Enzymatic Methylation of 23–29-kDa Bovine Retinal Rod Outer Segment Membrane Proteins

EVIDENCE FOR METHYL ESTER FORMATION AT CARBOXYL-TERMINAL CYSTEINYL RESIDUES*

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A group of 23–29-kDa polypeptides in the membranes of bovine rod outer segments are substrates for S-adenosylmethionine-dependent methylation reactions. The bulk of the methyl group incorporation is in base-labile ester-like linkages, and does not appear to be due to the widespread D-aspartyl/L-isoaspartyl methyltransferase (EC 2.1.1.77). To determine the site(s) of methylation, H3-methylated proteins separated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate were eluted and digested with papain, leucine aminopeptidase-M, and prolidase. After performic acid oxidation of the digest, a base-labile radioactive material was recovered that coeluted with a synthetic standard of cysteic acid methyl ester upon cation exchange and G-15 gel filtration chromatography, as well as in two thin-layer electrophoresis and two thin-layer chromatography systems. These results provide direct evidence for the methylation of the carboxyl-terminal group of a carboxyl-terminal cysteiny1 residue, a modification that has been proposed for the 21-kDa Ha-ras product and other cellular proteins (Clarke, S., Vogel, J. P., Deschenes, R. J., and Stock, J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4643–4647).

Protein carboxyl methyltransferases catalyze the transfer of methyl groups from S-adenosylmethionine to carboxyl groups in proteins. One enzyme, present only in chemotactic bacteria, methylates a specific set of L-glutamyl residues in membrane chemoreceptors to modulate their signaling function (Simms et al., 1987). A second enzyme, widely distributed in nature, is a D-aspartyl/L-isoaspartyl protein carboxyl methyltransferase that specifically methylates abnormal L-isoaspartyl and D-aspartyl residues in peptides and proteins and appears to participate in their repair or degradation (Clarke, 1985). The existence of a third class of methyltransferases was suggested by the structure of peptidyl sex factors from the jelly fungi Tremella mesenterica (Sakagami et al., 1981) and Tremella brasiliensis (Ishibashi et al., 1984) and from the a mating factor from the yeast Saccharomyces cerevisiae (An- dereg et al., 1988). These peptides contain a modified carboxyl-terminal cysteinyl residue that is both lipidated at the sulfhydryl moiety and methyl esterified at its α-carboxyl group. Since the genes for the S. cerevisiae a mating factor encode an additional three amino acids at the carboxyl terminus (Val-Ile-Ala; Brake et al., 1985), the post-translational processing of this factor includes the removal of these 3 residues.

Methylation of the carboxyl-terminal carboxyl group may also occur in proteins. Similarities between the carboxyl-terminal regions of the yeast a mating factor and the ras oncogene products suggested that the carboxyl terminus of these proteins are methylated. For example, the human and rat Ha-ras genes encode a product with a Cys-Val-Leu-Ser carboxyl-terminal sequence, and these proteins also appear to be lipidated at this cysteinyl residue (Barbacid, 1985; Buss and Sefton, 1986). Recently, it was found that ras oncogene products are in fact carboxyl methylated in nearly stoichiometric amounts in transformed rat embryo fibroblasts (Clarke et al., 1988) in the yeast Saccharomyces cerevisiae (Deschenes et al., 1988). Based on these results, it was suggested that the site of methylation was the exposed cysteinyl residue, although limited amounts of material precluded direct chemical analysis.

Carboxyl-terminal structures containing a Cys-Xaa-Xaa-Xaa sequence occur in several other proteins suggesting that a wider variety of proteins may be modified in similar ways. These include the α subunits of the large G proteins Gα, the γ subunit of transducin, members of the lamin A and lamin B family, the cGMP phosphodiesterase of retinal rods (see Clarke et al., 1988), as well as in the rho (Yamamoto et al., 1988) and ral (Chardin and Tavitian, 1986) members of the 21–24-kDa ras small G-protein superfamily. Evidence has been presented that at least two of these proteins, lamin B (Chelsky et al., 1987) and cGMP phosphodiesterase (Swanson and Applebury, 1983), are methyl-esterified.

To determine whether methyl esterification can occur at a carboxyl-terminal cysteinyl residue, we focused on the extensively methylated 23–29-kDa polypeptides in the membranes of bovine retinal rod outer segments (Swanson and Applebury, 1983). We demonstrate that in fact a significant portion of the methyl esters in these proteins occur at such sites, and thus provide clear evidence for a new class of protein carboxyl methyltransferases.

EXPERIMENTAL PROCEDURES

Preparation of Rod Outer Segments—Bovine retinal rod outer segments were kindly provided by H. Yamane and B. K.-K. Fung from the Departments of Ophthalmology and Pharmacology at the Jules Stein Eye Institute at UCLA. Rod outer segments were prepared from dark-adapted bovine retinas obtained from an abattoir (J. A. and W. L. Lawson Co., Lincoln, NE) according to the procedure of Hong and Hubbell (1973).

Methylation of Rod Outer Segments—For samples that were applied to 6.5% tube gels, 60 μl of rod outer segments, diluted from 6.5 to 2.25 mg/ml in rhodopsin with incubation buffer (50 mM sodium
HEPES,¹ 100 mM sodium chloride, 5 mM magnesium chloride, pH 7.0) was incubated with 30 μl of [3H]AdoMet (Amersham Corp., 69.2 Ci/mmol, 14.3 μM in dilute sulfuric acid (pH 2.5-3.5)) for 1 h at 37 °C under fluorescent lights. Membranes were obtained by centrifugation for 1 min at 13,600 × g at room temperature in a microcentrifuge and were washed twice with 400 μl of incubation buffer to remove any residual [3H]AdoMet.

Preparative Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—Methylated rod outer segment proteins were separated in tube gels of 6.5% acrylamide, 0.22% bis-acrylamide. ²H-Methylated membranes from 60 μl of rod outer segments were combined with 80 μl of sample buffer (3.3% sodium dodecyl sulfate, 2.5 M 2-mercaptoethanol, 21 mM Tris-HCl, 20% glycerol, 0.025% bromophenol blue, pH 6.8) and heated at 100 °C for 3 min. This material was applied to a gel that was 12.5 cm in length and had a diameter of 0.6 cm and was electrophoresed using the buffer system of Laemmli (1970) at room temperature for ~1.5 h at 150 volts. In a typical gel, the polypeptide of rhodopsin migrated 7.1 cm from the top of the gel.

To elute the methylated proteins the gels were immediately cut into 2.5-mm slices without fixing and placed in propylene glycol test tubes (12 × 78 mm) with 0.5 ml of 50 mM sodium acetate, pH 5.2, and 0.25% Triton X-100, capped, and incubated for 20-24 h with shaking at room temperature. At least 85% of the base-labile radioactive methyl group content in gel slices from the 25-29-kDa region was eluted from these gels (see below).

Determination of [3H]Methyl Radioactivity Incorporated into Rod Outer Segment Polypeptides—The amount of total radioactivity in the methylated proteins eluted from the 6.5% gel was determined by directly counting 30 μl of the eluted material in 5 ml of scintillation fluid (Amersham Corp., ACS II). The amount of base-labile volatile radioactivity eluted from the 6.5% gel was determined in a vapor diffusion assay by incubating 40 μl of the eluted sample with 40 μl of 2 M sodium hydroxide to generate methanol as described by Clarke et al. (1988) except that the volatile radioactivity was transferred into 5 ml of ACS II. These samples were then counted as described above.

Enzymatic Digestions—The digests were performed according to a protocol modified from that of Kleene et al. (1977). Fractions containing [3H]methyl radioactivity eluted from 6.5% gels were pooled, and this material (2.4 ml) was incubated with 2 units of papain (Sigma, papaya latex, 24 units/mg protein, 1 unit hydrolyzes 1.0 μmol of α-N-benzoyl-L-arginine ethyl ester/min at pH 6.2, 25 °C), 7 μl of 0.1 M potassium EDTA, pH 7.0, and 35 μl of 0.5 M 2-mercaptoethanol for 30 min at 37 °C. After this incubation period, 1 ml of water and 200 μl of 10% trichloroacetic acid (Pierce Chemical Co., high performance liquid chromatography/spectro grade), and the sample was lyophilized.

The lyophilized material was resuspended in 1.5 ml of 30 mM CaCl₂ and 30 mM MnCl₂, and the pH was adjusted to 7.0 with dilute sodium hydroxide. Tri-HCl (130 μl, 0.5 M, pH 8.0) was used to suspend 100 μg of aminopeptidase-M (Sigma, porcine kidney microsomes type IV, S, 3.3 L-leucine-p-nitroanilide units; 1 unit hydrolyzes 1.0 μmol of L-leucine-p-nitroanilide to L-leucine/min at pH 7.2 at 37 °C) and 200 μg of prolidase (Sigma, porcine kidney, 38 units; 1 unit hydrolyzes 1.0 μmol of Gly-Pro/min at pH 8.0 at 37 °C). The proteases were obtained as ammonium sulfate suspensions at pH 7.7 and 8.0, respectively, and were used after centrifugation. The digestion was allowed to proceed for 4 h at 40 °C. The digestion mixture was then centrifuged and the supernatant was removed. The supernatant was quenched with 50 μl of 10% trichloroacetic acid (TCA) and lyophilized.

Performic Acid Oxidation—The lyophilized enzymatically digested material was oxidized with performic acid. Formic acid (88%) and hydrogen peroxide (30%) were mixed together in a 9:1 ratio (v/v) and allowed to form the peracid at room temperature for 1 h. This peracid solution was cooled on ice for 10 min and 1.5 ml was added to the lyophilized sample. The mixture was incubated with shaking at 4 °C for 22.5 h and then lyophilized to dryness.

**RESULTS**

Methylation of Rod Outer Segment Membranes in Vitro—Rod outer segment membranes prepared from bovine retinas were incubated with [3H]AdoMet and assayed for incorporation of [3H]methyl groups after separating the proteins in sodium dodecyl sulfate-polyacrylamide gels at pH 8.5. Under these conditions, D-aspartyl and L-isoaspartyl methyl esters would generally be hydrolyzed, and the population of carboxyl-terminal methyl esters would be enhanced (Stephenson and Clarke, 1989). Confirming results obtained from Swanson and Applebury (1982), we found that the major methyl incorporation was into polypeptides of molecular mass 23-29 kDa (Fig. 1). Vapor-phase equilibrium assay of the gel slices revealed that at least 60% of the radiolabel associated with proteins in this molecular weight range is base-labile and volatile and suggests that this fraction is incorporated into methyl ester linkages (Fig. 1). We found that 85% of the base-labile radiolabeled [3H]methyl group incorporation could be inhibited by the addition of 125 mM S-adenosylhomocysteine, a potent inhibitor of methyltransferase enzymes. We also found that the methylation of these proteins could not be competitively inhibited by a synthetic peptide substrate for the L-protein.

**FIG. 1. S-Adenosylmethionine-dependent methyl esterification of bovine rod outer segment membrane proteins.** Rod outer segments (30 μl) at a concentration of 2.55 mg/ml in rhodopsin were incubated with 560 μl of incubation buffer (50 mM sodium HEPES, 100 mM sodium chloride, 5 mM magnesium chloride, 96 μM phenylmethylsulfonyl fluoride, and 0.096 mM dithiothreitol/dithioerythritol, pH 7.0) and incubated with 20 μl of [3H]AdoMet (Amersham Corp., 15 Ci/mmol, 66.7 M in dilute sulfuric acid (pH 2.5-3.5)-ethanol (8:1)) for 82 min at 37 °C under fluorescent lights. Membranes were obtained by centrifugation at 13,800 × g for 5–6 min at room temperature in a microcentrifuge and the polypeptides were separated by SDS polyacrylamide gel electrophoresis. Methylated rod outer segment membranes (from 30 μl of rod outer segments) were combined with 75 μl of sample buffer (see "Experimental Procedures") and heated at 100 °C for 3 min. Samples (25 μl) were applied onto two separate 0.6 cm wide lanes in a slab gel (separating gel: 15% acrylamide, 5 cm tall gel) and were electrophoresed in parallel lanes. The total [3H]methyl radioactivity incorporated into rod outer segment polypeptides (△) was assayed by incubating gel slices from the separating gel (approximately 1 cm wide, 2 mm high) in 100 μl of water and 800 μl of NCS tissue solubilizer (Amersham Corp.) overnight at room temperature and counting the radioactivity in 9.5 ml ACS II fluor (Amersham Corp.). The amount of base-labile methyl groups in the polypeptides (O) was determined as described by Clarke et al. (1988) except that gel slices were incubated with 30 μl of 5 M sodium hydroxide and these samples were equilibrated in 5 ml of ACS II fluor (Amersham Corp.) in 20-ml scintillation vials. The migration positions of standards including lysozyme, 14.4 kDa; soybean trypsin inhibitor, 21.5 KDa; carbonic anhydrase, 31 kDa; ovalbumin, 42.7 kDa; bovine serum albumin, 66.2 kDa; and phosphorylase b, 97.4 kDa are shown by arrows.

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¹ The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTP·S, guanosine 5'-O-(3-thiotriphosphate).
Protein Carboxyl-terminal Methylation

<table>
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<tr>
<th>Sample</th>
<th>Radioactivity in methyl esters and methanol&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Radioactivity in methanol&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Radioactivity in esters</th>
<th>Yield of [H]methyl esters</th>
<th>%</th>
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<tr>
<td>Eluted 23–29 kDa proteins</td>
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<td>4,911</td>
<td>34,822&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Papain digested proteins (no papain control)</td>
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<td>9,556&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>27.1</td>
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<tr>
<td>Periformic acid oxidized sample</td>
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<td>8,269&lt;sup&gt;f&lt;/sup&gt;</td>
<td>23.8</td>
<td>6.8</td>
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<td>Cysteic acid methyl ester peak upon cation exchange chromatography</td>
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<td></td>
<td>1,802&lt;sup&gt;g&lt;/sup&gt;</td>
<td>5.2</td>
<td></td>
</tr>
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</table>

<sup>a</sup> Determined by vapor-phase equilibration assay of the base-treated samples as described in “Experimental Procedures.”

<sup>b</sup> Determined by vapor-phase equilibration assay of non-base-treated samples as described in “Experimental Procedures.”

<sup>c</sup> Determined by subtracting radioactivity as methanol (column 2) from radioactivity as methanol and methyl esters (column 1).

Fig. 2. Isolation of cysteic acid [H]methyl ester from proteolytically digested and oxidized [H]-methylated 23–29 kDa proteins from bovine rod outer segments by cation-exchange chromatography. [H]-Methylated polypeptides, eluted from 65% gels, were digested with papain and then with leucine aminopeptidase-M and prolidase, and the resulting supernatant was oxidized with performic acid and lyophilized as described under “Experimental Procedures.” The lyophilized sample was resuspended in 100 µl of water and 150 µl of pH 2.2 sodium citrate sample buffer (0.2 M in sodium ion; Pierce Chemical Co., pHIX). This suspension was centrifuged to remove particulate matter and the supernatant was combined with 2 µmol of an L-cysteic acid methyl ester standard synthesized by the method of Rosowsky et al. (1984). The sample was applied to a cation exchange column (0.9 × 54 cm) consisting of sulfonated polystyrene amino acid analysis resin (Beckman AA-15) equilibrated in pH 3.25 sodium citrate (0.2 M in sodium ion; Pierce Chemical Co., pHIX) at 56°C. One minute fractions were collected at a flow rate of 1.1 ml/min. The fractions were assayed for total radioactivity (µ) by counting 30 µl of each fraction in 5 ml of fluor (Amersham, ACS II) and for the ninhydrin reactivity (C) of the cysteic acid methyl ester standard using 25 µl of each fraction as described by Moore (1968). The cysteic acid methyl ester standard eluted in fractions 18 and 19 (17–19 min) with 2354 cpm of the [H]-methylated sample. On this column aspartic acid elutes at about 55 min, aspartic acid γ-methyl ester elutes at about 60 min, and glutamic acid γ-methyl ester elutes at about 90 min.

<table>
<thead>
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<th>Volume (ml)</th>
<th>0.03</th>
<th>0.06</th>
<th>0.11</th>
<th>0.18</th>
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<tbody>
<tr>
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<td>-0.02</td>
<td>-0.01</td>
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<tr>
<td>N,N-dimethylformamide, µmol</td>
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<td>-0.01</td>
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<tr>
<td>N,N-dimethylpropionamide, µmol</td>
<td>-0.02</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.02</td>
</tr>
</tbody>
</table>

Fig. 3. Sephadex G-15 gel filtration chromatography of isolated cysteic acid [H]methyl ester. The fractions containing radiolabel with the L-cysteic acid methyl ester standard from cation exchange chromatography (Fig. 2; fractions 18 and 19) were pooled and analyzed by Sephadex G-15 gel filtration chromatography. Additional cysteic acid methyl ester standard (1 µmol) was mixed with the sample before applying to the column. The column, 1.5 × 81 cm, was equilibrated in 0.1 N acetic acid and the sample eluted at a flow rate of 0.4 ml/min. The fractions (2.5 ml) were assayed for total radioactivity (µ) by counting 50 µl in 5 ml of fluor (Amersham Corp., ACS II) and 25 µl of each fraction was assayed with ninhydrin reagent (C) to locate the standard.

Proteins from Rod Outer Segments as a Carboxyl-terminal Cysteineyl Residue—Our strategy in isolating carboxyl-methylated cysteine residues was to first digest the methylated proteins into small peptides and then to completely digest these fragments to free amino acids without hydrolyzing the [H]methyl ester group. We digested the [H]-methylated 23–29 kDa proteins eluted from a 6.5% gel with papain for 18 h at 37°C. Further digestion with leucine aminopeptidase and prolidase were performed (4 h at 40°C) to sequentially hydrolyze peptide bonds from the amino terminus of the peptide fragments. We recovered 27.1% of the methyl esters after these steps. We found that a considerable amount of [H]methyl ester was formed during the incubation with papain both by an apparently spontaneous hydrolysis mechanism and by enzyme-catalyzed ester hydrolysis (Table I).

The digested proteins were then treated with performic acid to fully oxidize the sulfur of the cysteine residue to the sulfonic acid in preparation for chromatographic analyses. This step removes the reactive sulfhydryl group while maintaining the methyl ester linkage. This procedure should also remove acyl groups attached to cysteineyl residues in thioester linkages.

isoaspartyl/d-aspartyl protein carboxyl methyltransferase. This peptide, Lys-Met-Asp-Lys-Thr-Asp-Ser-Glu-Glu-Glu-Ile-Arg, has a K<sub>s</sub> for the erythrocyte methyltransferase of 20 µM<sup>2</sup> and showed no inhibition of methylation when incubated at a final concentration of 750 µM with the rod outer segments (data not shown).

Determination of the Site of Methylation in 23–29 kDa

<sup>2</sup> J. Lowenson and S. Clarke, unpublished results.
Fig. 4. Confirmation of the identity of the proteolytic product as cysteic acid [³H]methyl ester by thin-layer electrophoresis. The two peak fractions of radioactivity from gel filtration chromatography co-migrated with the L-cysteic acid methyl ester standard were lyophilized and each fraction was resuspended in 50 µl of water. An aliquot (5 µl) of each fraction was applied to the center of a 20-cm cellulose sheet (Eastman Kodak 13254). Approximately 0.1 µmol of the amino acid standards L-lysine, L-glutamic acid, L-cysteic acid and cysteic acid methyl ester were applied in parallel lanes. The samples were electrophoresed at room temperature in either of two mobile phases: A, water:pyridine:acetic acid (225:25:1, by volume), pH 6.5, (electrophoresed at 400 V, 20 mA for 30 min); or B, water:formic acid:acetic acid (900:25:75, by volume) that was titrated to pH 3.35 with pyridine (electrophoresed at 400 V, 120 mA for 25 min). Data are shown for the radio labeled material eluting at 76 ml, and similar results were obtained for the material eluting at 74.8 ml on the Sephadex G-15 column. The positions of the L-cysteic acid methyl ester (hatched bars) were determined by ninhydrin spray.

Fig. 5. Confirmation of the identification of cysteic acid [³H]methyl ester by thin-layer chromatography. Samples from the gel filtration column were prepared as described above for thin-layer electrophoresis. The samples (5 µl) were applied to a 7 x 115-cm-long cellulose sheet (Eastman Kodak 13254) and the chromatograms developed at room temperature in 600-ml covered chambers containing either of two solvent systems, 1-propanol:water (7:3, v:v) (A) or 1-butanol:acetic acid:water (4:1:1, v:v:v) (B). The positions of the base-labile radiolabel (hatched bars) and the synthetic cysteic acid methyl ester standard within these samples were determined as described for the thin-layer electrophoresis samples (Fig. 4). Data are shown for the fraction that eluted from gel filtration chromatography at 74.8 ml. Similar results were obtained for the adjacent fractions containing the material eluting at 72.6 and 76.0 ml.

Fig. 6. Coelution of the acetylated [³H]-radiolabeled digestion product with acetylated synthetic cysteic acid methyl ester. A portion of the radiolabeled material from the 74.8-ml fraction of the Sephadex G-15 column effluent was lyophilized and resuspended in 1 ml of water and acetylated with 0.6 µmol of acetic anhydride (Aldrich, ACS reagent). The sample was incubated overnight at room temperature with gentle shaking. 5 µmol of synthetic L-cysteic acid methyl ester standard was acetylated using the same procedure. The acetylated [³H]-methylated sample was combined with 2 µmol of unacetylated cysteic acid methyl ester standard and spotted on silica plates (TLC plastic sheets, silica gel, layer thickness 0.2 mm, E. Merck, Darmstadt, West Germany) that were developed in 1-propanol:water (7:3, by volume). Acetylated and unacetylated cysteic acid methyl ester standards were chromatographed in parallel lanes. The migration positions of the base-labile [³H]methyl groups and the cysteic acid methyl ester standard in this sample lane were determined as described in the legend to Fig. 4. The migration positions of the acetylated cysteic acid methyl ester (Acetyl-CAME) and cysteic acid methyl ester (CAME) in parallel lanes were determined by a ferric hydroxylamine color reaction (stippled ellipses) (Zweig and Sherma, 1972).
Protein Carboxyl-terminal Methylation

**Discussion**

Bovine rod outer segments have been shown to enzymatically incorporate base-labile methyl esters into the α subunit of cGMP phosphodiesterase as well as a group of proteins in the molecular mass range 23–29 kDa (Swanson and Applebury, 1983). The apparent stability of at least a portion of these methyl esters under the conditions of Laemmli gel electrophoresis at pH 8.5 suggested that they may represent carboxyl-terminal methyl esters such as those that are present in the fungal peptide-mating factors (Sakagami et al., 1981; Ishibashi et al., 1984; Anderegg et al., 1988). In each of these peptideyl factors, the methyl group was present on the α-carboxyl group of a cysteiny1 residue whose hydrophilic group was linked to a lipid moiety. Based on structural similarities at the carboxyl terminus, we proposed that the mammalian ras oncogenes product and other proteins might be methylated in a similar fashion (Clarke et al., 1988). We now present chemical evidence that mammalian cells have enzymes that can specifically modify carboxyl-terminal cysteiny1 residues.

To determine the residue methylated in the 23–29-kDa polypeptides of the rod outer segment, we labeled these membrane-associated proteins with 3H-methyl groups derived from S-adenosylmethionine and utilized a protocol for complete enzymatic digestion. After we oxidized this digest with performic acid to stabilize the sulfhydryl group of cysteine residues during chromatography, we isolated a product identified as cysteic acid [3H]methyl ester. The yield of cysteic acid [3H] methyl ester from these polypeptides (7%) represents a minimal content of these modified residues because protease-catalyzed and spontaneous hydrolysis of these esters occurred during the isolation procedure (Table I). These results show that a significant portion of the methyl groups in the 23–29-kDa polypeptide fraction of bovine rod outer segments appears to be present at the α-carboxyl group of a carboxyl-terminal cysteiny1 residue. It is unclear if these proteins are also modified by covalent lipidation in the retina. Lipids attached via thioether linkages to the cysteine sulfhydryl group should be cleaved by the performic acid oxidation process. Although one would not expect that lipids in thioether linkages would be quantitatively cleaved, evidence has been presented for the formation of cystine and cysteic acid from farnesylated cysteinyl residues in peptide mating factors under acidic conditions (Kmiiya et al., 1979; Ishibashi et al., 1983). It is thus possible that retinal proteins containing either S-acylated or S-farnesylated cysteiny1 methyl esters could give rise to the cysteic acid methyl ester product seen in this work.

The identities of the methylated proteins are unknown, but based on their cysteine methylation, their membrane localization and their molecular weight, it is possible that they represent a class of small G proteins such as those in the ras superfamily including those in the ras, rho, and rab branches (Touchot et al., 1987; Chardin, 1988). The cDNA sequences of the proteins in the rho branch contain a Cys-Xaa-Xaa-Xaa sequence at the carboxyl terminus that is also found in the ras branch and in the a-mating factor and may signal carboxyl-terminal modification reactions (Powers et al., 1986; Clarke et al., 1988). The amino acid sequence at the carboxyl end of the ras subfamily of proteins, including YPT1 and SEC4, also contains cysteyn1 residues, but in the sequence Cys-Cys (Haubruck et al., 1987; Salminen and Novick, 1987). The ras-related smg proteins have a carboxyl-terminal sequence that is Cys-Xaa-Cys (Matsui et al., 1988; Narumiya, 1988). It is unknown whether these proteins undergo similar carboxyl-terminal processing reactions. Membrane bound proteins in a mouse macrophage cell line have been found whose carboxyl methylation is stimulated by nonhydrolyzable GTP analogues (Backlund and Aksamit, 1988). These proteins are distinct from the ras oncoproteins suggesting that G proteins other than ras may be targets of carboxyl-terminal methylation. However, we did not observe GTPγS enhanced methylation of the group of 23–29-kDa rod outer segment proteins examined in this study (data not shown).

The physiological functions of carboxyl-terminal methylation reactions are not clear at present. It has been proposed that methylation may promote the association of lipidated proteins with the membrane by neutralizing the charge on the carboxyl residue or it may inhibit any reactivity of the carboxyl group toward the acyl groups linked to cysteine (Clarke et al., 1988). In addition, the methylation of membrane-associated proteins such as ras and other G proteins may have a regulatory role. The GTPγS enhanced carboxyl methylation of the 21–23-kDa proteins in mouse macrophage cells (Backlund and Aksamit, 1988) suggests that methylation may modulate or be modulated by GTP binding and/or the GTPase activity of these proteins.

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