Amino Acid Sequence Adjacent to a Sulfhydryl Group Exposed on Illumination of Bovine Rhodopsin*

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Mahin Khatami, Ruth H. Angeletti, and John H. Rockey

From the Department of Ophthalmology, Scheie Eye Institute, and the Department of Neuropathology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Two sulfhydryl groups of bovine rhodopsin, available for chemical modification only after bleaching, were specifically labeled with radioactive iodoacetamide. The labeled protein was extensively reduced and alkylated and digested with pronase, and peptides were purified by gel filtration chromatography, anion and cation exchange chromatography, and high pressure liquid chromatography. Purified peptides were detected by their radioactivity, peptide fragmentation in detergent solutions of rod outer segment membranes with thermolysin (12), the F2 fragment produced by digestion of rhodopsin in rod outer segment membranes with thermolysin (12). The F2 fragment was directly exposed on photobleaching (6, 7, 12, 13, 14). A dipeptide of sequence alanyl-(retinal) lysine, containing the chromophore binding site lysyl residue, has been isolated from the primary structure of bovine rhodopsin. The retinal binding site lysyl residue has been located in the smaller F2 fragment produced by digestion of rhodopsin in rod outer segment membranes with thermolysin (12). The F2 fragment also contains the sites of light-dependent phosphorylation, a dark-reactive cysteinyl group, and the COOH-terminal amino acid sequence (5, 13, 14). The larger F1 fragment produced by thermolysin digestion of membrane-bound rhodopsin contains the NH2-terminal amino acid sequence, the sites of attachment of the carbohydrate residues, and four sulfhydryl groups, two of which are available for chemical modification only after bleaching (10, 12, 16). The sulfhydryl groups exposed on bleaching also have been located in a smaller fragment of apparent Mr = 13,000 produced by chemotryptic cleavage of labeled bovine rhodopsin in rod outer segment membranes (10).

The present investigations establish the amino acid sequence of a tripeptide of bovine rhodopsin containing a light-exposed sulfhydryl group selectively labeled with radioactive iodoacetamide in detergent solution.

MATERIALS AND METHODS

Rod outer segments were isolated from dark-adapted frozen bovine retina (Hormel, MN) by isopycnic discontinuous sucrose density gradient ultracentrifugation. Rhodopsin was extracted from rod outer segments by homogenization in 1.5% (w/v) cetyl(hexadecy1)trimethyl ammonium bromide (Eastman) in 5 mM EDTA, 50 mM Tris-HCl buffer, pH 7.8, at 4 °C, and purified by Bio-Gel A-1.5m chromatography. The activity was added and the sample was bleached. The radioactive alkylation of SH groups in the smaller F2 fragment produced by digestion of rhodopsin in rod outer segment membranes with thermolysin (12) was determined by the addition of a large excess of nonradioactive iodoacetamide and the protein was passed over a column of Sephadex G-25 in 50% acetic acid. Labeled opsin was added with a 50-fold molar excess of iodo-thioretrool in 6 M guanidine-HCl, 2 mM EDTA, 0.5 mM Tris-HCl buffer, pH 8.5, under nitrogen at 30 °C for 16 h, and alkylated with a 55-fold molar excess of iodo[14C]acetamide (specific activity, 22.1 mCi/mmol, ICN) and lyophylized. Alkylated opsin was digested with pronase (2-4% of opsin concentration; Streptomyces griseus protease, B Grade, Calbiochem) in 2% digitonin, 0.1 M Na-phosphate buffer, pH 7.2, at 37 °C (17, 18). Additional pronase was added every 6-8 h and the reaction was terminated after 32 h by dilution with distilled water and lyophilization.

Radiolabeled pronase peptides of opsin were isolated by sequential gel filtration in 22% formic acid on columns of Bio-Gel P-30, P-10, P-4, and P-2 (Bio-Rad). Peptides were further purified by HPLC on a C18 reverse phase column (Waters Associates Model 660 solvent programmer). Anion exchange chromatography was performed on DAX-2 resin (Dionex) and cation exchange chromatography on Dowex 50 (AA-15) resin. Peptides in the ion exchange chromatography effluent were detected by reacting a small aliquot of each fraction with fluorescamine (Fluram, Roche) (11, 19) in 0.1 M Na-phosphate buffer, pH 8.0, and determining the fluorescence in an Amino-Bowman Ratio Recording spectrophotofluorometer (excitation, 392 nm; emission, 475 nm).

Labeled proteins and purified peptides were hydrolyzed at 110 °C in 6 N constant-boiling HCl in vacuo (less than 50 μmol of residual pressure) for 24 h (20). Amino acid analysis was accomplished with a Durrum D-500, a Dionex Fluoropa MBF/SS, or a Technicon amino acid analyzer. S-Carboxymethylcysteine in acid hydrolysates was identified by analysis of the distribution of radioactivity in the effluent from the Technicon amino acid analyzer, or by high voltage paper electrophoresis. Authentic S-carboxymethylcysteine was mixed with the hydrolysates and run in parallel. High voltage electrophoresis was performed on Whatman 3MM paper in formic acid/acetic acid/water (1:4:28), pH 1.7, on a Savant flat plate apparatus (4,000 V applied for 45 min) at 0 °C. Ammonium acids were stained with ninhydrin and analyzed for radioactivity.

* The abbreviations used are: HPLC; high pressure liquid chromatography; PTH, phenylthiohydantoin.

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Amino acid sequence analysis was performed with a Beckman model 890 C Sequencer. Polybrene was used as a carrier for peptide sequencing (21). Phenylisothiocyanate was used for coupling with the amino acids. The PTH derivatives were identified by thin layer chromatography (Whatman paper, stripped plate LK6DF; solvent, ethylene dichloride/glacial acetic acid, 30:7); and by high pressure liquid chromatography (VARIANT Model 5000, 24 mM Na-acetate, pH 5.4, first eluent, followed by a linear gradient of methanol/acetone, 18:3). Ten per cent of each PTH fraction from the sequenator was taken for radioactivity determination.

RESULTS

Amino acid analysis and high voltage electrophoresis of hydrolyzed radiolabeled opsin demonstrated that 85 ± 10% of the radioactivity co-eluted or co-migrated with added authentic S-carboxymethylcysteine (Fig. 1). The ratio of nonradioactive iodoacetamide (added to rhodopsin in the dark) to iodo[14C]acetamide (added immediately prior to bleaching) was either 1:1 or 2:1. The specific activity of the iodo[14C]acetamide was corrected for dilution with nonradioactive io-

![Fig. 1. High voltage electrophoresis and amino acid analysis of S-carboxyl[14C]methylcysteine. A, high voltage electrophoresis of a hydrolysate of rhodopsin which had been reacted in the dark with nonradioactive iodoacetamide, prior to labeling with iodo[14C]acetamide after bleaching (1). Authentic S-carboxymethyl cysteine (CMC) was added to the hydrolysate to facilitate identification of CMC, and also run in parallel (2). The arrow indicates the point of origin. B, co-elution of the radioactivity of the hydrolysate of labeled rhodopsin with added authentic CMC upon amino acid analysis.](image1)

Fig. 2. High pressure liquid chromatography of radioactive peptides taken from a Bio-Gel P-2 column. The sample was dissolved in 0.5 ml of 0.1% H₃PO₄, and applied to a HPLC column (Cᵡ reversed phase, attached to a guard column; temperature, 30 °C; pressure, 2200 psi). Pump A, 0.1% H₃PO₄, 15 min; pump B, linear gradient (curve 6 on programmer), 0 to 100% of 60% CH₃CN and 40% of 0.1% H₃PO₄ (g). Flow rate, 2 ml/min; 1 min/fraction. Chart speed, 0.5 cm/min. Detection system, UV absorption at 210 nm; sensitivity, 2.0 absorbance units full scale; cpm/10 μl.

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![Fig. 3. Subfractionation of peak HP-1 of Fig. 2 by high pressure liquid chromatography. Pump A, 10 mM KH₂PO₄, pH 5.5, 25 min; pump B, 0 to 50% linear gradient of 10 mM KH₂PO₄, pH 5.5, and 50% methanol. Flow rate, 1 ml/min, 1 min/fraction collected; detection system, UV absorption at 210 nm; sensitivity, 1 absorbance unit full scale; cpm/10 μl. Peak HP3 was further purified for sequence analysis (Fig. 4). Further purification of peak HP2 did not yield a peptide suitable for sequence analysis.](image3)
The identification of the sequence carboxamido[14C]methylcysteinyl-prolyl-glycine for the prase peptide from labeled rhodopsin establishes the location of one of the two cysteinyl residues at positions 26, 27, and 33 (13, 14). The cysteinyl residue at position 33 has been reported to have a dark-reactive sulfhydryl group (13). More recently, a fourth cysteinyl residue has been identified in the sequence of a cyanogen bromide fragment of bovine rhodopsin (15). The nature of this cysteinyl residue and its placement within the primary structure of bovine rhodopsin has not yet been fully defined. The sequence Cys-Pro-Gly containing the light-exposed sulfhydryl group differs from all of the reported partial sequences for bovine rhodopsin thus far available.

The physicochemical mechanisms leading to exposure for chemical modification of the previously unreactive sulfhydryl groups of bovine rhodopsin, produced by photobleaching in rod outer segment membranes or detergent solution (9, 10), have yet to be fully understood. Membrane-labeling and proteolytic cleavage experiments have shown that the light-exposed sulfhydryl groups alkylated with iodo[14C]acetamide are located on the NH2-terminal F1 fragment (10). The retinal binding site lysyl residue and the phosphorylation sites are located on the COOH-terminal F2 fragment (12). The F1 and F2 fragments may occupy separate domains in the native visual pigment molecule (23). The light-exposed sulfhydryl groups of the F1 domain are thus far the only newly reactive residues of this portion of the rhodopsin molecule produced by photobleaching, and therefore may reflect a conformational change in a molecular domain distant from the chromophore binding site which may be of importance in visual transduction. A full understanding of the role of the sulfhydryl groups exposed upon bleaching awaits knowledge of their placement within the three-dimensional structure of the native and bleached rhodopsin molecules.

The postbleaching reactive sulfhydryl groups also furnish a useful focus for study of comparative amino acid sequences of rod and cone visual pigments from different species. The present work lays a foundation for such studies.

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