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FRACTIONATION AND CHARACTERIZATION OF PROTEIN C-TERMINAL PRENYL-CYSTEINE METHYLESTERASE ACTIVITIES FROM RABBIT BRAIN

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Summary: Reversible carboxyl methylation of the C-terminal geranylgeranylcysteine of G25K may regulate its activity and cellular localization. Brain homogenates were examined for enzyme activities which hydrolyze the methyl ester of [^{3}H]methyl-G25K to produce [^{3}H]methanol. Methylesterase activity was detected in both soluble and membrane fractions. The soluble activity was fractionated into at least two distinct activities. One soluble activity appears to be due to the lysosomal protease, cathepsin B, based on sensitivity to certain protease inhibitors, acidic pH optimum, size, and ability to cleave the peptide substrate Nα-CBZ-Arg-Arg-7-amido-4-methylcoumarin. A second soluble activity, associated with a protein of approximately 25 kDa, exhibits a neutral pH optimum, insensitivity to protease inhibitors, and inhibition by the esterase inhibitor, ebelactone B. The membrane fraction contains larger amounts of a similar methylesterase that may represent the physiologically relevant form of the enzyme.

G25K is a member of the rho sub-family of small GTP-binding proteins which are thought to be involved in various signal transduction pathways. Like many proteins synthesized with a C-terminal CXXX sequence, G25K undergoes the sequential post-translational modifications of isoprenylation, proteolysis, and methylation, which generate a geranylgeranylcysteine methyl ester residue at the G25K C terminus (1). These modifications may facilitate association of some GTP-binding proteins with their target cellular membranes, and the potential reversibility of the methylation has led to the suggestion that methylation may regulate GTP-binding protein action [see (2, 3) for review]. Methyl group turnover on mammalian small GTP-binding proteins has been inferred from the observation that GTP-γ-S induces both translocation to the membrane and methylation of several such proteins, including rac2 in human neutrophils (4), rap1 in human platelets (5) and G25K in rabbit brain (6). In the case of G25K, the methylated form has been

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Abbreviations: DTT, dithiothreitol; E-64, L-*trans*-epoxysuccinylleucylamido(4-guanidino)-butane; EDTA, ethylenediaminetetraacetic acid; FF(gg)CNH$_2$, Phe-Phe-(geranylgeranyl)Cys amide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES; 2(*N*-morpholino)ethane sulfonic acid; PMSF, phenylmethyl sulfonyl fluoride; TEDG buffer, 50 mM Tris-HCl, pH 7.5/1 mM EDTA/1 mM DTT/10 % glycerol; Tris, tris(hydroxymethyl)aminomethane.

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found in the membrane (1), but a soluble form has also been isolated and was found to be unmethylated (6) and associated with rho guanine-nucleotide dissociation inhibitor protein (7).

Direct evidence for turnover of the methyl group on C-terminal isoprenylcysteine residues is conflicting. No turnover of the methyl group was detected on p21H-ras over a 2 hour period in intact mammalian cells (8). In the yeast, *Saccharomyces cerevisiae*, slow turnover of some of the products of the STE14 isoprenylcysteine methyltransferase was noted over a 4 hour period in intact cells, but it was not established whether this turnover represented methyl ester hydrolysis or polypeptide degradation (9). *In vitro*, methyl esters on the γ subunit of transducin and on small (23-29 kDa) GTP-binding proteins undergo slow hydrolysis by an enzymatic activity in bovine rod outer segment membranes (10). This methylesterase activity, which also processed *N*-acetyl-*S*-farnesyl-L-cysteine methyl ester and *N*-acetyl-*S*-geranylgeranyl-L-cysteine methyl ester, was proposed to mediate turnover of the transducin methyl ester (11). A recent study found, however, that transducin is kept fully methylated in rod outer segments (12). The apparent methylesterase activity in rod outer segment membranes could result from a nonspecific protease or esterase. In a similar search for methylesterases in yeast, the vacuolar protease, carboxypeptidase Y, was found to hydrolyze *N*-acetyl-*S*-farnesyl-L-cysteine methyl ester (13). Farnesylcysteine methyl ester is hydrolyzed by pig liver esterase (14), and a recent study showed that pig liver esterase is capable of hydrolyzing the methyl ester of transducin as well (15).

Since the methylation status appears to affect the membrane localization of G25K, a putative methylesterase might play a role in regulating G25K function. Therefore, we looked for a G25K-specific methylesterase activity in rabbit brain using the substrate, [^{3}H]methyl-G25K. The results shown here describe the activities found in the soluble and membrane fractions.

MATERIALS AND METHODS

Materials. The methylesterase substrate, [^{3}H]methyl-G25K, was prepared as previously described (16) by methylating soluble G25K purified from rabbit brain using S-adenosyl[methyl-^{3}H]methionine (80 Ci/mmol, Amersham) and yeast STE14 methyltransferase that had been expressed in *E. coli*. Frozen rabbit brains were obtained from Pel-Freeze Biologicals. Inhibitors were from Boehringer Mannheim except for E-64 and the esterase inhibitor, ebelactone B, which were from Calbiochem. Bovine spleen cathepsin B and the peptide substrate Nα-CBZ-Arg-Arg-7-amido-4-methylcoumarin were from Sigma. Phe-Phe-(geranylgeranyl)-Cys-NH_2 was synthesized by reacting Phe-Phe-Cys-NH_2 with geranylgeranyl-bromide and purifying the product by reverse phase high pressure liquid chromatography using conditions described previously (17). All other materials were reagent grade and obtained from commercial sources.

Enzyme Assays. Methylesterase activity was determined as the release of volatile radioactivity from [^{3}H]methyl-G25K. The assay buffer consisted of 100 mM HEPES, pH 7.2/5 mM $MgCl_2$/5% glycerol/1 mg/ml bovine serum albumin/6.7 μM GDP. [^{3}H]methyl-G25K substrate (10,000 cpm, 0.2 pmol) was diluted into concentrated assay buffer, and reactions were initiated by addition of enzyme to a final volume of 75 μl. After incubation for 40 min at 37 °C, reactions were quenched by precipitation with trichloroacetic acid (5 %) on ice for 15 min, followed by centrifugation. Supernatants were assayed for [^{3}H]MeOH by a diffusion assay as described (18). Cathepsin B was assayed with the specific peptide substrate Nα-CBZ-Arg-Arg-7-amido-4-methylcoumarin as described (19) except that 50 mM MES, pH 6.0 was substituted for 0.4 M sodium acetate, pH 5.5 as the assay buffer.

Fractionation of Methylesterase Activities. All steps were at 4 °C. Young rabbit brains (100 g) were thawed in 50 mM Tris-HCl, pH 7.5/1 mM EDTA/5 mM $MgCl_2$/10 % sucrose/1 mM

DTT/0.2 mM PMSF/1 μg/ml leupeptin/2 μg/ml aprotinin/0.7 μg/ml pepstatin and homogenized 4 x 30 sec in a blender with 1 min intervals on ice between homogenization. The homogenate was centrifuged at 1000 x g for 15 min, and the supernatant was further clarified by ultracentrifugation at 100,000 x g for 1 h.

Ammonium Sulfate Fractionation--To the soluble (100,000 x g supernatant) fraction, solid ammonium sulfate was added slowly to 25 % saturation. After centrifugation at 20,000 x g, the supernatant was brought to 40 % saturation with solid ammonium sulfate and centrifuged as above. Successive supernatants were similarly increased in 20 % increments with ammonium sulfate up to 100 % saturation. The pellets representing the 5 ammonium sulfate fractions (AS1-5) were resuspended in 4 ml and dialyzed against 2 (1 L) changes of TEDG buffer (50 mM Tris-HCl, pH 7.5/1 mM EDTA/1 mM DTT/10 % glycerol) for 4 h each.

Anion Exchange and Hydroxyapatite Chromatographies--Two activities resolved by ammonium sulfate fractionation (AS2 and AS4) were partially purified as follows. AS2 was recombined with AS1 and loaded onto a 1.6 x 25 cm column of DEAE Sepharose Fast Flow (Pharmacia), equilibrated in TEDG buffer and eluted with a 300 ml linear gradient to 0.5 M NaCl in equilibration buffer at a flow rate of 1.5 ml/min. Fractions of 6 ml were collected and methyl-G25K methylesterase activity eluted as a single peak in 4 fractions at approximately 0.2 M NaCl. The pooled DEAE peak fractions were loaded directly onto a 1.6 x 10 cm column of HTP hydroxyapatite (BioRad) equilibrated in TEDG. Proteins were eluted with a 225 ml linear gradient to 0.15 M potassium phosphate (dibasic) in TEDG at a flow rate of 0.75 ml/min. Methyl-G25K methylesterase activity eluted as a single peak in 7 (4.5 ml) fractions at approximately 0.12 M potassium phosphate. These two columns resulted in a 73-fold purification of the AS2 methylesterase activity with a 29 % yield (data not shown). The AS4 fraction (60-90 % ammonium sulfate cut) was purified on DEAE sepharose equilibrated in 20 mM Tris-HCl, pH 8.0/1 mM EDTA/1 mM DTT/10 % glycerol and eluted with a 400 ml linear gradient to 0.5 M NaCl in equilibration buffer at a flow rate of 2 ml/min. Methyl-G25K methylesterase activity eluted as a single peak in 6 (6 ml) fractions at approximately 0.23 M NaCl. The pooled DEAE peak fractions were concentrated 18-fold by ultra filtration using an Amicon PM10 membrane and dialyzed against Mono Q equilibration buffer (25 mM sodium phosphate, pH 7.0/1 mM EDTA/1 mM DTT/10 % glycerol). The concentrated DEAE pool was loaded onto a Mono Q HR 5/5 column (Pharmacia) in equilibration buffer and eluted with a 60 ml linear gradient to 0.25 M NaCl in equilibration buffer at a flow rate of 0.75 ml/min. Under these conditions, methyl-G25K methylesterase activity did not bind to the Mono Q column and eluted in 6 (1 ml) fractions in equilibration buffer. These two columns resulted in a 58-fold purification of the AS4 methylesterase activity with a 4.5 % yield (data not shown). The partially purified preparations of AS2 and AS4 were then chromatographed at pH 7.5 on the Mono Q HR 5/5 column equilibrated in TEDG buffer (except with only 20 mM Tris-HCl for the AS4 preparation) and eluted with linear gradients of NaCl as shown in Fig 3.

Gel Filtration Size Analysis--Each peak of methylesterase activity from the Mono Q column (Fig 3) was concentrated approximately 10-fold in a Centricon 10 Microconcentrator (Amicon) and injected onto a Superose 12 HR 10/30 column (Pharmacia) equilibrated and eluted in TEDG buffer containing 0.2 M NaCl at a flow rate of 0.4 ml/min. Fractions of 0.4 ml were collected and assayed for methylesterase activity (Fig 4).

RESULTS

Ammonium sulfate fractionation resolves at least two methyl-G25K methylesterase activities in brain (Fig 1). The first appears predominantly in the 25-40 % fraction (AS2), and the second peaks in the 60-80 % fraction (AS4). Total recovery in all five fractions was 45 % for methylesterase activity and 75 % for protein. The pH optima of the two methyl-G25K methyl-esterase activities are shown in Fig 2. AS2 exhibits a broad neutral pH optimum in the range 6.5 to 7.0, whereas the AS4 activity has a more acidic optimum at pH 5.9. pH's below 5.5 could not be assayed because the samples precipitate.

Table I shows the sensitivity of the two methyl-G25K methylesterase activities to various inhibitors. The AS2 activity was insensitive to all protease inhibitors tested but was sensitive to the esterase inhibitor, ebelactone B. In contrast, AS4 exhibited sensitivity to the microbial, peptide

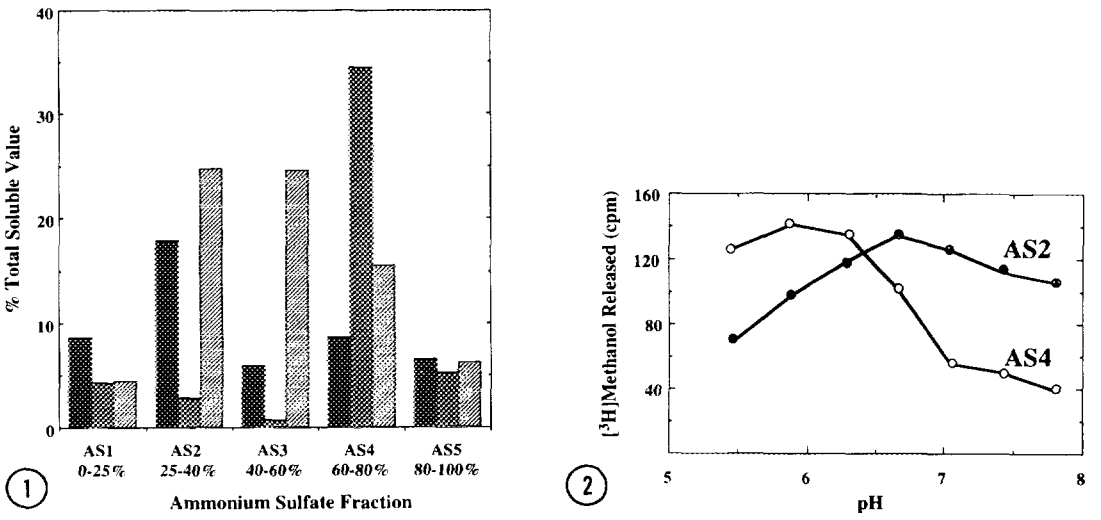


Figure 1. Ammonium sulfate fractionation of soluble activities capable of demethylating [^{3}H]methyl-G25K. Five ammonium sulfate fractions were prepared as described under "Materials and Methods" and assayed for methylesterase activity on [^{3}H]methyl-G25K at pH 7.2 (solid bars), peptidase activity on the cathepsin B substrate, Nα-CBZ-Arg-Arg-7-amido-4-methylcoumarin, at pH 6.0 (gray bars), and protein by the Bradford method (hatched bars). Results are expressed as the percentage of the total cytosolic methyl-G25K methylesterase activity (422 fmol/min), peptidase activity (11.2 nmol/min), and protein (2.3 g) recovered in each fraction.

Figure 2. pH dependence of methyl-G25K methylesterase activities. The methylesterase activity of the 25-40 % ammonium sulfate fraction (AS2, filled circles) and the 60-80 % ammonium sulfate fraction (AS4, open circles) were determined using [^{3}H]methyl-G25K (10,000 cpm) as substrate as described under "Materials and Methods" using either 100 mM MES for pH's from 5.5 to 6.3 or 100 mM HEPES for pH's from 6.7 to 7.8 in the assay buffer. All pH values were measured at 37 °C.

aldehyde, protease inhibitors (chymostatin, antipain, and leupeptin) which inhibit certain serine and thiol proteases. Additionally, the AS4 methylesterase was highly sensitive to E-64, a thiol protease inhibitor known to react irreversibly with a thiol at the active site of papain or cathepsin B (20). Neither methylesterase activity was affected by a geranylgeranylated peptide, Phe-Phe-(geranylgeranyl)Cys-NH_2, which mimics the C terminus of the γ_2 subunit of heterotrimeric GTP-binding proteins (21).

As shown in Fig 3, Mono Q chromatography resolves two methyl-G25K methylesterase activities (Q1 and Q2) in samples derived from either AS2 or AS4. Each of the four activities was further characterized by determining the apparent size on Superose 12 gel filtration chromatography (Fig 4). The smaller size of AS2-Q2 (22.0 kDa) relative to AS2-Q1 (24.4 kDa) suggests that the smaller might merely be an active proteolytic fragment of the larger. The two AS4 activities coeluted at a size of 26.8 kDa.

After DEAE sepharose chromatography and the first Mono Q chromatography, the AS4 activity readily hydrolyzed the methyl ester of G25K with an apparent K_m of 8 nM (data not shown), however, the acidic pH optimum and sensitivity to protease inhibitors led us to suspect that the AS4 activity resulted from a lysosomal protease, most likely cathepsin B. Reported pH optima for brain cathepsin B range from 4.0 to 6.5 depending on the substrate employed (22-25).

TABLE I

Effect of Various Inhibitors on Methyl-G25K Methylesterase Activities

Inhibitor	Concentration	% Activity Retained[a]		
		AS2[b]	AS4[c]	Membranes[b]
PMSF	0.2 mM	95	103	91
chymostatin	20 µg/ml	92	71	89
aprotinin	2 µg/ml	99	104	90
antipain	20 µg/ml	94	25	88
leupeptin	1 µg/ml	94	35	89
pepstatin	0.7 µg/ml	102	106	90
ebelactone B	20 µg/ml	16	91	6
E-64	10 µg/ml	90	11	90
$FF(gg)CNH_2$	2 µM	103	109	nd[d]
$FF(gg)CNH_2$	20 µM	93	102	90

[a]activity after preincubation with inhibitor for 30 min at 4 °C expressed as a percentage of activity after preincubation without inhibitor.

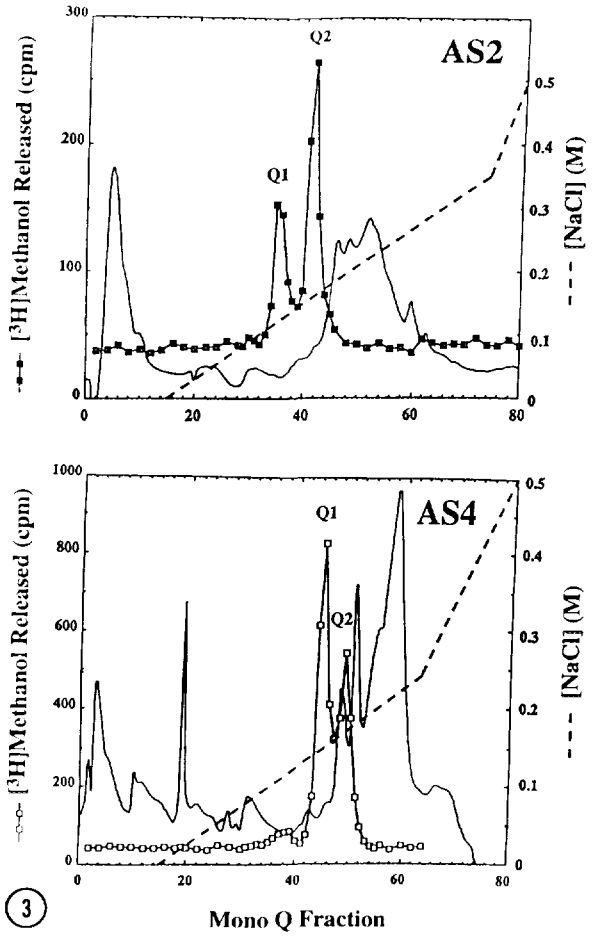
[b]assayed at pH 7.2.

[c]assayed at pH 6.4.

[d]not determined.

Cathepsin B is sensitive to the same inhibitors as the AS4 activity [reviewed in (19)]. The presence of two activities in AS4 further supports identification as cathepsin B because two isozymes of brain cathepsin B with different pI's of 6.1 and 6.8 have been reported (23). Furthermore, the size of 26.8 kDa correlates with the observation that the two bovine brain isozymes of cathepsin B coelute with chymotrypsinogen A at 25 kDa on a Sephadex G100 column (23). Consequently, we tested the five ammonium sulfate fractions for activity on the specific cathepsin B substrate, Nα-CBZ-Arg-Arg-7-amido-4-methylcoumarin, and found that cathepsin B appears to fractionate in AS4 (Fig 1). Total recovery of cathepsin B activity in all five fractions was 53 %. The Superose 12 purified AS4 enzymes retained the ability to cleave Nα-CBZ-Arg-Arg-7-amido-4-methylcoumarin (data not shown). Furthermore, bovine spleen cathepsin B purchased from Sigma exhibited methylesterase activity on [^{3}H]methyl-G25K under our assay conditions (data not shown).

Quantities of the AS2-derived methylesterase activities were insufficient for further purification after the four column procedure described above. Because the proteins, though small, precipitated in low concentrations of ammonium sulfate, they were likely to be highly hydrophobic. Consequently, a membrane fraction was prepared and checked as a potentially more plentiful source of methyl-G25K methylesterase activity. Rabbit brains (90 g) were homogenized as described under "Materials and Methods", and the membrane and soluble portions were separated by ultracentrifugation at 100,000 x g in the presence of four protease inhibitors: PMSF, leupeptin, aprotinin, and pepstatin A. Because leupeptin inhibits the methyl-G25K methylesterase activity of AS4, the results for the cytosolic fraction (Table II) are largely reflective of the AS2-type activity. The membrane fraction proved to contain nearly 10-fold more methyl-G25K methylesterase activity



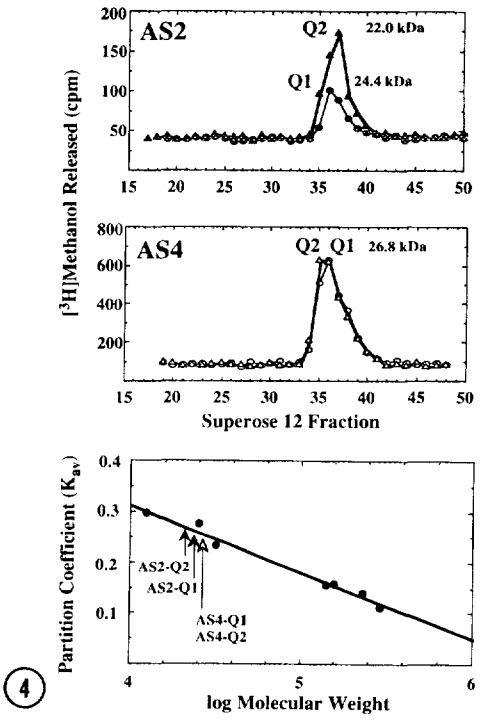


Figure 3. Mono Q chromatography of putative methyl-G25K methylesterases. Partially purified preparations derived from AS2 or AS4 were subjected to Mono Q chromatography as described under "Materials and Methods". The NaCl gradient is shown (dashed lines). Protein was monitored by absorbance at 280 nm (solid lines). Absorbance units full scale were 0.4 (upper panel) and 0.05 (lower panel). Fractions of 1 ml (1 min) were collected and assayed for release of volatile counts as [^{3}H]MeOH from [^{3}H]methyl-G25K (connected squares). Two peaks of methylesterase activity were resolved from both the AS2 (upper panel) and the AS4 (lower panel) samples.

Figure 4. Molecular weight of partially purified methyl-G25K methylesterase activities determined by gel filtration chromatography on Superose 12. The upper panel shows the elution of AS2-Q1 at 24.4 kDa (filled circles) slightly before AS2-Q2 at 22.0 kDa (filled triangles). AS4-Q1 (open circles) and AS4-Q2 (open triangles) coeluted at 26.8 kDa (center panel). Molecular weights were determined by preparation of a standard curve (lower panel) using cytochrome c (12.4 kDa), chymotrypsinogen A (25 kDa), myokinase (32 kDa), lactate dehydrogenase (142 kDa), aldolase (158 kDa), catalase (232 kDa), and glutamate dehydrogenase (290 kDa). The apparent partition coefficient (K_{av}, y-axis) is defined as $(V_e-V_o)/(V_t-V_o)$ where V_e is the elution volume, V_o is the void volume (determined with Blue Dextran 2000) and V_t is the total bed volume.

than the soluble fraction. Furthermore, washing the membranes with 1 M NaCl, which can remove some peripheral membrane proteins (26), failed to solubilize the majority of this activity (Table II). Characterization of the membrane-bound methylesterase revealed a pH optimum of 7.0 (data not

TABLE II

Distribution of Methyl-G25K Methylesterase Activities in Membrane and Soluble Fractions

Fraction	Total Protein (mg)	Total Activity (fmol/min)	Yield (%)	Specific Activity (fmol/min/mg)
Tissue Homogenate	4628	4667	100	1.00
Cytosol	2081	446	9.6	0.21
Membranes	2120	4020	86.1	1.90
NaCl Washed Membranes	1690	3047	65.8	1.80
NaCl Wash	319	122	2.6	0.38

shown), insensitivity to protease inhibitors, and sensitivity to the esterase inhibitor, ebelactone B (Table I).

DISCUSSION

At least two soluble enzymes capable of liberating the methyl group from methyl-G25K are present in brain. The following properties suggest that one of these, AS4, is the lysosomal protease cathepsin B: the ability to cleave the specific peptide substrate Nα-CBZ-Arg-Arg-7-amido-4-methylcoumarin, an acidic pH optimum with methyl-G25K of approximately 5.9, sensitivity to microbial, peptide aldehyde, protease inhibitors and to the specific inhibitor E-64, and the presence of two isozymes of different pI but identical size of 26.8 kDa by gel filtration. Furthermore, bovine spleen cathepsin B exhibited methylesterase activity on [^{3}H]methyl-G25K. The low yield of the AS4 activity may result from the instability of cathepsin B above pH 6 (27). Also, the total methylesterase activity of AS4 may be underestimated in this study because of the inclusion of leupeptin in the preparation of the original brain homogenate. The second soluble methylesterase, AS2, exhibits the following properties: a neutral pH optimum in the range 6.5 to 7.0, insensitivity to protease inhibitors but high sensitivity to the esterase inhibitor, ebelactone B, a size of 24.4 kDa on gel filtration, with either an active fragment or additional enzyme of 22.0 kDa, and low abundance. A membrane-bound methyl-G25K methylesterase with properties resembling those of AS2 is present in 10-fold higher amounts than the soluble activity.

The search for specific methylesterases that might be involved in methyl group turnover on GTP-binding proteins is complicated by the ability of certain non-specific proteases to hydrolyze methyl esters *in vitro*. Both cathepsin B, found here to hydrolyze the methyl-G25K methyl ester, and yeast carboxypeptidase Y, found to hydrolyze the *N*-acetyl-*S*-farnesyl-L-cysteine methyl ester (13), are sequestered within the lysosomes of mammalian cells or the vacuoles of yeast and would not be expected to play a role in methyl group turnover *in vivo*. The AS2-type activity described here, however, might be a specific methyl-G25K methylesterase because it is present in both the

cytosolic and membrane fractions, has a neutral pH optimum, is insensitive to protease inhibitors and appears to display some specificity for the G25K C terminus, Arg-Lys-(geranylgeranyl)Cys methyl ester (1, 28) based on the fact that the peptide, Phe-Phe-(geranylgeranyl)Cys amide, which mimics the C terminus of the γ_2 subunits (21), failed to inhibit the AS2 activity.

The possibility also exists that the AS2 activity results from a non-specific esterase. It was recently shown that pig liver esterase can hydrolyze farnesylcysteine methyl ester (14) and the transducin γ subunit methyl ester (15). The rod outer segment activity that was proposed to turnover the transducin γ subunit methyl ester was studied using simple isoprenylated cysteine derivatives (11). The recent report that transducin γ methyl ester is not turned over in rod outer segments (12) suggests the possibility that the putative rod outer segment esterase is not specific for GTP-binding protein methyl esters. The AS2 and membrane-bound activities identified here resemble the isoprenylcysteine methyl ester hydrolase of bovine rod outer segments described by Tan and Rando (11) in neutral pH optimum, insensitivity to protease inhibitors, and sensitivity to the esterase inhibitor, ebelactone B.

The AS2-type activities in the membrane and soluble fractions could be distinct, or the soluble activities may be derived from the membrane methylesterase by proteolysis or some other mechanism. Perhaps, as is thought for the substrate G25K (6), the methylesterase represents an "amphitrophic protein" that can cycle on and off the membrane (29). Further clarification of these issues will require successful solubilization and purification of the membrane bound activity.

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