The Amino- and Carboxyl-Terminal Sequence of Bovine Rhodopsin

Paul A. Hargrave and Shao-Ling Fong

School of Medicine and Department of Chemistry and Biochemistry, Southern Illinois University, Carbondale, Illinois 62901

The amino terminus of bovine rhodopsin is blocked and has the sequence x-Met-Asn(CHO)-Gly-Thr-Glu-Gly-Pro-Asn-Phe-Tyr-Val-Pro-Phe-Ser-Asn(CHO)-Lys-Thr-Gly-Val-Val-Arg, where CHO represents sites of carbohydrate attachment. The carboxyl-terminal sequence of rhodopsin is Val-Ser-Lys-Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala. Upon short-term digestion of rod outer segment (ROS) membranes with thermolysin, opsin ($\sim$ 35,000 daltons) is converted to a membrane-bound fragment O' ($\sim$ 30,500 daltons) and 2 peptides containing 12 amino acids are released from the carboxyl terminus of rhodopsin into the supernatant. Upon long-term digestion of ROS with thermolysin, opsin and O' are replaced by the membrane-bound fragments F_1 ($\sim$25,000 daltons),and F_2 ($\sim$9,500 daltons). When ^{32}P-ROS are digested, F_2 carries the [32]P. Both O' and F_1 contain the amino-terminal glycopeptide.

Rhodopsin is the photoreceptor protein of rod cells which are the specialized cells of the vertebrate retina responsible for black-and-white and dim-light vision. Rod outer segment membranes (ROS) contain about 50% by weight of both protein and lipid, and most of the protein (85 ± 5%) is rhodopsin (1). Rhodopsin is a glycoprotein of molecular weight 35,000–40,000 daltons (2–4). It contains 11-cis retinal in Schiff base linkage, which is important in its role as a light receptor (5). Several sulfhydryl groups in the protein are available to different chemical modification reagents (6) and cysteinyl residues have been suggested to be important in rhodopsin's cellular function (7). Rhodopsin becomes phosphorylated by a kinase in a light-dependent reaction, the role of which may be to modulate photoreceptor sensitivity (8). The only reported structural studies on rhodopsin to date are the sequence of a 9-amino acid glycopeptide (9) and the composition of the retinyl-lysine site (10). The use of proteolytic enzymes to probe the orientation of rhodopsin in its membrane environment (11) has been employed to map the location of these important functional sites of the protein in large proteolytic fragments (12). Pober and Stryer (12) have shown that thermolysin cleaves rhodopsin in the disk membrane into

2 large fragments: F_1, which contains carbohydrate, and F_2, which contains the retinyl-binding site.

This report summarizes our identification and sequence of the amino-terminal region of rhodopsin (13) and presents the sequence of the carboxyl-terminal tryptic peptide. We also present the current status of our efforts to localize these regions, and the phosphorylation site, in large fragments from rhodopsin.

MATERIALS AND METHODS

Preparation of Rhodopsin

Rod outer segment membranes were prepared under dim red light from frozen dark-adapted bovine retinas (Geo. Hormel and Co., Austin, Minn.) by the method of Papermaster and Dreyer (1). The membranes banding at the sucrose 1.11−1.13 g/ml interface were harvested and stored frozen at −20°C. In order to prepare chromatographically pure rhodopsin, ROS membranes were dissolved in 50 mM Tris acetate buffer (pH 7.8, 1 mM in Na_2 EDTA) containing 5% Ammonyx L0 detergent. Agarose gel filtration was performed as described previously (14).

The Amino-Terminal Tryptic Peptide of Rhodopsin

All materials and methods concerned with the preparation of the amino-terminal tryptic peptide of rhodopsin have been previously described (13).

The Carboxyl-Terminal Region of Rhodopsin

Rhodopsin for hydrazinolysis was reduced and aminoethylated (15), dialyzed, and lyophylized. The dry residue was delipidated with chloroform:methanol (3:2) and dried over P_2O_5 under oil pump vacuum in an Abderhalden dryer (64°C). Protein content of the dry material was determined by amino acid analysis on acid-hydrolyzed weighed samples. Hydrazinolysis was performed at 85°C for varying times according to the procedure of Braun and Schroeder (16). Amino acids were separated from hydrazides by chromatography on Amberlite CG-50 resin (16). Norleucine was employed as an internal standard.

Peptide T2 from rhodopsin was obtained by chromatography on P6 polyacrylamide gel in 5% acetic acid (13). It was obtained in pure form by rechromatography on the P6 column. Homogeneity was verified by paper chromatography in butanol:acetic acid:water (17) and by electrophoresis at pH 1.65 (18). Two-hundred nanomoles of T2 was linked to triethylenetetramine resin and subjected to the automated Edman degradation on a Sequemat Model 12 solid phase sequencer (Sequemat, Inc., Watertown, Mass.) by the method of Laursen (19). PTH-amino acids were produced by the method of Tarr (20) and identified both by chromatography on polyamide sheets (21) and on silica gel using chloroform:ethanol (98:2) (22). One-thousand nanomoles of peptide T2 (670 nmol/ml) was digested with 600 μg thermolysin (CalBiochem) in 50 mM N-ethylmorpholine acetate (pH 8.0) at 40°C for 2.5 h. The digestion mixture was applied to electrophoresis paper and subjected to 7 V/cm for 30 min followed by 36 V/cm for 3 h in formic acid, pH 1.65. Peptides were visualized with dilute ninhydrin spray, cut out, and eluted with 5% formic acid (17). Peptide sequences were determined by the dansyl-Edman method (23).

The carboxyl-terminal tryptic peptide of rhodopsin was prepared by a method designed to be selective for such a peptide (24). Briefly, the method involves reduction and

aminoethylation of the protein followed by esterification of all carboxyl groups of the protein with glycinamide. The modified rhodopsin is then digested with 10% by weight of trypsin, followed by carboxypeptidase B. The peptide mixture, in 8 M urea, is chromatographed on Dowex 1 resin at pH 11, and the peptide-containing eluant desalted by chromatography on P2 polyacrylamide gel. The purity of the carboxyl-terminal peptide was assessed by chromatography on a 1.0 × 215 cm column of P6 resin (100–200 mesh) equilibrated in 100 mM NH_4HCO_3. The peptide-containing eluant fractions from the column were further examined by preparative paper electrophoresis (as described above for peptide T2). Peptides were visualized with fluorescamine (25) and eluted from the paper as before.

Limited Proteolysis of ROS Membranes

Thermolysin digestion of ROS was performed according to Pober and Stryer (12) based on the procedure of Saari (11). Digestion was terminated either by addition of Na_2 EDTA (to 10 mM) or by addition of o-phenanthroline (to 1 mM). Samples of ROS were prepared for electrophoresis by dissolving in an SDS-cocktail (1) and were submitted to SDS-PAGE (26). Gels were scanned using a Varian Techtron 635 spectrophotometer attachment. Molecular weights were estimated graphically based on the mobility of 7 standard proteins.

Membranes from digestion experiments were pelleted by centrifugation (40,000 × g, 20 min), resuspended (10 mM Tris-acetate, pH 6.9, 5 mM in Na_2EDTA) and the centrifugation repeated. The supernatants were further analyzed by amino acid analysis and by preparative paper electrophoresis. Membranes were dissolved by stirring overnight in the dark in buffer containing Ammonyx L0. The detergent extract was clarified by centrifugation (40,000 × g, 30 min). The extract was made 1 mM in $MgCl_2$, $MnCl_2$, and $CaCl_2$ prior to loading on a column of Concanavalin A-Sepharose (Pharmacia). The column was eluted with 50 mM Tris-acetate buffer (pH 6.9, 0.3% in Ammonyx L0, 1 mM in $MgCl_2$, $MnCl_2$, and $CaCl_2$), followed by the same buffer containing 500 mM α-methyl glucoside (Sigma). Protein-containing eluates were pooled and concentrated by ultrafiltration. Following dialysis to reduce the detergent concentration, samples were digested with trypsin (13). Digests were made 50 mM in pyridine, titrated to pH 2.4 with acetic acid, and chromatographed on a 0.9 × 6.0 cm column of AG50WX8 resin in the same buffer. Additional peptides were eluted with a 200 mM pyridine acetate buffer (pH 3.1). Peptides were further separated on a preparative scale by paper electrophoresis.

Phosphorylation of ROS membranes was performed by the procedure of Kühn et al. (27) as modified by McDowell and Kühn (28). Gels to be sliced were fixed in solutions lacking Coomassie Blue (26). One-millimeter slices, prepared using a Mickle gel slicer, were dissolved by heating with 0.5 ml 30% H_2O_2 in capped vials at 50°C for 15 h (29). Radioactive counting was performed using a Triton X114-xylene scintillation cocktail (30).

RESULTS

Rhodopsin and its Tryptic Glycopeptide Lacks a Free Amino Terminus

When the tryptic peptides of aminoethyl-rhodopsin are subjected to cation-exchange chromatography at low pH, several acidic peptides are not retained by the column and are eluted in the early fractions from the column (Fig. 1, inset). Three peptides have been
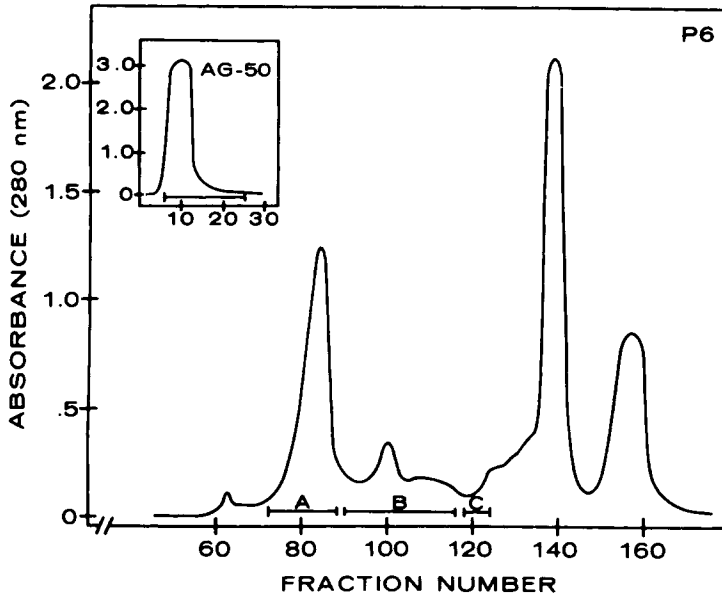
Fig. 1. Chromatography of the soluble tryptic peptides of aminoethyl rhodopsin. Inset) Soluble peptides from a tryptic digest of aminoethyl rhodopsin were chromatographed on a 0.9 × 60 cm column of AG50WX8 resin in 50 mM pyridine acetate buffer (pH 2.4) at 60 ml/h. 3.0 ml fractions were collected. Column frations which were found to contain glucosamine by amino acid analysis were pooled as indicated. Figure) Pooled glucosamine-containing fractions from chromatography on AG50 (inset) were concentrated and applied to 1.0 × 200 cm column of Biogel P6 (100–200 mesh) equilibrated in 5% acetic acid. 1.0 ml fractions were collected and their $A_{280 \, nm}$ determined. Peptides were detected by paper electrophoresis of hydrolyzed aliquots. No peptide material was found in the $A_{280 \, nm}$-absorbing peaks following pool C.

separated from this unbound material by gel filtration (Fig. 1). From pool A was obtained a 16-amino acid glycopeptide, T1 (Table I). Peptide T1 does not have a free amino terminus, as demonstrated by dansylation and by the Edman reaction.

Dansylation of rhodopsin prior to or following the Edman reaction shows no amino-terminal amino acid for the protein. Thus the amino-terminal blocked peptide T1 is a likely candidate for the amino-terminal peptide of rhodopsin. A method specific for isolating only the amino-terminal peptide from a protein (by tryptic cleavage at arginyl residues) produced a single peptide, T1′, from rhodopsin (Table I). This peptide (T1′) contained peptide T1 in its sequence. The primary sequence of this amino-terminal region of rhodopsin has been determined (13):

x-Met-Asn(CHO)-Gly-Thr-Glu-Gly-Pro-Asn-Phe-Tyr-Val-Pro-Phe-Ser-Asn(CHO)-Lys-Thr-
Gly-Val-Val-Arg.
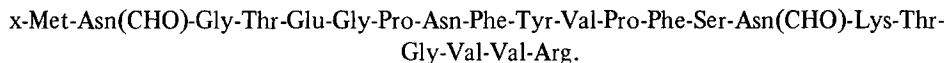
The nature of the amino-terminal blocking group is currently under investigation.

Identification of the Carboxyl-Terminal Region of Rhodopsin

When hydrazinolysis is performed on chromatographically-pure rhodopsin, alanine is identified as the carboxyl-terminal amino acid (Table II). A tryptic peptide

TABLE I. Amino Acid Analysis of Rhodopsin Peptides

Amino acid	T1[a]	T1'[a,b]	T2	T2-Th1[c]	T2-Th2[d]	T2'[e]	Th-196
Asx	3.0 (3)	3.0 (3)					
Thr	1.0	2.0 (2)	1.8 (2)		1.7 (2)	1.6 (2)	1.8 (2)
Ser	0.99 (1)	1.1 (1)	1.1 (1)		0.94 (1)	0.98 (1)	1.8 (2)
Glx	1.1 (1)	1.3 (1)	2.1 (2)		2.0 (2)	2.1 (2)	2.1 (2)
Pro	2.1 (2)	N.Q. (2)	1.1 (1)	1.0 (1)		0.97 (1)	
Gly	2.0 (2)	2.8 (3)				2.2 (2)	
Ala			2.0 (2)	2.0 (2)		2.0 (2)	
Val	0.95 (1)	3.1 (3)	1.2 (1)	0.73 (1)		1.0 (1)	1.0 (1)
Met	0.28 (1)[f]	0.60 (1)					
Tyr	0.90 (1)	N.Q.[g] (1)					
Phe	1.9 (1)	1.7 (2)					
Lys	0.89 (1)	1.1 (1)					1.0 (1)
GlcN	3.5	1.9					
% yield[h]	35	23	37	70	60	15	–

Numbers which are underlined are values used for normalization. Numbers in parentheses are integral values for the moles of amino acid present per mole of peptide. N.Q.) not quantitated but present.
[a]Hydrolyzed in the presence of phenol
[b]72-h hydrolysis
[c]Prepared by paper electrophoresis; mobility 53 cm
[d]Prepared by paper electrophoresis; mobility 41 cm
[e]Prepared by paper electrophoresis; mobility 23.6 cm
[f]Met = 0.88 when hydrolyzed in absence of phenol
[g]Could not be quantitated due to coelution with glucosamine in this analysis. Detected by paper electrophoresis.
[h]Percent yields are based on the starting material for that peptide, and do not take into account aliquots removed for analytical purposes.

TABLE II. Hydrazinolysis of Rhodopsin and its Carboxyl-Terminal Peptide

	Rhodopsin moles amino acid/mole rhodopsin		Peptide T2 moles amino acid/mole peptide	
Amino acid	60 h	80 h	60 h	100 h
Thr		0.10		
Ser		0.31		
Gly	0.07	0.20		
Ala	0.40	0.83	0.43	0.60

All yields are normalized to 100 % recovery of the norleucine internal standard.

(T2) which lacks a basic amino acid in its composition, was prepared from pool C, Fig. 1. It contains alanine (Table I) and it has an alanine carboxyl terminus as shown by hydrazinolysis (Table II). Upon digestion of T2 with thermolysin, 2 peptides were produced in good yield [T2-Th1 and T2-Th2 (Table I)]. Their sequences were determined by the dansyl-Edman technique to be Val-Ala-Pro-Ala and Thr-Glx-Thr-Ser-Glx respectively. Peptide T2, when analyzed by the solid-phase Edman method, gave the sequence

<p style="text-align:center">Thr-Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala.</p>

The carboxyl-terminal tryptic peptide of rhodopsin was then selectively prepared from glycinamide-modified rhodopsin. The purified peptide, T2', has the amino acid composition of T2 and 2 moles of glycine (Table I). One round of the dansyl-Edman reaction showed its amino-terminal sequence to be Thr-Glx.

Since both T2 and T2' are produced by a tryptic cleavage, the amino acid preceding the amino-terminal threonine must be basic. An overlapping peptide (Th-196) which extends this sequence has been prepared from a thermolytic digest of aminoethyl-rhodopsin (P. A. Hargrave, C. V. Barber, R. W. Siemens, K. A. Woodin and W. J. Dreyer, unpublished). Its composition is given in Table I. Its sequence, obtained by the dansyl-Edman method is

<p style="text-align:center">Val-Ser-Lys-Thr-Glx-Thr-Ser-(Glx).</p>

Proteolytic Digestion of Rhodopsin in ROS Membranes

The action of thermolysin on ROS membranes resulted in the production of 3 large membrane-bound fragments (Exp. 1, Fig. 2). Opsin (O, $\sim$ 35,000 daltons) is first converted to a smaller fragment O' ($\sim$ 30,500 daltons) in a reaction which is virtually complete in 5 min. In a separate experiment (Exp. 2), photographs of gels show the same conversion of O to O' (Fig. 3). O' is then converted to membrane-bound fragments F_1 ($\sim$ 25,000 daltons) and F_2 ($\sim$ 9,500 daltons). Opsin, O', and F_1 all stain with the PAS reagent, but F_2 does not (data not shown).

The supernatant from the 5-min digestion of ROS (Exp. 2), and its control, were further examined by amino acid analysis and by preparative paper electrophoresis. In the experimental sample, 6.5% of the 56.8 mg of ROS protein was solubilized, and in the control 0.55%. By paper electrophoresis 2 peptides from the digestion supernatant were detected with ninhydrin spray: Th-S1 (48-cm mobility) and Th-S2 (40.5-cm mobility). No additional peptides were subsequently detected using peptide-bond spray, and no peptides were found in the supernatant from control membranes incubated in the absence of thermolysin. Peptide Th-S1 had the composition of peptide Th-196 and also showed the amino-terminal sequence Val-Ser-Lys. Peptide Th-S2 had the composition of peptide T2-Th1 and also showed the amino-terminal sequence Val-Ala-Pro. More peptides were detected in the supernatant from the 15-h digestion, but they have not yet been completely characterized. Glucosamine is absent from their amino acid composition.

The protein composition of the membrane was also examined. Undigested ROS membranes, membranes after 5 min digestion (Exp. 2), and membranes after 15 h digestion (Exp. 1), were dissolved in Ammonyx L0 and chromatographed on Concanavalin A-Sepharose in the dark. The nature of the material prepared (rhodopsin, O', and F_1-F_2) was verified by SDS-PAGE. Following trypsin digestion the most acidic tryptic peptides were obtained by ion-exchange chromatography and further separated by preparative paper electrophoresis. All 3 protein samples were found to contain the amino-terminal tryptic peptide T1 (mobility 11.5–12 cm).
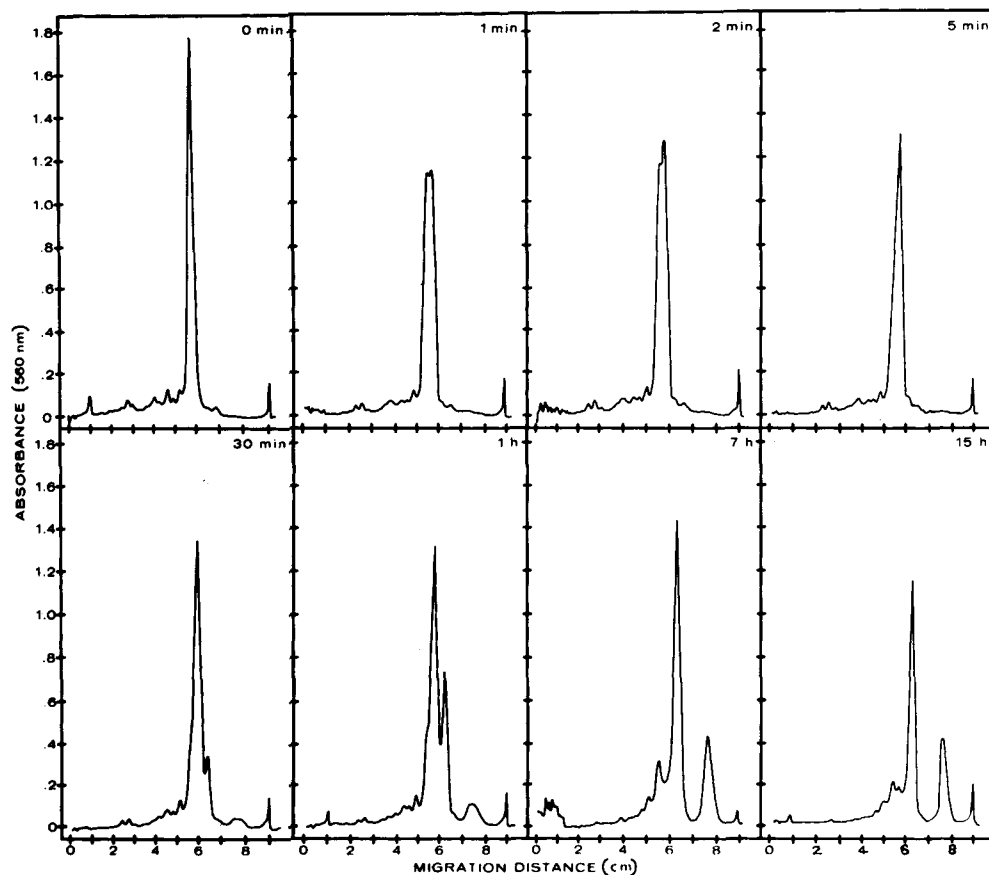
Fig. 2. Thermolysin digestion of ROS membranes: spectrophotometer tracings of stained SDS-PAGE gels. Gels were scanned at 560 nm using a Varian Techtron 635 spectrophotometer with gel scanner attachment. The spike in the tracings at 9 mm shows a nichrome wire which was inserted to mark the position of the tracking dye.

When rhodopsin, O', or the F_1-F_2 complex of rhodopsin was loaded on the Con-canavalin A-Sepharose column and eluted from it in the dark, protein recovery of greater than 90% was obtained. Pober and Stryer have shown that illuminating the F_1-F_2 complex on the column causes the dissociation of F_2, and F_1 may then be prepared by elution with α-methyl glucoside (12). However, when we reloaded the purified F_1-F_2 complex and attempted to prepare quantities of purified F_1 and F_2, poor yields were obtained. Figure 4 shows the results of an experiment in which an extract of ROS (digested to produce F_1-F_2) was chromatographed on Concanavalin A-Sepharose. Of the 26.9 mg protein loaded, 1.6 mg emerged in the unbound fraction (pool A), 1.1 mg was eluted following light ex-posure (pool B), and 0.95 mg was eluted with α-methyl glucoside (pool C). Examination by SDS-PAGE showed F_2 in pool B, the F_1 (and its aggregates) in pool C, as expected. Their amino acid analyses are in substantial agreement with those reported by Pober and Stryer (12).
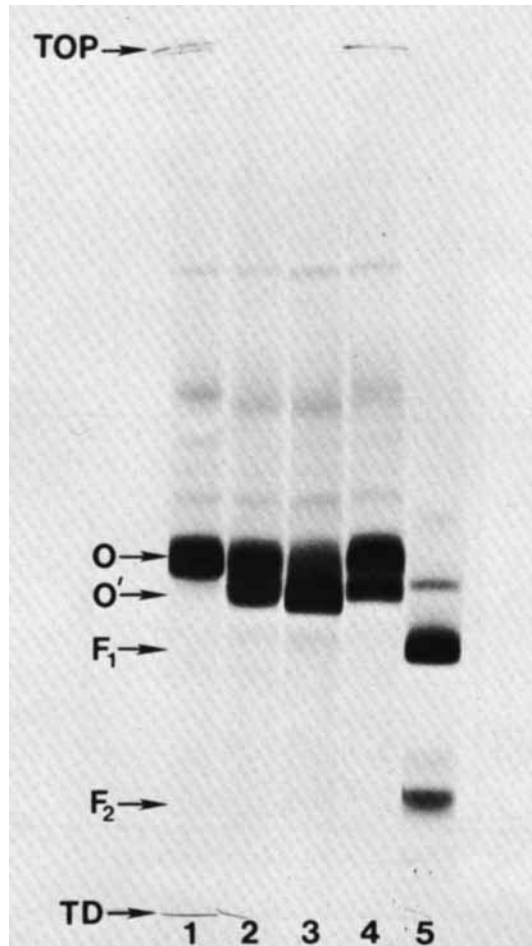
Fig. 3. Thermolysin digestion of ROS membranes: photograph of Coomassie Blue-stained SDS-PAGE gels. Gels 1–4 are from Exp. 2, a 5-min thermolysin digestion experiment in which gel 1 = 0 time, gel 2 = 1 min, gel 3 = 5 min, gel 4 = a mixture of 0-time and 5-min. Gel 5 is from a 15-h digestion of ^{32}P-ROS (Exp. 3). O = opsin, O′, F_1, and F_2 are digestion products of opsin. The nichrome wire at the base of the gels shows the original position of the tracking dye (TD).

The Location of the Phosphorylation Site(s)

Light-exposed phosphorylated ROS were submitted to SDS-PAGE before and after thermolysin digestion (Exp. 3, Fig. 5). All ^{32}P which was incorporated into ROS membrane proteins is incorporated into rhodopsin. Following proteolysis, ^{32}P migrates with the F_2 fragments and not with the large F_1. Heterogeneity in the F_2 region of the gel is shown by the radioactivity profile. In some digests of ROS we have observed a closely spaced Coomassie Blue-staining doublet in the F_2 region.
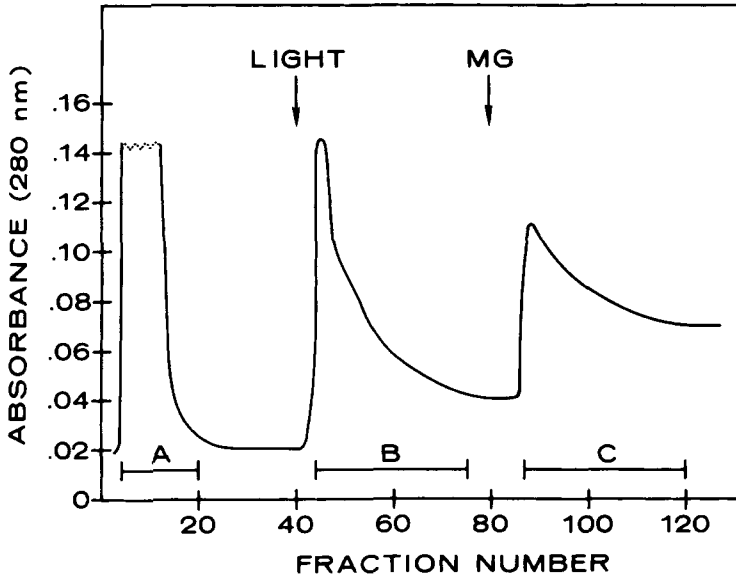
Fig. 4. Preparation of rhodopsin fragments F_1 and F_2 by chromatography on Concanavalin A-Sepharose. Thermolysin-digested ROS were solubilized in buffer containing Ammonyx L0 (see Methods) and chromatographed on a 0.9 × 17.5 cm column of Concanavalin A-Sepharose. Column flow of 12 ml/h was interrupted for 1 h during exposure to light. Flow was reduced to 6 ml/h during elution with α-methyl glucoside (MG). Fractions A, B, and C were pooled as shown.

DISCUSSION

Our results have shown that the amino-terminus of rhodopsin is blocked and that the blocked peptide T1 is the amino-terminus of rhodopsin (13). Peptide T1 is hydrophilic in composition and contains 2 sites at which carbohydrate is attached. The single carbohydrate site previously reported had been thought to account for all of rhodopsin's carbohydrate (9). This region of the rhodopsin molecule would be expected to be exposed at the membrane surface.

In contrast to a previous study in which hydrazinolysis was reported to yield no carboxyl-terminal amino acid for rhodopsin (31), we find alanine. Variable submolar amounts of glycine and serine were also produced from rhodopsin, and from lysozyme which we examined as a control. This has been reported to occur with other proteins (16). Due to its lack of a basic amino acid, the tryptic peptide T2 would be expected to be the carboxyl-terminal peptide of rhodopsin. Like rhodopsin, its carboxyl-terminal is alanine. The carboxyl-terminal status of T2 is further confirmed by its isolation using a method specific for preparing carboxyl-terminal peptides from proteins. The thermolytic peptide Th-196 contains the tryptic cleavage site and extends our sequence in the carboxyl-terminal region. This region of the molecule is hydrophilic in composition and might be expected to be exposed to an aqueous environment.

The first event in the limited thermolytic digestion of rhodopsin in ROS has been shown to be production of a membrane-bound fragment O' which has an apparent molecular weight of 30,500 daltons. This is accompanied by a release of 2 peptides into the
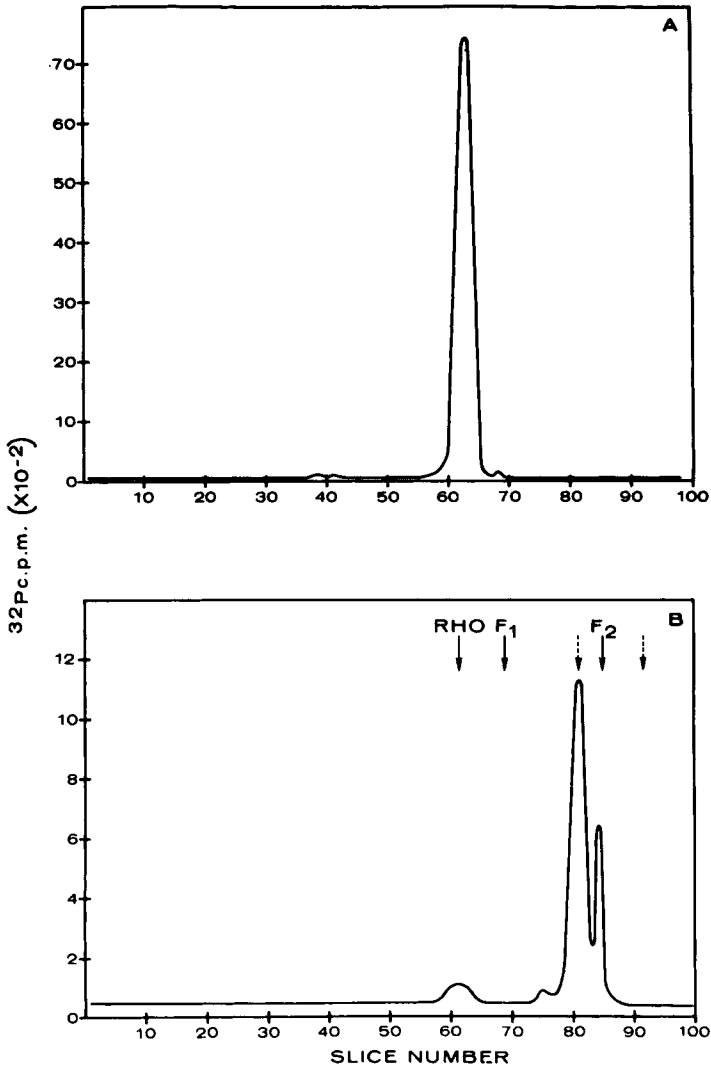
Fig. 5. ^{32}P distribution in ROS and thermolysin-digested ROS. Unstained SDS-PAGE gels of ^{32}P-ROS (A) and thermolysin-digested ^{32}P-ROS (B) were sliced in 1-mm pieces and their radioactivity determined. In B, solid arrows show the positions of rhodopsin and its F_1 and F_2 fragments in a stained duplicate gel. Dashed arrows show the positions of 2 very weakly staining bands.

supernatant. These 2 thermolytic peptides originate from the carboxyl-terminal region of rhodopsin and account for 1,200 daltons molecular weight. No glucosamine-containing peptides are released into the supernatant, and the amino-terminal tryptic peptide can be prepared from the purified O′ fragment. Additional thermolytic cleavage may have occurred at the carboxyl terminal of rhodopsin to produce small undetected membrane-bound fragments. However, the apparent molecular weights of rhodopsin and its fragments as determined by SDS-PAGE probably do not adequately reflect their true molecular weights.

The rapid production of an intermediate which may be comparable to O′ has been

observed by the action of subtilisin on ROS (32). Since these authors used membranes of native sidedness, this would imply that the carboxyl-terminal region of rhodopsin is located at the extradiskal surface of the disk membrane. It is probable that the amino-terminal region of rhodopsin is located at the intradiskal membrane surface: we have shown that the amino-terminal sequence has sites of carbohydrate attachment in rhodopsin, and carbohydrate has been cytochemically localized only at the inner disk surface (33).

Following the rapid conversion of opsin to O', O' is converted to F_1 and F_2 over a period of hours. We have made no attempt to quantitate this conversion, but Saari (J. Saari, personal communication) has found F_1 and F_2 to appear concurrently and in approximately equal molar quantities. We have observed the appearance of several additional peptides in the supernatant during this conversion. No glucosamine-containing peptides are released. F_1 still stains with PAS reagent, and the amino-terminal peptide T1 is still present in the F_1-F_2 complex. Thus, the soluble peptides must come either from the region of internal cleavage which produces F_1 and F_2 from O', or from the caboxyl-terminal region of F_2.

The retinyl site of rhodopsin has been shown to be located in the F_2 fragment (12, 32). We have located the phosphorylation site(s) also in F_2 [as has Saari (personal communication)]. F_2, by its staining and ^{32}P profile, does not appear to represent a unique single fragment but is probably a small family of overlapping peptides from the carboxyl-terminal region of rhodopsin.

It would be desirable to prepare fragments F_1 and F_2 in quantity in order to further investigate their primary structure. Although we were able to elute rhodopsin and its F_1-F_2 noncovalent complex from the Concanavalin-A Sepharose column in the dark in 90% yield, very low yields were obtained for F_1 and F_2 following light exposure on the column. Our experimental conditions were not identical to those of Pober and Stryer (12), however. Better preparative scale yields might be obtained by use of their lightly-substituted Concanavalin A-agarose gel and their detergent-buffer conditions.

ACKNOWLEDGMENTS

REFERENCES

1. Papermaster DS, Dreyer WD: Biochemistry 13:2438, 1974.
2. Hubbard R: J Gen Physiol 37:381, 1954.
3. Daemen FJM, de Grip WJ, Jansen PAA: Biochim Biophys Acta 271:419, 1972.
4. Lewis MS, Krieg LC, Kirk WD: Exp Eye Res 18:29, 1974.
5. Wald G: Science 162:230, 1968.
6. de Grip WJ, Bonting SL, Daemen FJM: Biochim Biophys Acta 396:104, 1975.
7. Wald G, Brown P, Gibbons I: J Opt Soc Am 53:20, 1963.
8. Kühn H, Bader S: Biochim Biophys Acta 428:13, 1976.
9. Heller J, Lawrence MA: Biochemistry 9:864, 1970.
10. Bownds D: Nature 216:1178, 1967.
11. Saari JC: J Cell Biol 63:480, 1974.
12. Pober JS, Stryer L: J Mol Biol 95:477, 1975.
13. Hargrave PA: Biochim Biophys Acta 492:83, 1977.
14. Hargrave PA: Vision Res 16:1013, 1976.

15. Cole RD: In Hirs CHW (ed): "Methods in Enzymology." New York: Academic Press, 1967, vol 11, p 315.
16. Braun V, Schroeder WA: Arch Biochem Biophys 118:241, 1967.
17. Bennett JC: In Hirs CHW (ed): "Methods in Enzymology." New York: Academic Press, 1967, vol 11, p 330.
18. Dreyer WJ, Bynum E: In Hirs CHW (ed): "Methods in Enzymology." New York: Academic Press, 1967, vol 11, p 32.
19. Laursen RA, Bonner AG, Horn MJ: In Perham RN (ed): "Instrumentation in Amino Acid Sequence Analysis." Academic Press, 1975, p 73.
20. Tarr GE: Anal Biochem 63:361, 1975.
21. Summers MR, Smythers GW, Oroszlan S: Anal Biochem 53:624, 1973.
22. Horn MA: Personal communication.
23. Gray WR, Smith JF: Anal Biochem 33:36, 1970.
24. Hargrave PA, Wold F: Int J Peptide Protein Res 5:85, 1973.
25. Mendez E, Lai CY: Anal Biochem 65:281, 1975.
26. Fairbanks G, Steck TL, Wallach DFH: Biochemistry 10:2606, 1971.
27. Kühn H, Cook JH, Dreyer WJ: Biochemistry 12:2495, 1973.
28. McDowell JH, Kühn H: Biochemistry (In press).
29. Richards JC: Personal communication.
30. Anderson LE, McClure WO: Anal Biochem 51:173, 1973.
31. Heller J: Biochemistry 7:2906, 1968.
32. Gaw JE, Dratz EA, Vandenberg CA, Slaughter SE, Kelton EA, Lipschultz MJ, Schwartz S: Biophys J 17:80a, 1977.
33. Rölich P: Nature (London) 263:789, 1976.