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Functional Rhodopsin Complex Consisting of Three Noncovalently Linked Fragments†

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ABSTRACT: A simple method for the routine preparation of both bovine rod outer segments and rhodopsin of good purity in exceptionally high yields is described. Incubation of rod outer segment disc membranes with the proteolytic enzyme, papain, resulted in the production of a complex in which three fragments of apparent molecular weights 23 000, 15 500, and 6000, referred to as H, M, and L (heavy, medium, and light) fragments, were identified. The H fragment was reduced by 2-mercaptoethanol to give a mixture of polypeptides of molecular weight 9000–4000 showing that it contained the disulfide bond(s) of rhodopsin. To investigate the relationship of the three fragments to the native rhodopsin molecule, several strategic sites on the rhodopsin molecule were specifically labeled. The active site was labeled with 11-*cis*-[15-^{3}H]retinal and SH groups with *N*-ethyl[2,3-^{14}C]maleimide or iodo[^{3}H]-acetamidosalicylate. The L fragment contained an SH group which was modified by either *N*-ethyl[2,3-^{14}C]maleimide or iodo[^{3}H]acetamidosalicylate while the H fragment contained an SH group which was modified only by *N*-ethyl[2,3-^{14}C]-maleimide. The M fragment contained the retinal binding site. The three fragments arose without any significant loss in amino acid residues from rhodopsin and they were inseparable when the digested rod outer segments were solubilized in the detergent lauryl dimethylamine *N*-oxide and then chromatographed on calcium phosphate. The characteristic spectral, bleaching, and regeneration properties of native rhodopsin were fully maintained in cleaved rhodopsin. Assuming that papain can only digest those parts of the rhodopsin molecule that are normally exposed to the aqueous phase, then the mode of cleavage of rhodopsin yielding, after thiolysis, at least five large polypeptides favors a model in which the rhodopsin polypeptide chain alternately threads in and out of the membrane.

Rhodopsin, the photoreceptor protein of bovine retinae, consists of a glycoprotein opsin covalently bonded to 11-*cis*-retinal by a Schiff base linkage (Akhtar et al., 1965, 1967, 1968; Bownds, 1967). Light activation photoisomerizes the retinal chromophore from the 11-cis to the all-trans form (Wald, 1968) and this initiates spectrally defined changes in the visual pigment and concomitant protein conformational changes that may alter the membrane conductance leading to visual excitation. In order to describe these processes in molecular terms a knowledge of the structure of rhodopsin and its vectorial organization in the membrane is required.

A number of physicochemical approaches are currently being used to determine the molecular organization of rhodopsin in photoreceptor membranes. One such technique which is specifically aimed at pin-pointing regions of rhodopsin that protrude from the membrane into the aqueous environment involves the limited proteolysis of rod outer segments (ROS)[1] (Bonting et al., 1974; Daemen et al., 1974; Trayhurn et al., 1974a,b; Saari, 1974; Pober and Stryer, 1975; van Breugel et al., 1975; Klip et al., 1976; Towner et al., 1977). We have now subjected ROS, radioactively labeled at various strategic sites on the rhodopsin molecule, to papain treatment and have produced a complex consisting of at least three noncovalently linked fragments which retains many of rhodopsin's characteristic properties. The cleavage mode sheds light on selected aspects of the structure of rhodopsin as it exists in the disc membrane.

Experimental Section

Materials. Fresh cattle eyes were obtained from a local slaughter house; the retinae were immediately removed and stored at −18 °C in the dark. Tween 80 and papain (2× crystallized) were purchased from Sigma Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Ammonyx-LO (a 30% solution of mostly lauryl dimethylamine *N*-oxide) was obtained from Venture Chemicals, Reading, Berks, U.K. Butyl-PBD [5-(4-biphenyl)-2-(4-*tert*-butylphenyl)-1-oxa-3,4-diazole] and NE260 were purchased from Koch-Light Laboratories, Colnbrook, Bucks, U.K., and Nuclear Enterprises, Sighthill, Edinburgh, Scotland, U.K., respectively. NCS tissue solubilizer was obtained from Hopkins and Williams, Chadwell Heath, Essex, U.K. NaB^3H_4, iodo[^{3}H]acetic acid, and *N*-ethyl[2,3-^{14}C]maleimide were purchased from the Radiochemical Centre, Amersham, Bucks, U.K. All other chemicals were reagent grade. Iodo[^{3}H]acetamidosalicylate was synthesized according to the procedure of Holbrook et al. (1973).

Preparation of Rod Outer Segments (ROS). ROS were isolated by modification of the procedure of McConnell (1965). All appropriate operations were carried out under dim red light in a cold room at 4 °C. The buffer solution was 0.067 M potassium phosphate, pH 7. All sucrose solutions contained the buffer.

Retinae (100–200) were thawed, homogenized by two passes

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[1] Abbreviations used: ROS, rod outer segments; [^{3}H]retinal-labeled ROS, rod outer segments labeled with 11-*cis*-[15-^{3}H]retinal; butyl-PBD, 5-(4-biphenyl)-2-(4-*tert*-butylphenyl)-1-oxa-3,4-diazole.

with a Potter Elvehjem homogenizer (clearance 0.5 mm) in 45% (w/v) sucrose (1.5 mL/retina), and centrifuged at 9000*g* for 20 min. The floating ROS were loosened from the sides of the tubes with a spatula; the supernatant was removed by decantation, diluted with an equal volume of the buffer, and then centrifuged at 38 000*g* for 45 min (step 1). The ROS pellet was resuspended in 34% (w/v) sucrose with one pass of the homogenizer and 17 mL of the resulting suspension layered onto a four-step discontinuous sucrose gradient which consisted of 2-mL portions of 40%, 38%, 36%, and 34% (w/v) sucrose. The ROS from up to 200 retinae could be layered upon three gradients which were centrifuged at 128 000*g* for 1 h in the 3 × 25 mL swing out rotor of an MSE Superspeed 65 centrifuge. At the end of this period good separation of ROS from mitochondria was obtained in the four-step gradient provided it was not overloaded. The ROS, usually present at the 34%/36% (w/v) sucrose interface, were recovered and diluted fourfold with the buffer and the ROS sedimented by centrifugation at 38 000*g* for 45 min. They were then washed twice by resuspension in the buffer and recentrifugation (step 2). The final pellet of ROS was further purified by Tween washing (Zorn and Futterman, 1973). ROS from step 2 were suspended in 10% (w/v) Tween 80 with one pass of the homogenizer at a concentration of 2–4 A_{500} units/mL. After vigorous shaking at 20 °C for 30 min, the mixture was diluted fourfold with the buffer and sedimented by centrifugation at 108 000*g* for 30 min. The pellet was then washed twice by resuspension in the buffer and recentrifugation (step 3). The final pellet of Tween washed ROS was stored at −18 °C.

Solubilization of ROS and Purification of Rhodopsin. ROS were solubilized in Ammonyx LO and then column purified by modification of the method of Applebury et al. (1974). Thawed ROS (step 3) were washed twice with distilled water and collected by centrifugation at 108 000*g* for 30 min after each washing. The pellet was solubilized by homogenization in 2% (w/v) Ammonyx LO containing 0.01 M imidazole buffer, pH 7 (3–5 A_{500} units/mL), followed by vigorous shaking for 30 min and then centrifugation at 100 000*g* for 20 min to give a clear extract (step 4). The solubilized rhodopsin was purified by calcium phosphate chromatography under dim red light at 20 °C. Calcium phosphate was freshly prepared by the method of Mathews et al. (1964) and 10 × 1 cm columns were equilibrated with 25 mL of 2% (w/v) Ammonyx LO containing 0.01 M imidazole buffer (pH 7). Five to 10 A_{500} units of the solubilized rhodopsin (in 2–4 mL) were applied to the column which was eluted with a 60-mL gradient from 5 to 300 mM potassium phosphate (pH 7) containing 2% (w/v) Ammonyx LO. The fractions (3–5 mL) were analyzed spectrophotometrically and those containing rhodopsin were pooled (step 5).

Preparation of ROS Containing Tritium at the Active Site of Rhodopsin. 11-*cis*-[15-^{3}H]Retinal of various specific radioactivities was synthesized and purified as described (Akhtar et al., 1968; Hirtenstein and Akhtar, 1970). ROS from step 2 were homogenized at 3–4 A_{500} units/mL in 0.067 M potassium phosphate buffer (pH 7.0) bleached with a 495-nm interference filter, band width 9 nm, incorporated into a 150-W light source and regenerated by adding a twofold molar excess of 11-*cis*-[15-^{3}H]retinal in Tween 80 (Hirtenstein and Akhtar, 1970). 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 distilled water, 2% (w/v) hydroxylamine, 1% (v/v) formaldehyde, and distilled 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. The [^{3}H]retinal-labeled ROS were then Tween washed as described above. Although Tween washed ROS from step 3 regenerated equally well, the Tween washing of ROS after regeneration helped remove excess tritiated retinal oxime as well as solubilizing some unwanted protein.

Alkylation of SH Groups. ROS from step 3 and [^{3}H]retinal-labeled ROS were modified with *N*-ethyl[2,3-^{14}C]-maleimide to produce singly and doubly labeled ROS, respectively. The ROS were suspended in 0.2 M potassium phosphate buffer, pH 7 (2–4 A_{500} units/mL), and incubated with a 20-fold molar excess of *N*-ethyl[2,3-^{14}C]maleimide (the specific radioactivity used was variable and is stated in the figure legends) for 8 h at 20 °C as described by De Grip et al. (1975).

ROS were also doubly labeled with iodo[^{3}H]acetamidosalicylate and *N*-ethyl[2,3-^{14}C]maleimide. ROS from step 3 were suspended in 0.2 M potassium phosphate buffer, pH 7 (2–4 A_{500} units/mL), and first incubated with a 20-fold molar excess of iodo[^{3}H]acetamidosalicylate (specific radioactivity, 3.47×10^6 dpm/μmol) for 8 h at 20 °C. After the ROS were washed twice with 0.2 M potassium phosphate buffer (pH 7), they were resuspended in 0.2 M potassium phosphate buffer, pH 7 (2–4 A_{500} units/mL), and then incubated with *N*-ethyl[2,3-^{14}C]maleimide for 8 h at 20 °C.

The variously labeled ROS were then washed twice with distilled water and collected by centrifugation (100 000*g* for 30 min) after each washing.

Treatment of Variously Labeled ROS with Papain. The variously labeled ROS were digested with papain using the incubation conditions as described (Trayhurn et al., 1974a). Ten A_{500} units of ROS was suspended in 10 mL of 0.067 M potassium phosphate buffer (pH 7) containing 5 mM cysteine and 2 mM EDTA. One milligram of papain was added and the mixture incubated at 37 °C for 3 h. The digestion was terminated by the addition of iodoacetamide to a concentration of 10 mM; the ROS were collected by centrifugation at 190 000*g* for 15 min and washed twice by recentrifugation. No loss in 500-nm absorbance occurred during proteolysis.

Column Chromatography of Variously Labeled ROS. To determine the stoichiometry of the incorporation of the various labels into the rhodopsin, the preparations were purified from unbound label. Samples of the variously labeled ROS were solubilized in Ammonyx LO and chromatographed on calcium phosphate as described above for unlabeled rhodopsin except that the column was washed with 25 mL of 2% (w/v) Ammonyx LO containing 0.01 M imidazole buffer (pH 7) to remove unbound radioactive label before elution of the rhodopsin with the phosphate gradient. The fractions were analyzed spectrophotometrically and the radioactivity was determined in 0.2-mL aliquots as described under Radioactivity Measurement by using NE 260. The number of moles of label bound per mole of rhodopsin was calculated from the 500-nm absorbance peak using ϵ_{500} = 40 600 (Wald and Brown, 1953–1954).

Column Chromatography of Variously Labeled ROS Treated with Papain. Variously labeled ROS treated with papain were also column chromatographed. They could be solubilized in Ammonyx LO as described for untreated ROS without any loss in 500-nm absorbance to yield a solution of papain-cleaved rhodopsin. Papain-cleaved rhodopsin was more strongly adsorbed to the calcium phosphate column than rhodopsin and the two species were completely separated by using a gradient from 5 to 500 mM potassium phosphate (pH 7) containing 2% (w/v) Ammonyx LO (Figures 5 and 6b).

Amino Acid Analysis. Rhodopsin and papain-cleaved rhodopsin labeled with *N*-ethyl[2,3-^{14}C]maleimide were purified by chromatography on calcium phosphate. Samples were

then dialyzed against cold water and the proteins precipitated by addition of 20 volumes of methanol (−18 °C, 18 h). The precipitate was collected by centrifugation at 12 000*g* for 10 min, washed by recentrifugation, and then suspended at about 2 mg/mL in distilled water. The molarity of the suspensions was calculated from the specific radioactivity of the *N*-ethyl[2,3-^{14}C]maleimide covalently bound to the parent column protein and also from the 280-nm absorbance of an aliquot solubilized in 5% (w/v) sodium dodecyl sulfate using ϵ_{280} = 69 020. Both methods gave similar answers. Twenty-nanomole samples were analyzed, and the internal standard, norleucine, was added before hydrolysis to correct for manipulative losses. HCl (6 M) (0.5 mL) containing 0.1% (v/v) thioglycolic acid and 0.1% (w/v) phenol was added. The samples were hydrolyzed in vacuo for 36 h at 108 °C. HCl was then removed by rotary evaporation at 20 °C. The residue was dissolved in 0.01 M HCl (5 mL) and analyzed on an automatic amino acid analyzer. Cysteine and tryptophan were not recovered.

Tryptophan was determined by *N*-bromosuccinimide titration (Spande and Witkop, 1967) in the absence of retinal by using protein precipitates solubilized in 1% (w/v) sodium dodecyl sulfate containing 100 mM sodium acetate buffer, pH 4. Oxidation of tryptophan was followed spectrophotometrically by the addition of 10-μL aliquots of *N*-bromosuccinimide dissolved in 100 mM sodium acetate buffer, pH 4, to both sample and reference cuvettes at 20 °C. At least 20 min was allowed for completion of reaction after each addition of *N*-bromosuccinimide. The extent of tryptophan oxidation was calculated from the decrease in 280-nm absorbance, after correction for dilution, by the method of Spande and Witkop (1967).

Polyacrylamide Gel Electrophoresis and Comments on the Reliability of Molecular Weight Determination. [^{3}H]Retinal-labeled ROS and those incubated with papain were suspended in distilled water (2 A_{500} units/mL) and irradiated for 2 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 (Akhtar et al., 1968). Nonfixed tritiated retinal was removed by suspending the ROS in methanol (1 mg of ROS protein/mL) and then collecting the ROS by centrifugation at 12 000*g* for 15 min. The pellet was suspended in water (1 mg of ROS protein/mL) and recentrifuged to remove methanol. Fixation, 70–80%, of tritium to the protein was obtained. Samples of the fixed [^{3}H]retinal-labeled ROS and other variously labeled ROS including those treated with papain were then solubilized at 20 °C in 5% (w/v) sodium dodecyl sulfate, 50 mM sodium carbonate in the absence or presence of 10% (v/v) 2-mercaptoethanol as indicated to give a final protein concentration of 2 mg/mL. Protein (40–150 μg) was applied to gels (6.3 × 70 mm dimensions) containing 12.5% (w/v) acrylamide, 0.416% (w/v) bisacrylamide, 0.1% (w/v) sodium dodecyl sulfate, and 6 M urea (Swank and Munkes, 1971). Electrophoresis was performed at 5 mA/gel for 6 h. The gels were stained with Coomassie blue and destained by the method of Weber and Osborn (1969) and scanned at 265 nm in a Joece Loebl densitometer (Joece Loebl and Co., Gateshead, U.K.). It was not possible to use this instrument to scan gels at 550 nm, although subsequent experiments have shown that the 550-nm scans yield results comparable to those obtained at 265 nm.

Apparent molecular weights were estimated by parallel electrophoresis with reference proteins, namely, ovalbumin (43 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), myoglobin (17 200), lysozyme (14 400), cytochrome *c* (11 700), and insulin (β chain = 3280; α chain = 2110). The mobility was linearly related to the logarithms of the molecular weights between 43 000 (ovalbumin) and 2110 (α chain of insulin).

The assignment of molecular weights by sodium dodecyl sulfate–polyacrylamide gel electrophoresis is based on two assumptions: first, that the polypeptides under investigation adopt a rod-like structure in the presence of sodium dodecyl sulfate and, secondly, that they bind sodium dodecyl sulfate directly in proportion to their masses. Owing to the presence of disulfide bonds in rhodopsin and its derivatives, the first of these conditions is not strictly fulfilled when the electrophoresis is performed in the absence of mercaptoethanol. Regarding the second assumption, in view of the hydrophobic nature of rhodopsin, whether the fragments arising from the various regions of the molecule would bind detergent and hence migrate strictly according to their absolute masses is not certain. Other workers have obtained erroneous molecular weights for some integral membrane proteins and in particular glycoproteins (see, for example, Grefraths and Reynolds, 1974; Frank and Rodbard, 1975). In view of these reservations the molecular weights assigned to the various species in the present paper may not represent their true molecular weights and should be regarded as apparent sodium dodecyl sulfate molecular weights.

Generation of Meta I Derivatives. ROS from step 3 and those treated with papain were solubilized in 2% (w/v) digitonin containing 0.067 M potassium phosphate (pH 7.8) by homogenization followed by shaking for 3 h. The digitonin extracts were cooled to 0 °C and irradiated with light of 500 ± 5 nm for 5 min to generate meta I (Matthews et al., 1963).

Measurement of Rhodopsin. The rhodopsin content of ROS suspensions was determined by measuring the difference spectrum at 500 nm using a Pye Unicam SP8000 spectrophotometer in which the samples could be placed near to the photomultiplier. The test cell contained the ROS suspension and the reference cell, an identical sample that had been bleached. Thus the rhodopsin content was determined in A_{500} units (1 A_{500} unit/mL = difference spectrum absorbance of 1 OD at 500 nm measured in a 1-cm path length cell). The spectra of solubilized preparations were recorded on a Cary 118 spectrophotometer. Where appropriate, an ϵ_{500} = 40 600 (Wald and Brown, 1953–1954) was used to calculate the molarity of rhodopsin.

Radioactivity Measurement. Aqueous samples (0.2 mL) and methanol samples (1 mL) were counted in 15 mL of NE260. Polyacrylamide gels containing radioactive bands were sliced into 2-mm discs. Discs from gels containing only ^{3}H radioactivity were analyzed using a Packard TriCarb sample oxidizer. Discs from gels containing ^{14}C or ^{14}C and ^{3}H radioactivity were swollen with 0.8 mL of 90% (v/v) NCS tissue solubilizer by incubating overnight at 50 °C in tightly capped vials. Ten milliliters of butyl-PBD (8 g/L in toluene) scintillant was then added.

The vials were then counted either in a Phillips scintillation counter or in an Intertechnique SL40 counter programmed to automatic quench correction. The recovery of the radioactivity originally applied to the gels was greater than 80%.

Results

Preparation of Unlabeled ROS and Rhodopsin. By combining the meritorious features of several preparations into one, a protocol has been developed that allows an efficient processing of bovine retinae to give either ROS or solubilized rhodopsin of good purity in exceptionally high yields. Yields of between 1.4 and 2.0 μmol of purified rhodopsin/100 retinae were routinely obtained. This compares with ranges of 0.7–1.0,

TABLE I: Rhodopsin Purification from 116 Retinae.

		Vol (mL)	Protein[a] (mg/mL)	Total protein	Total A_{500}	Yield (%)	A_{280}/A_{500}[b]
	Homogenate	240	35	8400	120	100	
Step 1	ROS from a 4-step sucrose gradient	30	54	1620	114	95	+10
Step 2	ROS from a 4-step sucrose gradient	16	11.5	184	103	86	2.5
Step 3	ROS washed with Tween 80	18	6	108	87	72	1.9
Step 4	Solubilized rhodopsin (in Ammonyx LO)	18.5	4.65	86	81	68	1.9
Step 5	Column purified rhodopsin[c]	186	0.374	70	75	62.5	1.6

[a]Protein was determined using the biuret procedure of Gasbarro et al. (1972) with bovine serum albumin as standard. [b]The A_{280}/A_{500} ratio of ROS was determined after solubilization in Ammonyx LO. [c]Rhodopsin was column purified in 10-A_{500}-unit batches.

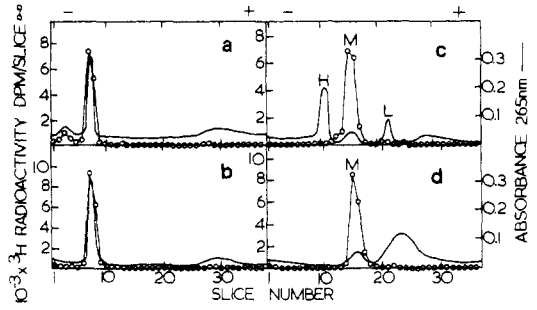

FIGURE 1: Gel electrophoresis of ROS containing tritiated *N*-retinylopsin derivatives. ROS specifically labeled at the active site were prepared by regeneration from 11-*cis*-[15-^{3}H]retinal[1] (specific radioactivity, 24 × 10^6 dpm/μmol) and a sample incubated with papain. Both batches were $NaBH_4$ fixed and methanol washed as described in the Experimental Section. Protein (40 μg) containing 18 000 dpm of ^{3}H derived from native (a and b) and papain-treated ROS (c and d) was then solubilized in sodium dodecyl sulfate in the absence (a and c) or presence (b and d) of 2-mercaptoethanol and electrophoresed. The open circles show the radioactivity in 2-mm slices and the continuous lines the A_{265} scans. Of the radioactivity originally applied, 87%, 91%, 91%, and 92% were recovered in the protein bands in a, b, c, and d, respectively. The rapidly migrating band is phospholipid and is present on all gels.

0.3–0.9, and about 0.9 μmol of rhodopsin/100 retinae reported by Shichi et al. (1969), Applebury et al. (1974), and Papermaster and Dreyer (1974), respectively. The results of a typical preparation are shown in Table I.

ROS obtained from the four-step sucrose gradient had A_{280}/A_{500} ratios of 2.4–3.0. Tween washing improved this ratio to about 1.9–2.4 by removing unwanted proteins. Such Tween washed ROS were used for treatment with papain. In a systematic study it was found that non-Tween-washed ROS on treatment with papain gave an overall fragmentation pattern similar to that for their Tween-washed counterpart, but the higher purity of the latter type of ROS made them the obvious choice for more critical analytical studies. The Tween-washing step may be omitted when the ROS are solubilized in Ammonyx LO for calcium phosphate chromatography without any loss in the quality of the rhodopsin prepared.

Preparation and Papain Digestion of [^{3}H]Retinal-Labeled ROS. ROS were regenerated with 11-*cis*-[15-^{3}H]retinal and a sample incubated with papain to produce cleaved rhodopsin as described in the Experimental Section. The ROS containing the radioactive rhodopsin or cleaved rhodopsin were separately solubilized in Ammonyx LO and chromatographed on calcium phosphate columns to establish their radiochemical homogeneity. Rhodopsin eluted from the column with about 100 mM potassium phosphate, while cleaved rhodopsin was relatively strongly adsorbed and eluted at about 350 mM potassium phosphate (for example, see Figure 6). Based on the 500-nm absorbance peak and using ϵ_{500} = 40 600 (Wald and Brown, 1953–1954), 1.02 and 1.04 mol of [^{3}H]retinal were bound to rhodopsin and cleaved rhodopsin, respectively, suggesting that the ^{3}H was specifically incorporated into their active sites. The spectra of purified labeled rhodopsin and cleaved rhodopsin were similar to that of native rhodopsin and had A_{280}/A_{500} ratios of about 1.7.

The labeled ROS prepared as above were used to investigate the structural aspects of papain cleaved rhodopsin. In order to take full advantage of the active site label, the tritiated retinal moiety in both ROS and papain treated ROS was first fixed to the protein with $NaBH_4$ to produce the corresponding *N*-retinyl opsin derivatives. The resulting preparations were then solubilized in sodium dodecyl sulfate either in the absence or presence of 2-mercaptoethanol for nonreductive and reductive electrophoresis, respectively. Theoretically, the solubilization of a protein in sodium dodecyl sulfate alone should result in the denaturation of the protein and the dissociation of noncovalently linked polypeptides whereas in the presence of 2-mercaptoethanol in addition the reduction of inter- as well as intramolecular disulfide linkages may also occur.

Electrophoresis of $NaBH_4$ fixed [^{3}H]retinal-labeled ROS under both nonreducing (Figure 1a) and reducing conditions (Figure 1b) showed that in both cases most of the radioactivity on the gel was present in the 36 000 molecular weight region which corresponds to the position of bovine opsin. When $NaBH_4$-fixed, papain-treated [^{3}H]retinal-labeled ROS were electrophoresed, the apparent molecular weight of the ^{3}H-labeled *N*-retinyl polypeptide obtained under both nonreducing (Figure 1c) and reducing conditions (Figure 1d) was 15 500 (M fragment) confirming our previous observations (Towner et al., 1977). Two additional protein bands of apparent mol wt 23 000 and 6000 were also resolved when cleaved rhodopsin was analyzed under the conditions of nonreductive electrophoresis (Figure 1c). We shall refer to these fragments of apparent molecular weights[2] ~23 000, ~15 500, and ~6000 as H, M, and L (heavy, medium, and light) fragments. The electrophoretic behavior of cleaved ROS under reductive conditions (Figure 1d) is investigated in depth below.

The Relationship of the Retinal Binding Polypeptide (M Fragment) to the H and L Fragments. A great deal of attention has been focused on the SH groups of rhodopsin (Wald and Brown, 1951–1952; Wu and Stryer, 1972; De Grip et al., 1973, 1975; Delmelle and Virmaux, 1977) and they have been implicated in a disulfide interchange as part of the structural changes that occur following light adsorption by rhodopsin (McDowell and Williams, 1976). ROS contain two accessible SH groups (De Grip et al., 1973). To investigate their location in papain cleaved rhodopsin both SH groups of rhodopsin were

[2] See comments on the reliability of apparent molecular weights determined by sodium dodecyl sulfate gel electrophoresis in the Experimental Section.

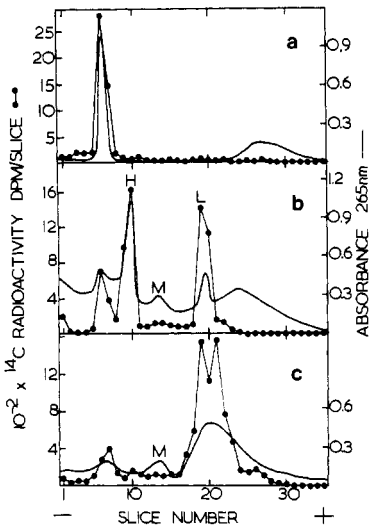

FIGURE 2: Gel electrophoresis of ROS containing rhodopsin or papain-cleaved rhodopsin labeled with *N*-ethyl[2,3-^{14}C]maleimide. ROS were labeled with 1.98 mol of *N*-ethyl[2,3-^{14}C]maleimide (specific radioactivity, 1.1 × 10^6 dpm/μmol) per mol of rhodopsin. The stoichiometry of the incorporation into rhodopsin was determined by chromatography of solubilized ROS on calcium phosphate as described in the Experimental Section. These ^{14}C-labeled ROS (100 μg) were electrophoresed under nonreducing conditions (a), a sample was treated with papain, and portions of protein (140 μg) were electrophoresed under non-reducing (b) and reducing (c) conditions. Of the radioactivity originally applied to the gels in a, 90% coincided with the opsin band (denatured rhodopsin) of apparent molecular weight 36 000; in b 17%, 36%, and 35% coincided with undigested opsin, the H fragment, and the L fragment, respectively; in c, 82% was recovered in two peaks of apparent molecular weights 6000 and 4500 that were part of a broad band of protein in the 9000–4000 molecular weight region.

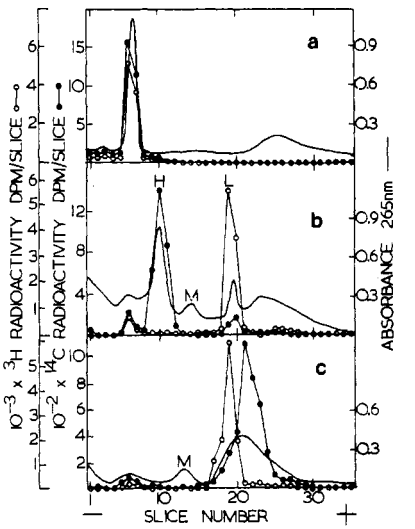

FIGURE 3: Gel electrophoresis of ROS containing rhodopsin or papain-cleaved rhodopsin doubly labeled with iodo[^{3}H]acetamidosalicylate and *N*-ethyl[2,3-^{14}C]maleimide. ROS were first incubated with iodo[^{3}H]-acetamidosalicylate (specific radioactivity, 3.47 × 10^6 dpm/μmol) followed by *N*-ethyl[2,3-^{14}C]maleimide (specific radioactivity, 1.1 × 10^6 dpm/μmol). ^{3}H label, 1.04 mol, and 1.01 mol of ^{14}C label were incorporated per mol of rhodopsin (^{3}H/^{14}C ratio = 3.23) as determined by calcium phosphate chromatography. These ROS (110 μg) were electrophoresed under nonreducing conditions (a) and a sample was treated with papain and portions of protein (145 μg) electrophoresed under both nonreducing (b) and reducing conditions (c). Of the radioactivity originally applied to the gels 93%, 88%, and 91% was recovered in the protein bands in a, b, and c, respectively. In a the opsin band had an ^{3}H/^{14}C ratio of 3.12. In b the H fragment contained mostly ^{14}C but little ^{3}H, while the L fragment contained almost exclusively ^{3}H. In c the apparent molecular weights of the ^{3}H- and ^{14}C-labeled polypeptides were estimated as 6000 and 4500, respectively.

labeled by incubating ROS with *N*-ethyl[2,3-^{14}C]maleimide. Electrophoresis of ROS labeled with 1.98 mol of *N*-ethyl[2,3-^{14}C]maleimide under nonreducing conditions showed that most of the ^{14}C radioactivity on the gel was present in the 36 000 molecular weight region which corresponded to the position of bovine opsin (Figure 2a). These ROS were incubated with papain and electrophoresed. Under nonreducing conditions the ^{14}C radioactivity was almost equally distributed between the H and L fragments (Figure 2b), whereas under reducing conditions (Figure 2c) the ^{14}C-label shifted from the H region into two peaks of apparent molecular weights 6000 and 4500 contained within a broad protein band (9000–4000).

Two possibilities existed to explain the presence of ^{14}C radioactivity in the H and L fragments in the experiment of Figure 2b carried out under nonreductive conditions. Either in the cleaved rhodopsin the two SH groups modified by *N*-ethyl[2,3-^{14}C]maleimide reside in two different fragments (H and L) that arise from different domains of rhodopsin, or the L fragment represents a degradation product of the H fragment. To differentiate between these possibilities two of the SH groups of rhodopsin were modified by two different reagents. By incubating ROS with iodo[^{3}H]acetamidosalicylate, which only alkylates one SH group (Wu and Stryer, 1972), followed by *N*-ethyl[2,3-^{14}C]maleimide, which now labels another SH group, doubly labeled ROS were prepared. One such preparation contained 1.04 mol of the ^{3}H label and 1.01 mol of the ^{14}C label per mol of rhodopsin and had a ^{3}H/^{14}C ratio of 3.23. It is likely, but not yet proved, that the two SH groups modified by this stepwise procedure are the same as those modified by *N*-ethyl[2,3-^{14}C]maleimide alone. Electrophoresis of these ROS under nonreductive conditions showed that the opsin band had a ^{3}H/^{14}C ratio of 3.12 (Figure 3a), whereas in an identical experiment where papain-treated ROS were treated similarly the two isotopes clearly separated, the ^{3}H label was present in the L fragment and the ^{14}C label in the H fragment (Figure 3b). This experiment proves that the L fragment is not a degradation product of the H fragment and that the two fragments are present in a molar ratio of 1:1 in papain-cleaved rhodopsin.

Electrophoresis of the papain-cleaved, doubly labeled membranes was also performed under reductive conditions (presence of 2-mercaptoethanol) which revealed the absence of any protein or radioactivity in the H region. Instead there was a broad band in the 9000–4000 molecular weight region in which the apparent molecular weight of the polypeptide containing the SH group modified by *N*-ethyl[2,3-^{14}C]-maleimide was 4500 (Figure 3c). The H fragment must, therefore, consist of several polypeptide chains linked by the two disulfide bonds which have been suggested to be present in rhodopsin (De Grip et al., 1973). The L fragment which was labeled with iodo[^{3}H]acetamidosalicylate was judged to be unaffected by 2-mercaptoethanol because the apparent molecular weight of the ^{3}H radioactive peak was the same, 6000, under reductive as well as nonreductive electrophoretic conditions (compare Figure 3b with Figure 3c).

Next, papain-treated ROS doubly labeled with 2.0 mol of *N*-ethyl[2,3-^{14}C]maleimide and 1.04 mol of 11-*cis*-[15-^{3}H]retinal were fixed with $NaBH_4$ and subjected to reductive electrophoresis. Figure 4 shows that the ^{3}H representing the retinyl binding site was present in the 15 500 molecular weight region and in total this peak contained 6225 dpm of ^{3}H. All the ^{14}C, amounting to 3280 dpm, was found to be in a band corresponding to the molecular weight region 4000–9000. The

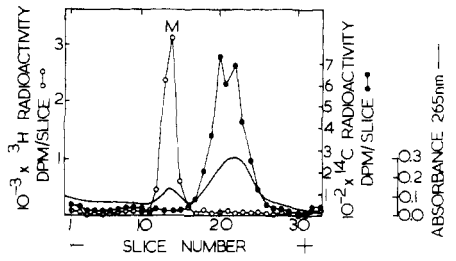

FIGURE 4: Gel electrophoresis of papain-treated ROS doubly labeled with 11-*cis*-[15-^{3}H]retinal (specific radioactivity, 4 × 10^6 dpm/μmol) and *N*-ethyl[2,3-^{14}C]maleimide (specific radioactivity, 0.84 × 10^6 dpm/μmol). ^{3}H label, 1.04 mol, and 2 mol of ^{14}C label were present per mol of cleaved rhodopsin (^{3}H/^{14}C ratio = 2.39) as determined by calcium phosphate chromatography (see Figure 6). These papain-treated ROS were $NaBH_4$ fixed and methanol washed. 82% fixation of ^{3}H to the protein was obtained and the protein now had a ^{3}H/^{14}C ratio = 1.94. Ninety micrograms was electrophoresed under reducing conditions and 88% of the applied radioactivity was recovered in the protein bands. The ^{3}H label was present in the M fragment and the ^{14}C label in the broad band of apparent molecular weight 9000–4000. The ratio of ^{3}H in the M fragment:^{14}C in the broad band was 1.9.

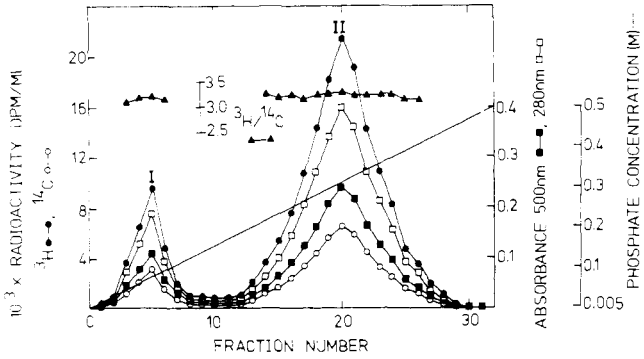

FIGURE 5: Column chromatography of solubilized ROS containing papain-cleaved rhodopsin doubly labeled with iodo[^{3}H]acetamidosalicylate and *N*-ethyl[2,3-^{14}C]maleimide. Doubly labeled ROS that contained 1.04 mol of ^{3}H label and 1.01 mol of ^{14}C label and had a ^{3}H/^{14}C ratio of 3.23 were prepared as in the legend to Figure 3, treated with papain, and solubilized in Ammonyx LO, and 6 A_{500} units was loaded onto a calcium phosphate column. Elution with a 100-mL gradient from 5 to 500 mM potassium phosphate (pH 7) containing 2% (w/v) Ammonyx LO separated undigested rhodopsin (0.8 A_{500} unit, peak I) from papain-cleaved rhodopsin (4.8 A_{500} units, peak II). Three-milliliter fractions were collected. Peak I had a ^{3}H/^{14}C ratio of 3.2 ± 0.1 and contained 1.02 mol of ^{3}H label and 1.01 mol of ^{14}C label per mol of rhodopsin. Peak II had a relatively constant ^{3}H/^{14}C ratio also of 3.2 ± 0.1 in all the constituent fractions and contained 1.03 mol of ^{3}H label and 0.99 mol of ^{14}C label per mol of papain-cleaved rhodopsin. The A_{280}/A_{500} ratios of the fractions of peak I and II were about 1.7.

ratio of the ^{3}H:^{14}C radioactivity in these bands was 1.9 and this compares well with the ^{3}H:^{14}C ratio of 1.94 found in the $NaBH_4$-fixed sample of the original ROS. Thus the cleaved rhodopsin contains one retinyl binding site present in the M fragment and two SH groups modified by *N*-ethylmaleimide located in the band of apparent molecular weight 4000–9000 which represents the L fragment and remnant species derived by the thiolysis of the H fragment (cf. Figure 2c). Since it has already been shown that in cleaved rhodopsin the H:L fragments are present in a molar ratio of 1:1, the cumulative experiments described above allow the conclusion to be drawn that H:M:L fragments must be in the ratio of 1:1:1.

Column Chromatography of Papain-Cleaved Rhodopsin. How do the H, M, and L fragments exist in ROS containing cleaved rhodopsin? There appear to be two possibilities. Firstly, that they interact to form a single noncovalent complex or, secondly, that they exist as distinct entities which are not held

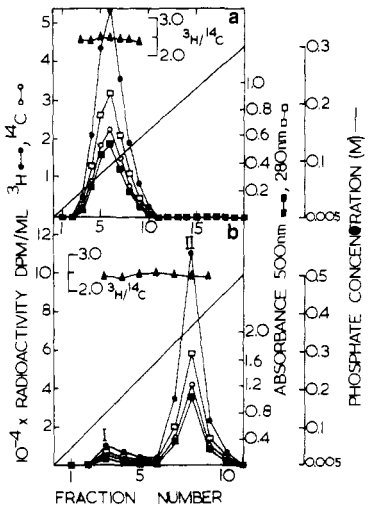

FIGURE 6: Calcium phosphate column chromatography of solubilized ROS containing rhodopsin (a) or papain-cleaved rhodopsin (b) doubly labeled with 11-*cis*-[15-^{3}H]retinal (specific radioactivity, 4 × 10^6 dpm/μmol) and *N*-ethyl[2,3-^{14}C]maleimide (specific radioactivity, 0.84 × 10^6 dpm/μmol). In a, purified rhodopsin (A_{280}/A_{500} = 1.68) had an ^{3}H/^{14}C = 2.39 and contained 1.02 mol of ^{3}H label and 2 mol of ^{14}C label per mol of rhodopsin. In b, all the fractions constituting peak I (undigested rhodopsin) and peak II (purified, cleaved rhodopsin, A_{280}/A_{5000} = 1.66) had an ^{3}H/^{14}C ratio of 2.39 ± 0.1; the cleaved rhodopsin contained 1.04 mol of ^{3}H label and 2 mol of ^{14}C label per mol. The recovery of visual pigment was about 90% in both columns. Three- and 5-mL fractions were collected in a and b, respectively.

within an organized complex. To investigate these possibilities, a sample of the papain-treated ROS doubly labeled with-iodo[^{3}H]acetamidosalicylate on the L fragment and *N*-ethyl[2,3-^{14}C]maleimide on the H fragment as used in Figure 3b was solubilized in Ammonyx LO and analyzed by calcium phosphate column chromatography. Figure 5 shows that the ^{3}H/^{14}C ratio of the parent undigested rhodopsin (peak I) and all the fractions constituting the cleaved rhodopsin (peak II) were similar. The failure of the two isotopes to separate, and, therefore the H and L fragments, when taken in conjunction with the fact that they were dissociated by sodium dodecyl sulfate alone (Figure 3b) suggests that the two fragments interact noncovalently and that these interactions are maintained in Ammonyx LO.

Next doubly labeled ROS containing [^{3}H]retinal at the active site and ^{14}C on the two SH groups, modified by *N*-ethyl[2,3-^{14}C]maleimide, were solubilized in Ammonyx LO and subjected to calcium phosphate chromatography (Figure 6a). Both isotopes and the 280- and 500-nm absorbance peaks of rhodopsin coeluted. The ^{3}H/^{14}C ratio of the pure rhodopsin was 2.39. Treatment of these ROS with papain followed by solubilization and chromatography showed that the ^{3}H/^{14}C ratio across the cleaved rhodopsin peak was relatively constant (2.39 ± 0.1) and identical with the parent rhodopsin (Figure 6b). Since the electrophoretic experiments have shown that, in cleaved rhodopsin the ^{3}H is present in the M fragment and ^{14}C in the H and L fragments, the coelution of the three species is consistent with cleaved rhodopsin being an organized complex consisting of three noncovalently linked fragments.

Amino Acid Analysis. The retention of all the *N*-ethyl[2,3-^{14}C]maleimide label bound to rhodopsin in cleaved rhodopsin, and a knowledge of the specific radioactivity of the proteins purified by column chromatography, determined as in Figure 6, provided a convenient and unambiguous approach for the determination of the molarity of these two species in denatured protein samples. Consequently it was possible to determine the absolute amino acid contents of column purified

TABLE II: Absolute Amino Acid Content of Rhodopsin and Papain Cleaved Rhodopsin.

Amino acid	Residues/mol of rhodopsin, nearest integer[a]	Residues/mol of papain-cleaved rhodopsin, nearest integer[a]
Lys	10	11
His	6	7
Arg	9	9
Asp	22	22
Thr	24	23
Ser	12	11
Glu	25	25
Gly	21	22
Ala	25	23
Val	22	24
Met	10	10
Ile	15	15
Leu	24	24
Tyr	16	16
Phe	25	25
Pro	18	18
Trp[b]	10	10
Total:	294	295
Min mol wt[c]	33370	33562

[a]Each value is the average of the analyses of three preparations of rhodopsin and papain-cleaved rhodopsin. [b]Trp was determined by *N*-bromosuccinimide titration. [c]Minimum molecular weight excludes cysteine and carbohydrate which were not determined.

rhodopsin and papain cleaved rhodopsin. The results obtained are shown in Table II. From the data minimum molecular weights (excluding carbohydrate and cysteine which were not determined) of 33 370 and 33 562 can be calculated for rhodopsin and papain-cleaved rhodopsin, respectively. These results forcefully emphasize that, within the reliability of the technique, no significant loss of amino acid residues from rhodopsin occurred during the papain digestion of ROS. The protein molecular weight based on the amino acid content of rhodopsin is similar to that of Ebrey and Honig (1975), although values of 28 600, 38 240, and 39 100 ± 900 have been reported by Shields et al. (1967), Plantner and Kean (1976), and Daemen et al. (1972).

Tryptophan was determined by the method of Spande and Witkop (1967) using *N*-bromosuccinimide. Tryptophan residues, 9.5 and 9.7, were detected per mol of rhodopsin and cleaved rhodopsin, respectively, which is similar to that reported by Cooper and Hogan (1976) for rhodopsin using the *N*-bromosuccinimide method.

Properties of Papain-Cleaved Rhodopsin. Figure 7 shows that neither the rate nor the extent of regeneration of bleached ROS from exogenous 11-*cis*-retinal was impaired by papain proteolysis of ROS. For studying the meta I to meta II conversion, ROS and those treated with papain were Tween washed, as described in the Experimental Section, to enable direct solubilization in 2% (w/v) digitonin containing 0.067 M potassium phosphate buffer (pH 7.8) giving preparations of $A_{280}/A_{500} = 2.2 \pm 0.2$. Figure 8 shows the preparation of an equilibrium mixture containing about 70% of the meta I derivatives of both rhodopsin and cleaved rhodopsin. Since a similar proportion of the meta I derivatives was stabilized, it may be concluded that the meta I → meta II equilibrium constant for cleaved rhodopsin is similar to rhodopsin. The λ_{max} of the meta I derivative of cleaved rhodopsin was 478 nm, identical with metarhodopsin II.

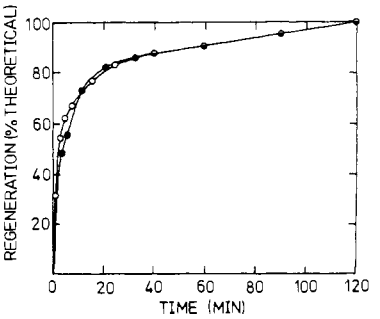

FIGURE 7: Regeneration of native and papain-treated ROS. ROS and those incubated with papain were suspended in 67 mM potassium phosphate buffer, pH 7 (4 A_{500} units/mL), and bleached. The decrease in A_{500} was taken as the theoretical amount of visual pigment present. A twofold molar excess of 11-*cis*-retinal in Tween 80 was then added and the mixture incubated at 37 °C for 2 h. The regeneration (% theoretical) was determined from spectra recorded at time intervals. Native ROS (○); papain-treated ROS (●).

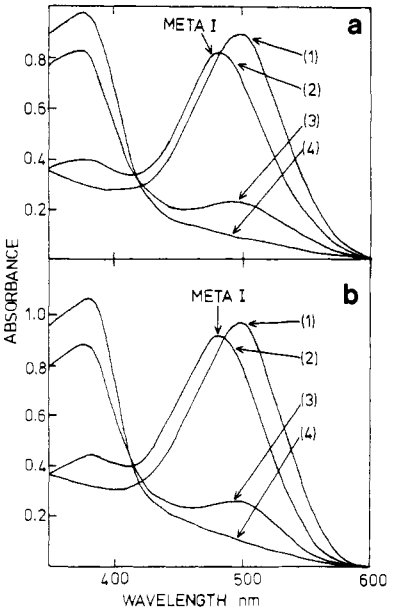

FIGURE 8: Stabilization of meta I derivatives of rhodopsin (a) and papain-cleaved rhodopsin (b). ROS and those incubated with papain were solubilized in 2% (w/v) digitonin containing 0.067 M potassium phosphate buffer (pH 7.8) and cooled to 0 °C (curve 1). (Curve 2) After illumination at 0 °C for 5 min with 500 ± 5 nm light to produce the meta I derivatives of $\lambda_m = 478$ nm. (Curve 3) The preceding solution was allowed to warm to 20 °C; the decay of the λ_{max} nm peaks on warming proves that they were the meta I derivatives. (Curve 4) After bleaching.

Discussion

This investigation has identified the presence of three component fragments (H, M, and L) in papain-treated ROS. To establish their relationship to the native rhodopsin molecule, a radioactive approach was adopted whereby rhodopsin in disc membranes was specifically labeled at several strategic sites. It was shown that the retinal binding site resides in the M fragment of apparent molecular weight 15 500 (Figure 1c) confirming our previous work (Towner et al., 1977) and that the H and L fragments of apparent molecular weights 23 000 and 6000, respectively, each contains one of the two SH groups which can be chemically modified in unbleached rhodopsin. The L fragment contains an SH group which is labeled by iodoacetyl amidosalicylate or *N*-ethylmaleimide, whereas the H fragment contains an SH group which is labeled by *N*-ethylmaleimide but not iodoacetyl amidosalicylate. The experiment shown in Figure 3b where ROS were doubly labeled with iodo[^{3}H]acetamidosalicylate and *N*-ethyl[2,3-^{14}C]maleimide, treated with papain, and electrophoresed under nonreducing

conditions rigorously excludes the possibility that the L fragment is a degradation product formed by the further digestion of the H fragment. The H, M, and L fragments arise without any significant loss in amino acid residues from rhodopsin as illustrated in Table II.

Earlier reports (Trayhurn et al., 1974a,b) suggested that papain removed about one-third of the polypeptide chain from rhodopsin in ROS to yield a functionally active membrane bound fragment termed rhodopsin core. Our results clearly show that this is not the case. Instead, proteolytic digestion of rhodopsin in ROS disc membranes by papain merely results in the cleavage of the molecule to produce a complex, three constituent fragments of which have been identified and shown to be present in a 1:1:1 stoichiometry. The fragments represent different parts of the rhodopsin molecule. These results dramatize the danger of deducing general conclusions from the Coomassie blue staining pattern of gels since the importance of the fragments corresponding to M and L in the present paper was not realized by previous workers.

The results described in this paper may be compared with those reported in several important papers published recently; for example, in the spin-labeling experiments of Delmelle and Virmaux (1977), it was assumed that the retinal binding site and the two reactive SH groups of rhodopsin were present in the papain-cleaved fragment of molecular weight 24 800 (which corresponds to the H fragment of the present paper). We now know that this is not the case.

Pober and Stryer (1975) proposed that thermolysin treatment of ROS results in the formation of a single chain polypeptide of apparent molecular weight 26 000–30 000 (F1) and a retinal binding polypeptide of apparent molecular weight 13 000–18 000 (F2). Pober and Stryer (1975) asserted that similar fragmentation patterns were obtained for both papain- and thermolysin-treated ROS. With respect to the identification of the retinal binding polypeptide, our results are similar to those of Pober and Stryer. However, the H fragment was reduced by 2-mercaptoethanol as had also been observed previously (Trayhurn and Virmaux, 1974) into polypeptides of apparent molecular weight 9000–4000, whereas Pober and Stryer's equivalent species (F1) was resistant to this treatment. It seems likely that the H fragment contains the two S–S bonds suggested to be present in rhodopsin and that papain cleaves the molecule within these disulfide loops. Also Pober and Stryer (1975) located an SH group modified by *N*-(iodoacetamidoethyl)-1-aminonaphthalene-5-sulfonate in F2. According to Wu and Stryer (1972), this SH group is also labeled by iodoacetamidosalicylate, which we observed to be located in the hitherto unrecognized L fragment. It seems likely that these differences could either be due to the differing specificities of papain and thermolysin or due to differences in the experimental conditions used in the two laboratories.

The nature of the interactions between the three fragments was investigated by solubilizing papain-treated ROS in Ammonyx LO and subjecting the cleaved rhodopsin to chromatography on calcium phosphate. The three fragments were inseparable suggesting that they exist as a single organized noncovalent complex.

In vitro an "ideal" sample of rhodopsin is characterized by several properties including its 500-nm absorption band, its regenerability, and the metarhodopsin I $\rightarrow$ metarhodopsin II interconversion. Whereas the 500-nm absorption band of rhodopsin is maintained when ROS are solubilized in a variety of detergents and is independent of the presence of phospholipids, the regeneration of visual pigment and the meta I $\rightarrow$ meta II equilibrium of rhodopsin solutions are relatively sensitive parameters and are easily impaired. It is therefore remarkable that both these functions are fully maintained in the multiple cleaved rhodopsin complex. The noncovalent interactions that hold the complex together must indeed be very strong in order that they be preserved during the many different conformational changes involved in the bleaching–regeneration sequence of cleaved rhodopsin. Thus papain cleaved rhodopsin is a complex consisting of at least three noncovalently linked fragments which retains some of the characteristic properties of rhodopsin.

Assuming that papain can only attack those parts of rhodopsin that are normally exposed to the aqueous environment, then the presence of several polypeptides in cleaved rhodopsin shows that the rhodopsin molecule must fold in and out of the membrane creating a series of "bulge" regions that extend outside the membrane. That rhodopsin in ROS can be cleaved by papain in several places is fortunate because, due to its extreme hydrophobic nature, it has so far eluded sequencing attempts. Thus the availability of many fragments produced by papain proteolysis may help the elucidation of the primary amino acid sequence of rhodopsin. Also, localization of the "bulge" regions in the primary structure of rhodopsin may assist in assigning the vectorial orientation of rhodopsin in the disc membrane.

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