CHARACTERIZATION OF A PHOTOEXPOSED SULFHYDRYL GROUP OF BOVINE RHODOPSIN AVAILABLE FOR CHEMICAL MODIFICATION*

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SUMMARY: Alkylation of rhodopsin in 40 mM cetyltrimethylammonium bromide revealed that the ratio of the light:dark formation of [^{14}C]carbamidomethylated-opsin was 1.0:0.1. A rhodopsin photoexposed sulfhydryl group was demonstrated in rod outer segment membranes with [^{14}C]parachloromercuribenzoate and [^{14}C]iodoacetamide. Amino acid analysis of native and S-carbamidomethylcysteinyl-opsin, before and after performic acid oxidation, indicated that 1.2-1.3 sulfhydryl groups were available for alkylation per 4.9 total half-cystinyl residues in illuminated rhodopsin.

Previous workers, using amperometric silver titration or spectrophotometric titration (1-8), have reported that bovine rhodopsin contains 0-4 native and 0-4 photoexposed sulfhydryl groups. The limitations of these indirect methods are well known (9-11). We have re-investigated the sulfhydryl chemistry of native (dark) and illuminated rhodopsin by studying chemically modified cysteinyl residues of highly purified opsin.

MATERIALS AND METHODS: Frozen, dark-adapted, bovine retinas were homogenized at 4° in 4 mM $CaCl_2$-0.2 M Tris HCl buffer (pH 8), and centrifuged at 20,000 rpm in a Spinco SW 25L rotor. The precipitate was mixed with 2.22 M sucrose-2 mM $CaCl_2$-10 mM Tris HCl buffer (pH 8) and rod outer segments (ROS)† were isolated by a discon-

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†Abbreviations: ROS, rod outer segments; CTAB, cetyltrimethylammonium bromide; CMC, S-carboxymethylcysteine

tinuous sucrose density gradient ultracentrifugation procedure modified from an unpublished method of D. S. Papermaster. ROS were washed three times with 67 mM Na phosphate buffer (pH 7), suspended in 0.2 M Tris HCl buffer (pH 8), and regenerated with 11-cis-retinal (Hoffman-LaRoche) for outer segment labelling experiments. Visual pigment was extracted from washed ROS with 40 mM CTAB-67 mM Na phosphate buffer (pH 7) and purified by Sephadex G-200 gel filtration according to Heller (4).

Kinetics of alkylation of rhodopsin: A CTAB solution of visual pigment was made 0.2 M Tris HCl-5 mM EDTA (pH 8) and a 5 molar excess (over rhodopsin) of [^{14}C]iodoacetamide (11.8 mCi/mmole) was added in the dark. The solution was divided into three parts. Part one was bleached. Aliquots were removed from parts one and two at 5, 10, 15, 30, 60 and 120 minutes. An aliquot was removed from part three in the dark at 60 min, part three was bleached, and further samples were removed. The alkylation reaction was stopped by swamping each aliquot with 0.5 ml of 0.1 M non-radioactive iodoacetamide and acidifying with 0.5 ml glacial acetic acid. The modified opsin was separated from the unreacted reagent by gel filtration through Sephadex G-25 (Pharmacia) in 50% acetic acid, and assayed for absorptivity at 280 nm and radioactivity.

A photoexposed sulfhydryl group of visual pigment was detected in rod outer segment membrane suspensions using [^{14}C]-iodoacetamide and [^{14}C]parachloromercuribenzoate (10.1 mCi/mmole). Regenerated ROS suspensions were divided into three parts. Part one was treated with non-radioactive reagent for two hours in darkness, washed free of unreacted reagent, and then exposed to radioactive reagent and bleached for two hours. Part two was first treated with radioactive reagent, washed repeatedly, and

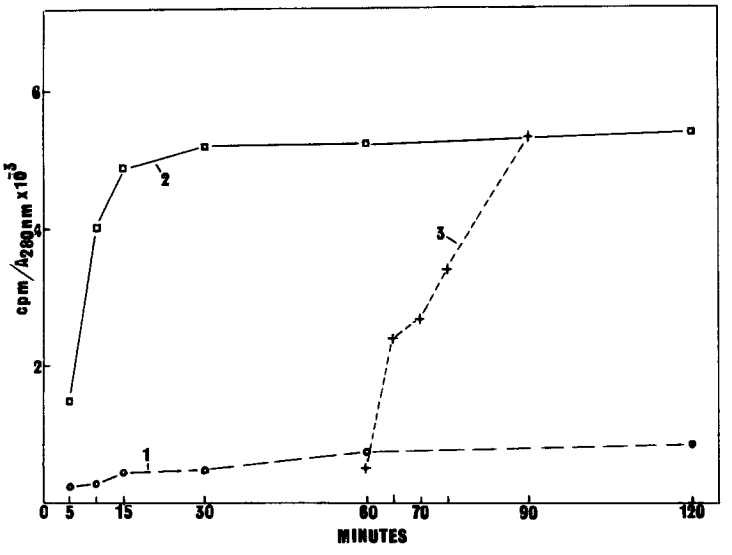

Figure 1. The kinetics of alkylation of bovine rhodopsin with radioactive iodoacetamide, in 40 mM CTAB-0.2 M Tris HCl-5 mM EDTA (pH 8) at 23°. Rhodopsin A_{280nm}/A_{500nm} ratio, 1.89. A 5 molar excess of [^{14}C]iodoacetamide over rhodopsin was added at time zero. Curve 1 (●—●) incorporation of radioactivity into rhodopsin maintained in the dark. Curve 2 (□—□), incorporation of radioactivity into rhodopsin bleached continuously with a bright white light, commencing at 2 minutes. Curve 3 (+--+), incorporation of radioactivity into rhodopsin maintained in the dark for 60 minutes and then bleached.

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then exposed to cold reagent and light. Part three was treated as part one, except that it was not bleached. Visual pigment was extracted with CTAB, purified by Sephadex G-25 gel filtration, and examined for absorptivity at 280 nm and radioactivity. Purified ROS opsin also was hydrolyzed with 6 N HCl and analyzed for radioactive CMC by high voltage paper electrophoresis.

The effect of alkylating illuminated bovine rhodopsin upon the recovery of half-cystinyl residues as cysteic acid after performic acid oxidation was determined by amino acid analysis. Rhodopsin in 40 mM CTAB-0.2 M Tris HCl-5 mM EDTA (pH 8.0), was divided into two fractions. A 20 molar excess (over rhodopsin)

TABLE I. Chemical modification of bovine rhodopsin sulfhydryl groups in CTAB and rod outer segments (ROS).

Labelled rhodopsin[a]	Iodoacetamide CTAB cpm/A_{280}[b]	Iodoacetamide ROS cpm/A_{280}[b]	Iodoacetamide ROS cpm/CMC[c]	PCMB ROS cpm/A_{280}[b]
Cold/hot (illuminated)	373	413	394	197
Hot/cold (illuminated)	67	5	10	63
Cold/hot (dark)	36	7	16	42

[a]Sequentially incubated for two hours at 20° with either cold or hot (radioactive) reagent.
[b]Radioactivity, normalized to absorptivity at 280 nm, of protein purified by Sephadex G-25 gel filtration in 50% acetic acid.
[c]Radioactivity recovered as S-carboxymethylcysteine (CMC) upon high voltage paper electrophoresis of an acid hydrolysate of the purified ROS opsin.

of iodoacetamide (1:10, hot:cold) was added to fraction one, the preparation was bleached, and alkylation was continued for 52 hours at 4° in the light. Fraction two was similarly bleached, but not alkylated. Each fraction was purified by Sephadex G-25 gel filtration in 50% acetic acid and by paper chromatography in tert-butyl alcohol-formic acid-water (70:15:15 by volume). One-half of each fraction was performic acid oxidized at 4° for 2.5 hours after the method of Hirs (12). Proteins were hydrolyzed *in vacuo* at 110° for 24 hours in 6 N HCl, and subjected to amino acid analysis (13). Cysteic acid values were corrected for a 10% loss (12). Cysteic acid or S-carboxymethylcysteine (CMC) values were normalized to 8 methionine sulfones (14, 15) or to the average recovered aspartic acids, respectively.

RESULTS AND DISCUSSION: The kinetics of alkylation of rhodopsin in CTAB are shown in Figure 1. The post-bleach reaction was com-

TABLE 2. Amino acid analysis of illuminated and chemically modified bovine rhodopsin.

Treatment of Visual Pigment	Amino Acids			
	Cysteic Acid	S-Carboxymethyl Cysteine	Aspartic Acid	Methionine Sulfone
Performic acid oxidized	4.9	-	17.4	8.0
Alkylated[a] and oxidized	3.6	-[b]	16.2	8.0
Alkylated[a]	-	1.2	16.8	-

[a]Alkylated with a 20 molar excess of iodoacetamide, in 40 mM CTAB-0.2 M Tris HCl-5 mM EDTA (pH 8), at 4° for 52 hours.
[b]S-carboxymethylcysteine was destroyed by performic acid oxidation.

plete by 30 min and at that time the ratio of light:dark formation of [^{14}C]carbamidomethylated-opsin was 1.0:0.14. The small uptake of radioactive label in the dark may represent thermally bleached visual pigment. A rhodopsin photoexposed sulfhydryl group also was demonstrated in rod outer segment suspensions with [^{14}C]iodoacetamide and [^{14}C]parachloromercuribenzoate (Table 1). The specificity of the iodoacetamide labelling was established by recovering radioactive S-carboxymethylcysteine upon high voltage paper electrophoresis of ROS opsin hydrolysates (Table 1). The amino acid analysis data of Table 2 indicated that prolonged alkylation of illuminated rhodopsin with iodoacetamide generated 1.2 S-carbamidomethylcysteinyl residues and reduced the recovery of cysteic acids (after performic acid oxidation) by 1.3 residues (data normalized to 8 recovered methionine sulfones).

These results furnish direct evidence that a rhodopsin sulfhydryl group becomes available for chemical modification only after illumination. A cyanogen bromide fragment containing the photoexposed sulfhydryl group has been isolated.

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