Partial purification of protein farnesyl cysteine carboxyl methyltransferase from bovine brain

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Abbreviations: G-protein, GTP binding protein; GTPase, Guanosine triphosphatase; PFCCMT, protein farnesyl cysteine carboxyl methyltransferase; FPLC, fast protein liquid chromatography; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); [*methyl*-³H]SAM, S-adenosyl-L-[*methyl*-³H] methionine; PM II, protein methylase II; AFC, N-acetyl-S-farnesyl-L-cysteine; CHAPS, 3[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; DEAE-cellulose, diethylaminoethyl-cellulose; ND-PAGE, non-denaturing polyacrylamide gel electrophoresis

Abstract

C-terminal farnesyl cysteine carboxyl methylation has been known to be the last step in the post-translational modification processes of several important signal transduction proteins in eukaryotes including ras related GTP binding proteins and the γ -subunit of heterotrimeric G proteins. Protein farnesyl cysteine carboxyl methyltransferase (PFCCMT; EC, 2.1.1.100) catalyzing the reaction is well characterized as being stimulated by guanosine 5'-O-(3-thiotriphosphate) (GTP γ S) and suppressed by N-acetyl-S-farnesyl-L-cysteine (AFC). As an initial step to understand the physiological significance of the process, we attempted to purify the enzyme, which was partially purified 130-fold (specific activity, 143 pmol of methyl group transferred/min/mg of protein) with yield of 1.8% after purification by fast protein liquid chromatography (FPLC) on a Superdex 75 column. The enzyme was further purified with non denaturing polyacrylamide gel electrophoresis (ND-PAGE) and sodium dodecyl sulfate polyacrylamide gel electro-

phoresis (SDS-PAGE). The molecular weight of PFCCMT was determined to be about 30 kDa based on Superdex 75 FPLC as well as photoaffinity labelling with S-adenosyl-L-[*methyl*-³H] methionine ([*methyl*-³H]SAM). The partially purified enzyme (Superdex 75 eluate) was found to be characteristically affected by GTP γ S, being activated about 40-fold in 2 mM, in contrast to ATP which did not show any effect on enzyme activity. Meanwhile, the enzyme was found to be markedly inhibited by AFC, reaching 0 activity in 2 mM. These observations strongly suggested that the partially purified enzyme was PFCCMT.

Keywords: G-proteins, partial purification of farnesylated cysteine carboxyl methyltransferase, GTP γ S, AFC

Introduction

GTP-binding regulatory proteins (G proteins) belong to a family that are present in all eukaryotic organisms from yeast to human. Mature G proteins are located on the inner side of the plasma membrane, and their biochemical properties include binding and exchange of guanine nucleotides and GTPase activity leading to the regulation of intracellular cAMP level independent of their phylogenetic origin. Similarities in cellular location, structure, and biochemistry among them suggest that they play a role in transduction of signals from the cell (Schaefer *et al.*, 1990). Included in this family are two classes of the G protein superfamily, the ras-related GTP-binding proteins and the γ -subunit of heterotrimeric G proteins (Pillinger *et al.*, 1994). The biochemical nature of their final product in signal transduction is not fully understood, particularly with respect to the maturation and processing of translational products. Since mutations should result in alterations not only in the subsequent transcription and translation but also the final maturation processing reactions, it is highly important to understand the enzymology and regulation of post-translational modification reactions (Kato *et al.*, 1992).

The carboxyl terminus of G proteins contains the consensus sequence CAAX, where C is cysteine, A is usually an aliphatic amino acid, and X is another amino acid. This is modified post-translationally in the order: (a) polyisoprenylation on sulfur atom of cysteinyl residue, (b) removal of 3 carboxyl-terminal amino acids by a carboxypeptidase, and (c) carboxyl methylation of the newly exposed terminal cysteine. These modifications have been shown to play a certain role for the translocation and membrane anchoring of G proteins and fungal mating factors (Clarke *et al.*, 1988; Gutierrez

et al., 1989; Hancock *et al.*, 1989). Of the post-translational methylation reported thus far, the cysteine-carboxyl methylation is the most recently discovered and appears to be specific for G-proteins. The modification is unique in that a poly-isoprenylation and proteolysis of the precursor methyl acceptor substrate are prerequisites for the subsequent carboxyl O-methylation. PFCCMT has been known to be responsible for the modification, stimulated by guanine nucleotides (Akbar *et al.*, 1991; Backlund, 1992) and inhibited by AFC (Scheer and Gierchik, 1995). Since the carboxyl methylation reaction is reversible (methyl-esterification/de-esterification), the process is likely to be important in controlling the activity of G-proteins. The regulatory significance of this reaction is suggested in various physiological activities, in both ras related signal transduction and neurotransmission (Fung *et al.*, 1990), and its specific activities in the brain, liver, kidney and heart increase with age. Particularly, its abundant presence in rat brain, especially in the nucleus of cerebellum and synaptosomes implies its close relevance to neurofunction (Baruch *et al.*, 1993). Saturating concentrations of AFC caused a more than 80% inhibition of ras related chemotactic responses of mouse peritoneal macrophages (Volker *et al.*, 1991). Pertinent observation that AFC also inhibited GTP γ S promoted base-labile methylation of several pancreatic islet small G proteins, which retarded physiological insulin release (Leiser *et al.*, 1995; Metz *et al.*, 1993), is in accord with the formulation that carboxyl methylation (unlike isoprenylation) is a potentially reversible regulatory step.

Therefore, it seems crucial to elucidate the post-translational modification processes by cloning the enzyme catalyzing the reaction in order to understand the regulatory mechanism of the physiologic as well as pathologic functions of G proteins. PFCCMT responsible for the modification has been cloned in yeast and *Xenopus laevis* (Imai *et al.*, 1997), and recently in HL60 (human myeloid) (Dai, Q., *et al.*, 1998). In this experiment, we attempted to purify PFCCMT from bovine brain, which could be involved in the regulation of neurofunction.

Materials and Methods

Materials

Superose 6 (1 $\times$ 30 cm), Superdex 75 (1 $\times$ 30 cm) columns and molecular marker proteins were Pharmacia (Uppsala, Sweden) products. DEAE-cellulose was the product of Whatman Inc. (Clifton, U.S.A.). [methyl- 3 H]SAM was purchased from Amersham Co. (Buckinghamshire, UK), and 3[(3-cholamidopropyl)-dimethylammonio]-l-propane-sulfonate (CHAPS) and GTP γ S were obtained from Sigma (St. Louis, U.S.A.). All other reagents were of analytical grades. Protein methylase II (PM II) (EC 2.1.1.24) purified

from bovine brain was the gift of Dr. Sangduk Kim.

Preparation of bovine brain membrane protein

Bovine brain was freshly obtained and kept at -70°C until use. Isolation of the brain membrane protein was performed according to the method of Sternweis and Robishaw (1984), and all the procedures were done at 4°C unless otherwise stated. Bovine brain was homogenized in 4 volumes of 1 mM ethylene diamine tetraacetic acid (EDTA), 0.1% β -mercaptoethanol, 5 mM MgCl $_2$, 10% sucrose, 0.2 mM phenyl methyl sulfonyl fluoride (PMSF), 10 mM Tris/HCl pH 7.5 buffer, and centrifuged at 13,500 *g* for 30 min. Pellets were resuspended in 0.5% CHAPS-5% glycerol in TEM buffer (1 mM EDTA, 0.1% β -mercapto-ethanol, 10 mM Tris/HCl pH 7.5) to extract membrane proteins. Supernatants were collected after centrifugation at 100,000 *g* for 30 min.

Purification of PFCCMT from bovine brain membrane protein

Purification of the enzyme was performed according to Boivin *et al.* (1993) with some modifications. All the procedures were done at 4°C unless otherwise stated. Membrane protein extract was titrated to pH 5.5 and subjected to centrifugation at 15,000 *g* for 10 min. Supernatants were adjusted to pH 7.4 and applied to DEAE-cellulose column (2.6 $\times$ 10 cm) equilibrated with buffer A (0.05% CHAPS, 10% glycerol, 1 mM EDTA, 1 mM β -mercaptoethanol, 1 μ g/ml leupeptin, 10 μ g/ml pepstatin, 2 μ g/ml aprotinin, 20 mM Tris/HCl pH 7.5). Column was eluted with buffer A containing 0.02 M NaCl at 33 ml/h. Pooled enzyme fractions from 50 runs of DEAE-cellulose were concentrated with ultrafiltration (Centriprep-10, Amicon), assigned to 15 runs of Superose 6 (1 $\times$ 30 cm) FPLC equilibrated with buffer B (0.01% CHAPS, 5% glycerol, 1 mM EDTA, 1 mM β -mercaptoethanol, 1 μ g/ml leupeptin, 10 μ g/ml pepstatin, 2 μ g/ml aprotinin, 20 mM Tris/HCl pH 7.5), and eluted with the same buffer at 0.25 ml/min. Pooled enzyme fractions from Superose 6 were assigned to 3 runs of Superdex 75 (1 $\times$ 30 cm) FPLC equilibrated with buffer B and eluted with the same buffer at 0.25 ml/min. Pooled enzyme fractions was further purified with 7.5% ND-PAGE and electroeluted from the gel using S & S Biotrap (Schleicher & Schuell). ND-PAGE eluate was applied to SDS-PAGE for further analysis (Laemmli, 1970).

Enzyme activity assay

PFCCMT assay was conducted as described previously (Park *et al.*, 1994). Briefly, a total mixture in a final volume of 0.1 ml containing 200 mM NaCl, 5 mM MgCl $_2$, 1.0 mM GTP γ S, 40 μ M [methyl- 3 H]SAM (specific activity 82 Ci/mmol), 100 mM Hepes pH 7.4 was incubated at 37°C for 1 h. For routine enzyme assay, [methyl- 3 H]SAM was

the only substrate added, and methyl acceptor was not added because it was found to coexist endogenously with the enzyme upto the final stage of purification. Reaction was stopped by adding 1 ml of cold ethyl acetate. The ethyl acetate layer was isolated by centrifugation and aliquots of 0.5 ml were mixed with 2.5 ml of scintillation cocktail solution (Luma gel safe) to assess radioactivity. Blank contained S-adenosyl-L-homocysteine, a competitive inhibitor of SAM. Specific activity of the enzyme is defined as picomoles of methyl groups transferred per minute per milligram of protein. Protein concentration was estimated by the Coomassie Plus Protein Assay Reagent (Pierce, IL, U.S.A.) with bovine serum albumin as the standard.

PM II activity was assayed using the method of Kim, S. (1984). The reaction mixture in a final volume of 0.1 ml contained 2 mM EDTA, 40 μ M [methyl- 3 H]SAM (specific activity 82 Ci/mmol), γ -globulin (20 mg/ml) as methyl acceptor, 0.02 mM citrate-phosphate buffer pH 6.0 (prepared by mixing 1 part of 0.5 M disodium phosphate and 0.6 part of 0.25 M citric acid), and was incubated at 37°C for 30 min. The amount of [methyl- 3 H] incorporation into the substrate was analyzed by extracting the alkali-hydrolyzed methanol from carboxyl methyl ester as follows. The incubation was stopped by the addition of 0.1 ml of 0.125 M sodium borate pH 10.0, kept at the same temperature for 1 min, and then transferred to ice. After awhile, 1 ml of isoamylalcohol was added, and the mixture was left on ice for an additional 20 min before being mixed vigorously for 15 sec in a vortex mixer. The layers were separated by centrifugation at 8,000 g for 10 min, and then 0.5 ml of the organic layer was mixed with 2.5 ml of scintillation cocktail solution to measure radioactivity incorporation.

Synthesis of AFC

Chemical synthesis of AFC was as follows. N-acetyl-L-cysteine (1.0 g, 6 mmol), guanidine carbonate (1.3 g, 7 mmol) and trans, trans-farnesyl bromide (1.7 g, 6 mmol) were dissolved in 75 ml of acetone. The resulting solution was stirred overnight at room temperature. After evaporating the solvent under reduced pressure, the residue was taken up in ethyl acetate, washed successively with 10% HCl and water, and dried over Na_2SO_4 . The resulting AFC was further purified by chromatography on a silica column eluted with ethyl acetate/methanol (4:1 to 2:1), which resulted in a colorless oil like fluid. For use, AFC was dissolved in Hepes buffer or dimethylsulfoxide. The type of solvent which dissolved AFC did not affect enzyme activity measurements (Tan *et al.*, 1991).

Photoaffinity labelling of methyltransferases with SAM

To identify the methyltransferases, SAM (methyl donor

substrate of methyltransferases) was covalently bound to the enzyme as described below. The reaction mixture containing ND-PAGE eluate, 136 pmol of [methyl- 3 H]SAM (10 μ Ci, specific activity 82 Ci/mmol), 0.1 M NaCl, 5 mM dithiothreitol, 0.1 M Hepes pH 7.5 in a total volume of 100 μ l was irradiated with ultraviolet light (254 nm) at 4°C at a distance of 1 cm for 1 h. The reaction was terminated by the addition of SDS-PAGE sample buffer. The mixture was allowed to stay at 37°C for additional 30 min, and then subjected to SDS-PAGE (Laemmli *et al.*, 1970). The gel was enhanced in Amplify™ (Amersham Co.), dried and exposed on Hyperfilm-MP (Amersham Co.) at -70°C for 7 days (Hurst *et al.*, 1984).

Results

Partial purification of PFCCMT and determination of molecular weight

PFCCMT was partially purified from bovine brain following the methyltransferase activity stimulated by GTP γ S and inhibited by AFC. This resulted in 130-fold purification (specific activity of 143 pmol of methyl group transferred/

Figure 1. DEAE-cellulose chromatography of PFCCMT. The supernatant from acid precipitation was applied to DEAE-cellulose column (2.6 X 10 cm) which was pre-equilibrated with buffer A (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM β -mercaptoethanol, 0.1% CHAPS, 10% glycerol, 1 μ g/ml leupeptin, 10 μ g/ml pepstatin, 2 μ g/ml aprotinin). The protein was eluted with buffer A containing 0.02 M NaCl, and fractions of 5 ml were collected. Endogenous PFCCMT activity was assayed in an incubation mixture containing 100 mM Hepes buffer (pH 7.4), 200 mM NaCl, 5 mM MgCl_2 , 1.0 mM GTP γ S, 40 μ M [methyl- 3 H]SAM (specific activity 82 Ci/mmol), and aliquots (50 μ l) from each fractions at 37°C as described under Methods.

Figure 2. Superose 6 fast protein liquid chromatography of PFCCMT. The enzymatically active fractions obtained from DEAE-cellulose chromatography were applied to a Superose 6 column (1×30 cm) which was pre-equilibrated with buffer B (0.01% CHAPS, 5% glycerol, 1 mM EDTA, 1 mM β -mercaptoethanol, 1 μ g/ml leupeptin, 10 μ g/ml pepstatin, 2 μ g/ml aprotinin, 20 mM Tris/HCl pH 7.5). The protein was eluted with buffer B at a flow rate of 0.25 ml/min. Fractions of 0.5 ml were collected and aliquots (10 μ l) from each fraction were assayed for PFCCMT activity as described under Methods.

Figure 3. Superdex 75 fast protein liquid chromatography of PFCCMT. The enzymatically active fractions obtained from Superose 6-FPLC were applied to a Superdex 75 column which was pre-equilibrated with buffer B (0.01% CHAPS, 5% glycerol, 1 mM EDTA, 1 mM β -mercaptoethanol, 1 μ g/ml leupeptin, 10 μ g/ml pepstatin, 2 μ g/ml aprotinin, 20 mM Tris/HCl pH 7.5). The protein was eluted with buffer B at a flow rate of 0.25 ml/min. Fractions of 0.5 ml were collected and aliquots (5 μ l) from each fraction were assayed for PFCCMT and PM II activities as described under Methods.

Table 1. Purification of PFCCMT from bovine brain.

Purification step	Protein (mg)	Specific activity (pmol/min/mg)	Total activity (pmol/min)	Purification (fold)	Yield (%)
Whole homogenate	3,690	1.10	4,060	1.00	100
Membrane	3,370	0.949	3,200	0.863	78.9
CHAPS extract	588	1.66	976	1.51	24.0
Acid preparation	456	1.57	716	1.43	17.6
DEAE - cellulose	8.61	12.8	110	11.6	2.71
Superose 6 - FPLC	2.50	28.9	72.3	26.3	1.78
Superdex 75 - FPLC	0.499	143	71.5	130	1.76

min/mg of protein) with an yield of 1.8% after FPLC on Superdex 75 column (Figure 1-3, Table 1). The enzyme was further purified by ND-PAGE, in which specific activity was not determined due to the limited amount of the enzyme obtained (Figure 4). Finally, active enzyme fractions were visualized on SDS-PAGE (Figure 5). PM II which catalyzes carboxyl methylation of aspartate and glutamate residues accompanied PFCCMT during the course of purification, though Superdex 75 profile indicated the difference in molecular weights, 30 kDa for PFCCMT and 25-26 kDa for PM II as previously reported (Kim, S., 1984) (Figure 3). By the way, PFCCMT migrated at the 86 kDa region on ND-PAGE in discordance with the molecular weight construed from Superdex 75 profile.

The discrepancy might be ascribed to chemical attributes of ND-PAGE procedure which features migration in a neutral environment, guaranteeing the presence of proteins in its natural state, and requires molecular weight as well as charge density on the surface of protein as determination factors for the migration rate. ND-PAGE eluates containing PFCCMT were found to contain multiple proteins on SDS-PAGE. Of those, 30 kDa protein (arrowed proteins in Figure 5) was most plausibly regarded as PFCCMT based on the molecular weight determined by Superdex 75 elution profile as well as SAM photoaffinity labelling of methyltransferases (Figure 6). In addition, it was this 30 kDa protein that the band intensity changed roughly in parallel with the

Figure 4. Non-denaturing polyacrylamide gel electrophoresis of PFCCMT. The enzymatically active fractions obtained from Superdex 75-FPLC was subjected to ND-PAGE. After electrophoresis, the gel was sliced into 5 mm sections and electro-eluted. The eluate was assayed for PFCCMT and PM II activities as described under Methods. Numbers of slices are for each 5 mm gel sections starting from the top of the gel. High endogenous activity of PFCCMT was obtained from slices I3 (6-6.5 cm) and I4 (6.5-7 cm).

Figure 6. Photoaffinity labelling of methyltransferases with [methyl-³H]SAM. Lane I shows [methyl-³H]SAM bound methyltransferases in the enzyme fraction from ND-PAGE. Lane 2 indicates the inhibition of [methyl-³H]SAM binding to methyltransferases from ND-PAGE by 1 mM S-adenosylhomocysteine. Lane 3 shows the pattern of [methyl-³H] bound PM II purified from bovine brain.

enzyme activities in the applied samples in contrast to other bands in the vicinity (Figure 5), thus was saved for future analysis.

Photoaffinity labelling of methyltransferases

[methyl-³H]SAM was bound to methyltransferases obtained from ND-PAGE under UV irradiation to visualize them,

Figure 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of PFCCMT from ND-PAGE. Samples are eluates from slices I2 (lane I), I3 (lane 2), I4 (lane 3) and I5 (lane 4) of ND-PAGE. Lane 5 shows the migration pattern of purified bovine brain PM II.

and 30 kDa and 26 kDa bands appeared on X-ray film (Lane I, Figure 6). Of these, the 30 kDa protein plausibly seemed to indicate PFCCMT according to the molecular weight calculated from FPLC on Superdex 75 column. Meanwhile, 26 kDa band was determined to be PM II, because bovine brain PM II applied to the same gel produced a major band at the same 26 kDa region (Lane 3, Figure 6) and the applied sample exhibited both PFCCMT and PM II activities.

Effect of GTP γ S on PFCCMT activities

The addition of GTP γ S elevated the enzyme activity of PFCCMT in a concentration dependent manner with more than 40-fold increase in 2 mM (Figure 7). This phenomenon suggests the possibility that binding of GTP to G proteins as endogenous methyl acceptor substrates causes alterations in the three dimensional structures of G proteins, which in turn enhances the methyl accepting capacities of G proteins and subsequently augments methyl transfer by PFCCMT. In case of PM II from bovine brain, GTP γ S did not show any influence on the regulation of methyl transfer activity of the enzyme (Figure 7). These findings confirmed that two enzymes are distinct entities even though both of them catalyze carboxyl methylation and existed concomitantly up to the final ND-PAGE stage of purification. In the meantime, ATP did not exhibit any effect on PFCCMT activity as was expected (Figure 7).

The effect of AFC on PFCCMT activity

AFC was found to be a potent inhibitor of PFCCMT in a concentration dependent manner using partially purified

Figure 7. The effect of GTP γ S on PFCCMT and PM II activities. (A) PFCCMT from Superdex 75-FPLC was assayed for endogenous methylation activity in the presence of indicated amounts of GTP γ S (●) and ATP (○) each using [methyl-³H]SAM as substrate as described under Methods. (B) PM II activity was assayed using purified bovine brain PM II (2 μ g), 40 μ M [methyl-³H]SAM and with γ -globulin, 0 (□) or 20 (■) mg/ml, in the indicated concentrations of GTP γ S (0, 1 and 2 mM) as described under Methods. All the measurements were averages of triplicates.

Figure 8. Inhibition of PFCCMT catalyzed endogenous methylation by AFC. PFCCMT activity was determined in the presence of 0, 0.8, 2 mM of AFC respectively using Superdex 75 FPLC eluate as an enzyme source as described under Methods.

enzyme (Superdex 75 eluates), completely suppressing enzyme activities in 2 mM (Figure 8). For routine enzyme assay, methyl acceptor was not administered exogenously because methyl acceptors coexisted with the enzyme up to the final stage of purification. The addition of AFC inhibited the methyl transfer to endogenous acceptors, which implied competition by AFC with endogenous methyl acceptors for the site of methyl acceptors on PFCCMT.

Discussion

In prokaryotes, the activities of membrane receptor-transducer proteins are regulated by carboxyl methylation of glutamate (Springer *et al.*, 1979). Recently, various significant signal transduction proteins of G protein superfamily in eukaryotes have been reported to be subject to post-translational C-terminal farnesyl cysteine carboxyl methylation. It has been suggested that the process may

play a regulatory role on the biological activities of G proteins (Sala *et al.*, 1991). Therefore, we partially purified the PFCCMT, catalyzing the reaction from bovine brain in an attempt to understand the biological significances of the processes. The fact that the partially purified enzyme, Superdex 75 eluates, is stimulated by GTP γ S and inhibited by AFC strongly supports that carboxyl methyltransferase purified was PFCCMT.

The major obstacle for purification has been the phenomenon that PFCCMT as a membrane bound enzyme loses its activity easily once it is detached from the membrane by various kinds of detergents, including Triton X-100, sodium cholate, Tween 20, etc. CHAPS, however, was found to preserve relatively high activity after extraction of the enzyme from membrane (Shi and Rando, 1992; Kang *et al.*, 1996). But, the purification of CHAPS extract with conventional procedures including DEAE-cellulose or Sephadex G-200 chromatographies did not work well, because proteins did not separate on preparatory columns due to aggregation even with high concentrations of CHAPS (data not shown). To override these difficulties, we introduced acid precipitation prior to column chromatographies, based on the finding that the pI of the enzyme is located on the alkaline side as revealed by isoelectric focusing (Kang *et al.*, 1996). Adjusting the solution to pH 5.5 kept the enzyme in solution while the proteins with acidic pI precipitated, resulting in the elimination of some proteins as well as phospholipids. The specific activity of the enzyme, however, decreased slightly after the procedure, which seems most probably ascribed to denaturation in an acidic environment. Consequently, the procedure effectively prevented macro-molecules from aggregation, thus enabling purification by DEAE-cellulose column chromatography (Table 1). With a new combination of protocol, we could partially purify PFCCMT to the extent that 30 kDa protein could be observed as a separate band on SDS-PAGE.

Even though the enzyme was purified 130-fold at Superdex 75 FPLC step and further purified with ND-PAGE, multiple bands were shown on SDS-PAGE. However, we chose the 30 kDa protein (indicated by the arrow in Fig. 5) as PFCCMT based on molecular weight and the increase of band intensities roughly in proportion to the enzyme activities. Many attempts have been made to purify and clone PFCCMT, and thus far the enzyme has been cloned in yeast, *Xenopus laevis*, and HL60 cells (human myeloid cell-line). The enzyme of *Schizosaccharomyces pombe* was found to have 236 amino acids, and that of *Xenopus* 288 amino acids (Imai *et al.*, 1997). We extracted 30 kDa protein from the gel, and found that it had 266 amino acids according to amino acid analysis (data not shown). HL60 PFCCMT has a homology with *ste 14*, PFCCMT of *Saccharomyces cerevisiae* and complemented a *ste 14* mutant (Dai *et al.*, 1998). The enzyme has molecular weight of 33 kDa, and was restricted to the endoplasmic reticulum, in contrast to bovine brain PFCCMT which was isolated from plasma membrane fraction and has molecular weight of 30 kDa. The possibility that the physiological significances as well as the structures of myeloid related and brain related isozymes are different needs to be elucidated.

Endogenous methyl acceptor substrates were found to be contained in the enzyme preparations up to the ND-PAGE stage of purification so that enzyme activity determinations were carried out without exogenous methyl acceptors, adding only SAM as substrates with enzyme preparations at all stages of purification. It seems likely that at least part of the proteins in ND-PAGE eluate shown on SDS-PAGE may represent endogenous methyl acceptors. The nature of the substrate which acted as methyl acceptor in this experiment has yet to be elucidated. Heretofore, it has been postulated that most of the endogenous methyl acceptor proteins of PFCCMT belong to the family of either ras related G-proteins or γ -subunit of heterotrimeric G-proteins with many of them being discovered and described (Klein *et al.*, 1994; Leiser *et al.*, 1995). In our experiment, dose dependent stimulation of PFCCMT by GTP γ S also indicates that unknown methyl acceptors might be G-proteins, which is compatible with the generally accepted concept that binding of GTP to G proteins affects enzyme-substrate interaction in favor of isoprenylated cysteine carboxyl methylation (Gingras *et al.*, 1993; Pillinger *et al.*, 1994; Leiser *et al.*, 1995). In addition, inhibition of methylation by AFC indicates that endogenous methyl acceptors may have CAAX motif, consensus C-terminal sequence of G protein superfamily.

At the moment, purified 30 kDa protein, putative PFCCMT, awaits verification of its entity exploiting protein internal microsequencing and subsequent cloning. This work is considered to have served for setting up the procedure to obtain a highly purified PFCCMT.

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References

- Akbar, H., Winegar, D. A. and Lapetina, E. G. (1991) Carboxyl methylation of platelet rap1 proteins is stimulated by guanosine 5'-(3-O-thio)triphosphate. *J. Biol. Chem.* 266: 4387-4391
- Backlund, Jr. P. S. (1992) GTP-stimulated carboxyl methylation of a soluble form of the GTP-binding protein G25K in brain. *J. Biol. Chem.* 267: 18432-18439
- Baruch, G. B., Paz, A., Marciano, D., Egozi, Y., Haklai, R. and Kloog, Y. (1993) The uniquely distributed isoprenylated protein methyltransferase activity in the rat brain is highly expressed in the cerebellum. *Biochem. Biophys. Res. Commun.* 195: 282-288
- Boivin, D., Gingras, D. and Beliveau, R. (1993) Purification and characterization of a membrane-bound protein carboxyl methyltransferase from rat kidney cortex. *J. Biol. Chem.* 268: 2610-2615.
- Clarke, S., Vogel, J. P., Deschenes, R. J. and Stock, J. (1988) Posttranslational modification of the Ha-ras oncogene protein: Evidence for a third class of protein carboxyl methyltransferases. *Proc. Natl. Acad. Sci. USA* 85: 4643-4647
- Dai, Q., Choy, E., Chiu, V., Romano, J., Slivka, S. R., Steitz, S. A., Michaelis, S. and Phillips, M. R. (1998) Mammalian prenylcysteine carboxyl methyltransferase is in the endoplasmic reticulum. *J. Biol. Chem.* 273: 15030-15034
- Fung, B. K., Yamane, H. K., Ota, I. M. and Clarke, S. (1990) The gamma subunit of brain G-proteins is methyl esterified at a C-terminal cysteine. *FEBS Lett.* 260: 313
- Gingras, D., Boivin, D. and Beliveau, R. (1993) Subcellular distribution and guanine nucleotide dependency of COOH-terminal methylation in kidney cortex. *Am. J. Physiol.* 265 (Renal Fluid Electrolyte Physiol. 34): F316-F322
- Gutierrez, L., Magee, A. I., Marshall, C. J. and Hancock, J. F. (1989) Post-translational processing of p21^{ras} is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis. *EMBO J.* 8: 1093-1098
- Hancock, J. F., Magee, A. I., Childs, J. E. and Marshall, C. J. (1989) All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell* 57: 1167-1177
- Hurst, J. H., Billingsley, M. L. and Lovenberg, W. (1984) Photoaffinity labelling of methyltransferase enzymes with S-adenosylmethionine: Effects of methyl acceptor substrates. *Biochem. Biophys. Res. Commun.* 122: 491-501.
- Imai, Y., Davey, J., Kobayashi, M. K. and Yamamoto, M. (1997) Genes encoding farnesyl cysteine carboxyl methyltransferase in *Schizosaccharomyces pombe* and *Xenopus laevis*. *Mol. Cell. Biol.* 17: 1543-1551
- Kang, M. S., Park, G. H. and Hwang, W. I. (1996) Study on purification of protein farnesyl cysteine carboxyl methyltransferase from bovine brain. *Korea University Medical Journal* 33: 1-13
- Kato, K., Cox, A. D., Hisaka, M. M., Graham, S. M., Buss, J. E. and Der, C. J. (1992) Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. *Proc. Natl. Acad. Sci. USA* 89: 6403-6407
- Kim, S. (1984) S-adenosyl methionine:protein-carboxyl O-methyltransferase (protein methylase II). *Methods Enzymol.* 106: 295-309
- Klein, Z., Ben-Baruch, G., Marciano, D., Solomon, R., Altaras, M. and Kloog, Y. (1994) Characterization of the prenylated protein methyltransferase in human endometrial carcinoma. *Biochim. Biophys. Acta.* 1226: 330-336
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227: 680-685.
- Leiser, M., Efrat, S. and Fleischer, N. (1995) Evidence that rap1 carboxyl methylation is involved in regulated insulin secretion. *Endocrinology* 136: 2521-2530

- Metz, S. A., Rabaglia, M. E., Stock, B. J. and Kowluru, A. (1993) Modulation of insulin secretion from normal rat islets by inhibitors of the post-trans-lational modifications of GTP-binding proteins. *Biochem. J.* 295: 31-40
- Park, K. S., Choi, S. Y., Park, G. H. and Kim, S. (1994) Studies on mammalian brain protein farnesyl-cysteine carboxyl-methyltransferase. *Korean J. Biochem.* 26: 47-57
- Pillinger, H. M., Volker, C., Stock, J., Weissmann, G. and Phillips, R. M. (1994) Characterization of a plasma membrane-associated prenylcysteine-directed α -carboxyl methyltransferase in human neutrophils. *J. Biol. Chem.* 269: 1486-1492
- Sala, D. P., Tan, E. W., Canada, F. J. and Rando, R. R. (1991) Methylation and demethylation reactions of guanine nucleotide binding proteins of retinal rod outer segments. *Proc. Natl. Acad. Sci. USA* 88: 3043-3046
- Schaefer, W. R., Trueblood, C. E., Yag, C. C., Mayer, M. P., Rosenberg, S., Pulter, C. D., Kim, S. H. and Rine, J. (1990) Enzymatic coupling of cholesterol intermediates to mating pheromone precursor and to Ras protein. *Science* 249: 1133-1139
- Scheer, A. and Gierschik, P. (1995) S-prenylated cysteine analogues inhibit receptor-mediated G protein activation in native human granulocyte and reconstituted bovine retinal rod outer segment membranes. *Biochemistry* 34: 4952-4961
- Shi, Y. Q. and Rando, R. R. (1992) Kinetic mechanism of isoprenylated protein methyltransferase. *J. Biol. Chem.* 267: 9547-9551
- Springer, M. S., Goy, M. F. and Adler, J. (1979) Protein methylation in behavioural control mechanisms and in signal transduction. *Nature* 280: 279-284
- Sternweis, P. C. and Robishaw, J. D. (1984) Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J. Biol. Chem.* 259: 13806-13813
- Tan, E. W., Perez-Sala, D., Canada, F. J. and Rando, R. R. (1991) Identifying the recognition unit for G-protein methylation. *J. Biol. Chem.* 266: 10719-10722
- Volker, C., Miller, R. A., McCleary, W. R., Rao, A., Poenie, M., Backer, J. M. and Stock, J. B. (1991) Effects of farnesylcysteine analogs on protein carboxyl methylation and signal transduction. *J. Biol. Chem.* 266: 21515-21522