala-Val-Tyr
1 2 3 4 5 6 7 8 9 10 11 12 13

These results have now been confirmed through the isolation of the same peptide from a purified sample of the M-fragment in 20% yield and its analysis through eleven cycles of the Edman degradation. Of the radioactivity associated with the retinyl-peptide, 85% was released at the eighth cycle of Edman degradation (Table 1). Since in these experiments a direct identification of the coloured thiazolinone derivative of [³H]retinyl-lysine was not possible, the structural assignment was made by using an alternative approach. The [³H]retinyl-peptide was allowed to react with O₃/H₂O₂ to convert the *N*-retinyl into an *N*-carboxymethyl-lysyl moiety (Mullen *et al.*, 1981). A portion of the modified peptide was then hydrolysed and subjected to h.v.p.e. to show that all the radioactivity was associated with [³H]carboxymethyl-lysine. The cumulative evidence has confirmed the assignment of the active-site peptide to the M-fragment.

The sequence of 83 amino acids from the C-terminal of bovine rhodopsin may be deduced from the structural information on two overlapping peptides already described in the literature and illustrated in Fig. 7. The 11 amino acids of the active-site peptide correspond to residues 60–50 from the C-terminal sequence, whereas the retinal-binding lysine residue is the 53rd amino acid from the C-terminus. In a previous study, alanylretinyl-lysine was isolated from a Pronase digestion of NaBH₄-treated rhodopsin, and it was suggested to correspond to the Ala-Lys sequence present in the sequence found by Pellicone *et al.* (1980) (Wang *et al.*, 1980) and subsequently assigned positions 54 and 53 from the C-terminus. We believe that the cumulative evidence described from our laboratory in the present paper and elsewhere (Mullen & Akhtar, 1981) provide a direct, unambiguous and quantitative identification of the retinal-binding site in the partial sequence of bovine rhodopsin. After the acceptance of this paper for publication the complete amino acid sequence of bovine rhodopsin was reported by Ovchinnikov *et al.* (1982). In the light of this important new information the positions of the strategic residues identified in the present paper have been redefined and the active-site lysine residue may now be assigned position 296 from the N-terminus of rhodopsin (Fig. 7).

considered possible that the extensive exposure of the L-fragment to formic acid, often under conditions that could promote formylation of amino groups, may have resulted in the blocking of the *N*-terminal residue. We were encouraged in this belief by Dr. J. S. Seehra, who had observed a similar phenomenon in the laboratory of Professor H. G. Khorana at the Massachusetts Institute of Technology (Cambridge, MA, U.S.A.). At his suggestion the L-fragment was treated with 200 mM-HCl at room temperature for 2 h in order to hydrolyse any *N*-formyl linkage. The pretreatment indeed resulted in the expected yield of the thiohydantoin derivative, thus providing indirect support for the above hypothesis (Table 2).

The first six cycles of the Edman degradation showed the following residues:

Gln/Phe-Phe/Arg-Arg/Asn-Asn/
CmCys-Xaa/Met-Met/Val

The sequence data were interpreted to suggest that the L-fragment consisted of two populations of peptides, arising through the cleavage of the rhodopsin chain between either residues 311 and 312 or 312 and 313 from the *N*-terminus (Fig. 7). This possibility was further examined through the determination of ^3H -radioactivity associated with the carboxymethylcysteine residues. There are three cysteine residues in the portion of the rhodopsin molecule constituting the L-fragment (Hargrave *et al.*, 1980), and one-third of the radioactivity associated with carboxymethylcysteine residues was released at turns 4 and 5 in the ratio of 2:1 (Table 2). It should be noticed that although carboxymethylcysteine was detected at the fifth cycle of the degradation, its positive identification could only be made from the radiochemical data. The data prove that the cleavage by papain, culminating in the formation of M- and L-fragments, occurs at peptide bonds located between residues 311 and 312, and 312 and 313, providing two species of L-fragment.

The cleavage profile of membrane-bound rhodopsin by papain has also been studied in a number of other laboratories (Trayhurn *et al.*, 1974; Pober & Stryer, 1975; van Breugel *et al.*, 1975; Albert & Litman, 1978; Fung & Hubbell, 1978; Zagalsky *et al.*, 1981). The unambiguous characterization of the various domains in the three purified fragments in the present paper and, in particular, the identification of the retinal-binding site in the M-fragment, should dispel the doubts cast on our original proposal by the results reported elsewhere (Trayhurn *et al.*, 1974; Pober & Stryer, 1975; Albert & Litman, 1978; Fung & Hubbell, 1978).

A model of rhodopsin in its membrane

In the light of the cumulative information now available, the organizational model of rhodopsin

previously proposed by us (Sale *et al.*, 1977; Mullen & Akhtar, 1981) may be refined by ascribing the portion of the membrane-bound rhodopsin which gives rise to the L-fragment an interdiscal cytosolic location leading to the assignment of residues 311 or 312 of native rhodopsin as the C-terminal amino acid of the M-fragment. The retinal-binding lysine residue at position 296 is then located in the membrane at a depth that maximally may correspond to 15 residues.

It is noteworthy that in the model for the organization of rhodopsin proposed by the Soviet group (Ovchinnikov *et al.*, 1982) the residues 311, 312 and 313 are shown to reside in the membrane. The demonstration in the present study that the peptide bonds between these residues are easily accessible to papain cleavage should necessitate reconsideration of the assumptions underlying the prediction.

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