Characterization of the phosphorylation of glycine N-methyltransferase

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Abstract

Glycine N-methyltransferase (GNMT) is a major protein of rat liver cytosol that binds 5-methyl-tetrahydrofolate polyglutamates *in vivo*. This enzyme is suggested to function in the regulation of the availability of a methyl donor, S-adenosylmethionine. Previously, the rat liver GNMT has been shown to be phosphorylated both *in vitro* by cAMP-dependent protein kinase (PKA) and in hepatocytes. Phosphorylation of GNMT seems to be one means of regulating the activity of the enzyme. Chemical analysis of phosphate showed that a small portion of GNMT is phosphorylated in vivo. Here the evidence showed that the rat liver GNMT is also phosphorylated *in vitro* by protein kinase C (PKC). Although phosphorylation of GNMT by PKA increases the GNMT activity, GNMT activity was not affected as a result of phosphorylation by PKC. At present, it is not known yet whether phosphorylation by PKC has a physiological role. Our data showed that the *in vivo* phosphorylation site was closer to the site phosphorylated by PKC rather than by PKA *in vitro*. Phosphorylation of GNMT may be affected by the regulatory control mechanisms of the enzyme.

Introduction

Glycine N-methyltransferase (GNMT; EC 2.1.1.20) catalyzes the methylation of glycine by S-adenosylmethionine (AdoMet) to form sarcosine and S-adenosylhomocysteine (AdoHcy). The enzyme was previously shown to be abundant in liver and pancreatic cytosol (Cook and Wagner, 1984, Yeo and Wagner, 1992, 1994). GNMT consists of four identical monomers and contains tightly bound folate polyglutamates *in vivo*. GNMT is allosterically inhibited by the folate polyglutamates, especially 5-methyl-tetrahydro-folate pentaglutamates ($5\text{-}CH_3\text{-}H_4PteGlu_5$). This represents a metabolic control mechanism which links the de novo synthesis of methyl groups to the methylating ability of the tissues (Yeo et al., 1999).

Xue and Snoswell (1985) showed that alloxan diabetes produced a 65-fold increase in the activity of glycine N-methyltransferase (GNMT) from sheep liver. They suggested that this is a response to elevated amino acid catabolism during diabetes. Noting this observation, preliminary experiments on the effect of alloxan diabetes and starvation on GNMT activity in rat livers were carried out and an approximate two-fold elevation of the enzyme activity was found in both cases (unpublished data). Indeed, rat liver GNMT was phosphorylated both *in vitro* by cAMP-dependent protein kinase (PKA) and in hepatocytes (Wagner et al., 1989). *In vitro* phosphorylation of the enzyme by PKA caused an increase in its activity approximately two-fold. In addition, the location of GNMT to the periportal region in liver and to the proximal convoluted tubules in kidney suggested that this enzyme plays a role in gluconeogenesis (Yeo and Wagner, 1994). GNMT might be activated by glucagon-cAMP mediated phosphorylation under gluconeogenic conditions. GNMT therefore be a key enzyme for gluconeogenesis from methionine through the transmethylation and transsulfuration pathways. In order to adequately describe the physiological regulation of GNMT, the phosphorylation of the enzyme must be further characterized in more

detail. In the present study, the amino acid residues of GNMT which were phosphorylated in hepatocytes and those phosphorylated *in vitro* by PKA were compared. Because protein kinase C (PKC) is known to be involved in hormonal regulation of many enzymes, *in vitro* studies with partially purified PKC were also performed. In addition, the amino acid residues of GNMT which were phosphorylated *in vitro* by PKC were also compared.

Materials and Methods

Materials

Male rats were purchased from Harlan Sprague-Dawley. Radioactive [γ-^{32}P]ATP (30 Ci/mmol) and inorganic [^{32}P]orthophosphate (1 Ci/mmol) were obtained from NEN-DuPont. For phosphoamino acid analysis, 6 N HCl obtained from Pierce. For HPLC analysis, acetonitrile was supplied by Baxter Healthcare Corp., and TFA was purchased from Pierce. Minimum essential medium (MEM) amino acid and non-essential amino acid, Gibco Laboratories; L-glutamine, Flow Laboratories, Inc.; 100 x vitamins for minimum essential medium eagle (modified), ICN Biomedicals Inc.; rapid Coomassie Stain (20x), Diversified Biotech. All reagents for SDS-PAGE including protein molecular weight standards were from Bio-Rad Laboratories. Purified catalytic subunit of PKA and cAMP analog (8-para-chloro-phenyl-thio-cAMP) were provided by Dr. J. Corbin, Department of Molecular Physiology and Biophysis, Vanderbilt University School of Medicine. PKC partially purified from bovine brain was the generous gift of Dr. K. Conricode, Howard Hughes Medical Institute at Vanderbilt University. Phosphatidyl serine was provided by Serdary Research Laboratories. Phosphoserine, phosphothreonine, protein A Sepharose beads, TPCK-treated trypsin, soybean trypsine inhibitor, insoluble alkaline phosphatase-agarose, 12-*O*-tetradecano- yl-phorbol-13-acetate (TPA), glucagon, X-Omat AR film, and Quanta Ⅲ intensifying screens (DuPont, Cronex) were purchased from Sigma Chemical Co.; collagenase (Type Ⅰ) was from Worthington Biochemical Corp. Thin layer cellulose plates for 2-D chromatography was obtained from Eastman Kodak Company.

Measurement of Phosphate in the GNMT Protein.

Liver GNMT was purified by a modification of the column-chromatographic method as described before (Suzuki and Wagner, 1980). In order to measure covalently bound phosphate, inorganic phosphate in the buffer was removed completely by repeated concentration and ultrafiltration of the GNMT sample (about 1 mg) in a Centricon 10 microconcentrator using 10 mM Tris-HCl, pH 7.5 as the replacement buffer. Removal of phosphate was monitored by chemical analysis of the filtrate. The protein was transferred to a thick-walled 10 x 70 mm Pyrex ignition tube and precipitated by the addition of 10% TCA. The precipitate was collected by centrifugation. The supernatant was decanted, and the pellet was dissolved in 500$\mu\ell$ of 0.1N NaOH. This step was completed in 1 min or less to prevent loss of phosphoserine by alkaline β-elimination. An aliquot of this solution (20$\mu\ell$) was used for protein assay and the remaining solubilized protein was reprecipitated by the addition of 480$\mu\ell$ of 20% TCA. The sample was centrifuged and the supernatant was removed. The protein pellet in the ignition tube was subjected to the phosphate assay as described by Buss and Stull (1983). The amount of protein was measured by a modification of the Bradford method (Bradford, 1976).

In vitro phosphorylation of GNMT by PKA.

GNMT was phosphorylated by the catalytic subunit of PKA in an *in vitro* phosphorylation

system as described (Wagner *et al.*, 1989). The reation mixture contained 5 μmol of HEPES (N-2-hydroxy-ethylpiperazine-N'-2- ethanesulfo nic acid) buffer, pH 7.4, 1 μmol of DTT, 0.5 μmol of $MgCl_2$, 10 nmol of cold ATP, 10 μCi of [γ-^{32}P]ATP, 0.3 μg of catalytic subunit of PKA, and 10 μg of purified GNMT in final volume of 100 $\mu\ell$. After incubation at 25℃ for 1 hr, the reaction was stopped by adding 100 $\mu\ell$ of a SDS sample buffer and heating for 5 min in the boiling water bath.

***In vitro* Phosphorylation of GNMT by PKC.**

Purified liver or pancereatic GNMT (1.5μg) was incubated at 30℃ for 30 min with 20 mM Tris-HCl (pH 7.5), 6 mM Mg acetate, 150 μM $CaCl_2$, 50 μg /ml of phosphatidylserine, 1 μg/ml TPA, 100 μM [γ-^{32}P]ATP (1 μCi/nmol) and 75 ng of partially purified PKC (1.0 unit/μg protein) in a volume of 50 $\mu\ell$. Phosphatidylserine and TPA were first mixed in chloroform and then dried under nitrogen. The residue was sonicated in a buffer solution to prepare lipid vesicles as described by Sekiguchi et al. 1988). Reactions were terminated with addition of 30 $\mu\ell$ of Laemmli SDS-sample buffer and heating for 5 min in the boiling water bath.

Isolation of Phosphorylated GNMT.

To further characterize the phosphorylation of GNMT by PKA or PKC, the phosphorylated GNMT protein was subjected to 9-10% SDS- PAGE. The gel was washed with distilled water three times and wrapped with GLAD Cling Wrap, and autoradiography was carried out using Kodak X-Omat AR film with one intensifying screen (DuPont Cronex-Quanta Ⅲ) at 4℃ for 3 hrs. The protein in the gel was stained with rapid Coomassie stain according to the manufacturer's direction except that the protein was not fixed with 12.5% TCA Solution. The radioactive bands on the gel corresponded to those of GNMT were excised with a scalpel on a glass plate and labeled GNMT was extracted by electroelution in a protein elution buffer containing 12.5 mM of Tris base, 96 mM glycine and 0.05% SDS for 3 hrs at room temperature using a Amicon micro-electroeluter at 200 V. The GNMT protein collected into a Centricon 10 microconcentrator (Amicon) was concentrated by spinning at 5,500 rpm in a Sorvall centrifuge with a SS34 rotor for one hour. The retentate was dialyzed against 10 mM ammonium bicarbonate and dried using a "Speed Vac" device. The labeled protein was subjected to phosphoamino acid and phosphopeptide sequence analysis as described below.

Phosphoamino Acid Analysis.

Modified GNMT was hydrolyzed in constant boiling 6N HCl at 110℃ for 2 hrs under nitrogen. The hydrolysate was recovered, mixed with phosphorylated amino acid standards (1 mg each of phosphoserine, phosphothreonine, and phosphotyrosine/ ml), and analyzed by two-dimensional thin-layer electrophoresis as described elsewhere (Cooper et al., 1983). The first dimension was run at 1000 V in acetic acid : formic acid: water (78:25:897, v/v), pH 1.9, for 90 min and the second dimension was at 1000 V in acetic acid : pyridine : water (50:5:945, v/v), pH 3.5, for 35 min on thin-layer cellulose plate (20 x 20 cm, Eastman). The internal standards were located by ninhydrin staining with a reagent containing 50 ml of 95% ethanol, 15 ml of glacial acetic acid and 0.13 g ninhydrin, and radiolabeled amino acid were visualized by autoradiography.

Phosphopeptide Sequence Analysis.

In vitro or *in vivo* phosphorylated GNMT was subjected to tryptic digestion after carboxyamido-methylation of free sulfhydryl groups with iodoacetamide. The tryptic peptides containing ^{32}P were then isolated by reverse phase HPLC with a SynChropak RP-P C18 column. The column for

HPLC was connected to a combined system of SP 8750 organizer module and SP 8700 solvent delivery system, and UV/VIS Monitor. Each peptide was collected manually and the radioactivity of ^{32}P was measured using a beta scintillation system. The amino acid sequence of the labeled peptide was determined in the common resource Protein Analytic Laboratory. Peptide sequence analysis was performed on an Applied Biosystems, Inc. model 470 A sequenator equipped with an on-line 120 PTH-derivatives were separated by reverse-phase HPLC over a PTH C18 column (220 x 2.1 cm). Peptides were modified on a TFA treated cartridge filter or a glass coated disk (Bio-Brene Plus).

Isolation of Rat Liver Hepatocytes.

Rat liver hepatocytes were isolated by a modification of the procedure described by Horne et al (1978). About 180 ml of perfusion medium (Ca free Kerbs-Ringer HEPES, pH7.4) was withdrawn to the perfusion medium chamber using a peristaltic pump, and equilibrated at 37℃ with 100% oxygen for 10-20 min. After anesthetizing the rat with 0.5 ml of sodium pentobarbital, the liver was perfused without recirculation at 14 ml/min for 7 min during which the chest was opened and the superior vena cava was severed, and then the ligature around the inferior vena cava was tightened. The remaining perfusate (approximately 80 ml) was recirculated and 20 mg of collagenase (type I) and 5 mg of soybean trypsin inhibitor were added to the perfusate. The collagenized liver was then removed, minced with scissors in a portion of perfusion medium, and shaken in a plastic Erlenmeyer flask aerated with a stream of 100% O_2. The cell suspension was passed through nylon mesh to remove connective tissue. The damaged cells were removed by a selective centrifugation procedure based upon the observation that viable cells were more dense than the damaged ones.

Phosphorylation of GNMT in Hepatocytes.

The packed hepatocytes were suspended in 3 volume of modified Krebs-Ringer HEPES containing no phosphate, 16 mM lactate and 4 mM sodium pyruvate as energy sources (instead of glucose), and 1.5% gelatin plus MEM essential and nonessential amino acids, vitamins, and 2 mM glutamine. To label the protein, 2 mCi of [^{32}P]phosphate was added and incubated at 37℃. After one hour incubation with [^{32}P] phosphate, 100 nM glucagon and 10 μM cAMP analog (8-para-chloro- phenyl-thio-cAMP) were added to enhance the *in vivo* phosphorylation reaction. After 5 min incubation at 37℃, the cells were washed twice with cold homogenization buffer and homogenized in the three volumes of the same buffer with a Teflon-glass homogenizer. The homogenate was centrifuged for 1 hr at 100,000 x g. GNMT in the supernatant was recovered by immunoprecipitation using affinity-purified anti-GNMT antibodies, analyzed by SDS-PAGE, and visualized by autoradiography. A band on the autoradiogram was located, excised, and extracted from the gel, and then subjected to the phosphoamino acid and phosphopeptide sequence analysis as described before.

Dephosphorylation of GNMT by Insoluble Alkaline Phosphatase-Agarose.

The phosphates contained in GNMT were removed by using insoluble alkaline phosphatase-agarose beads (100 unit/ 0.1 ml suspension). About 150 units alkaline phosphatase in 150 $\mu\ell$ suspension was spun for 1 min using an Eppendorf centrifuge and the supernatant was discarded. The beads were washed three times with 1 ml of 10 mM Tris-HCl, pH 9.0 buffer. To the beads, 30 μg of purified liver GNMT in 300 $\mu\ell$ of 10 mM Tris-HCl, pH 8.0, and 100 $\mu\ell$ of 1.5 mM $CaCl_2$ were added. After 45 min incubation at 37℃, the dephosphorylated GNMT in the supernatant was recovered by spinning the mixture

for 1 min using an Eppendorf centrifuge, and it was applied to *in vitro* phosphorylation with PKC and subsequent measurement of GNMT activity.

Measurement of GNMT Activity

The GNMT activity of the phosphorylated protein was measured by the addition of 20 μmol Tris, pH 9.0 buffer, 0.5 μmol of DTT, 0.4 μmol of glycine, and 0.02 μmol [methyl-^{3}H]AdoMet (94,880 dpm) in a final volume of 100 $\mu\ell$. After incubation at 25℃ for 30 min, the reaction was stopped by addition of 10% TCA and a suspension of acid-washed charcoal as described. GNMT activity was expressed as nmol/ mg protein/ min of sarcosine product formed during 30 min assay.

Results

Phosphate content of Intact Hepatic GNMT

To characterize further the *in vivo* phosphorylation of GNMT, the phosphate content of intact GNMT was measured by chemical analysis (Buss and Stull, 1983). The protein content of the solution was about 25 nmol subunit but the phosphate content of the solution was only 2.75 nmol. That is, GNMT contains 0.11 mol of phosphate per mol of subunit.

In vitro Phospharylation of Liver GNMT by PKA and Identification of Phosphorylated Amino Acyl Residues and Peptides

GNMT was phosphorylated by the catalalytic subunit of PKA and [^{32}P]ATP in an *in vitro* phosphorylation system (Wagner et al., 1989). When the modified GNMT was subjected to phosphoamino acid analysis, only phosphoserine was detected(Fig. 1).

In vitro phosphorylated GNMT was subjected to tryptic digestion after carboxyamido- methylation of free sulfhydryl groups with iodoacetamide. The tryptic peptides containing ^{32}P were then isolated by reverse phase HPLC with a SynChropak RP-P C18 column. Fig. 2 shows the chromatogram for tryptic

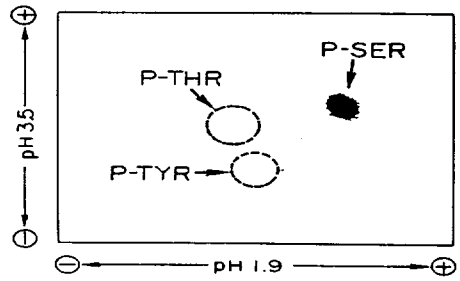

Figure 1. Phosphoamino acid analysis of GNMT phosphorylated *in vitro* by PKA. Purified rat liver GNMT(10 μg) was incubated with the catalytic subunit of PKA (0.3 μg). The labeled enzyme was further purified by 9% SDS-PAGE and recovered from the gel by electroelution. The [^{32}P]labeled GNMT was hydrolyzed in 6 N HCl and analyzed by two-dimensional thin-layer electrophoresis and autoradiography.

peptides of GNMT analyzed by reverse phase HPLC. By measuring the ^{32}P radioactivity of each peptide, it was found that only peak **a** contains the peptide labeled by ^{32}P. A sequence of 20 amino acids (Table 1) was obtained from the labeled peptide using the gas phase sequenator. It was exactly matched to the sequence of liver GNMT from residue 9 to residue 28. The position of the peptide is located in the protein as shown in Fig. 3. Since the *in vitro* phosphorylated GNMT contained only phosphoserine as judged by phosphoamino acid

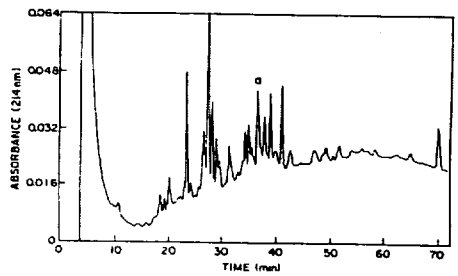

Figure 2. HPLC separation of tryptic digests of liver GNMT phosphorylated by PKA. Purified rat liver GNMT (150 μg) was phosphorylated by the catalytic subunit of PKA (3 μg) and the labeled protein was isolated by 9% SDS-PAGE. The eluted protein was modified by denaturation and carboxy- methylation, and digested by TPCK-treated trypsin. The digests were applied to a SynChropak RP-P C18 reverse phase HPLC column. Peak **a** contains a phosphopeptide.

Table 1 Amino acid sequence of a phosphopeptide derived from GNMT *in vitro* phosphorylated by PKA.

Cycle	Amino Acid	Yield (pmol)	Residue # on GNMT
1	Ser	58	9
2	Leu	70	10
3	Gly	82	11
4	Val	70	12
5	Ala	72	16
6	Ala	69	14
7	Glu	64	15
8	Glv	80	16
9	Ile	54	17
10	Pro	52	18
11	Asp	38	19
12	Gln	41	20
13	Tyr	35	21
14	Ala	39	22
15	Asp	34	23
16	Gly	50	24
17	Glu	33	25
18	Ala	26	26

(1) Val Asp Ser Val Tyr Arg Thr Arg $^{(9)}$**Ser* Leu Gly Val Ala Glu Gly Ile Pro Asp Gln Tyr Ala Asp Gly Glu Ala Ala Arg** Val Trp Gln Leu Tyr Ile Gly Asp Thr Arg Ser Arg Thr Ala Glu Tyr Lys Ala Trp Leu Leu Gly Leu Leu Arg Gln His Gly Cys His Arg Val Leu Asp Val Ala Cys Gly Thr Gly Val Asp Ser Ile Met Leu Val Glu Glu Gly Phe Ser Val Thr Ser Val Asp Ala Ser Asp Lys Met Leu Thr Ala Leu Lys Glu Arg Trp Asn Arg Arg Lys Glu Pro Ala Phe Asp Lys Trp Val Ile Glu Glu Ala Asn Trp Leu Thr Leu Asp Lys Asp Val Pro Ala Gly Asp Gly Phe Asp Ala Val Ile Cys Leu Gly Asn Ser Phe Ala His Leu Pro Pro Asp Ser Lys Gly Asp Gln Ser Glu His arg Leu Lys Asn Ile Ala Ser Met Val Arg Pro Gly Gly Leu Leu Val Ile Asp His Arg Asn Tyr Asp Tyr Ile Leu Thr Gly Cys Ala Pro Pro Gly Lys **Asn Ile Tyr Tyr Lys** $^{(196)}$**Ser**Asp Leu Thr Lys Asp Thr** Thr Ser Val Leu Thr Val Asn Asn Lys Ala His Met Val Thr Leu Asp Tyr Thr Val Gln Val Pro Gly Ala Gly Arg Asp Gly Ala Gly Arg Asp Gly Ala Pro Gly Phe Ser Lys Phe Arg Leu Ser Tyr Tyr Pro His Cys Leu Ala Ser Phe Thr Glu Leu Val Gln Glu Ala Phe Gly Gly Arg Cys Gln His Ser Val Leu Gly Asp Phe Lys Pro Tyr Arg Pro Gly Gln Ala Tyr Val Pro Cys Tyr Phe Ile His Val Leu Lys I . s Thr Gly$^{(292)}$

Figure 3. Localization of phosphopeptide derived from liver GNMT phosphorylated *in vitro*(*) by PKA and in hepatocytes(**) to the rat liver GNMT.
Sequence of rat liver GNMT was adapted from Ogawa et al.(1987)

analysis, serine at residue 9 might be a phosphorylation site for PKA. Because PKA requires basic residues preceding the phosphorylation site, the observed sequence (Arg-Thr-Arg-^{9}Ser(P)-) seems well fitted to the preferred recognition sequence for PKA.

Identification of Amino Acyl Residues and Peptides Phosphorylated in Heparocytes

GNMT labeled with [^{32}P]phosphate in hepatocytes and recovered by immunoprecipitation using immunopurified anti-GNMT antibody or column chromatographic methods was further purified by SDS-PAGE and recovered from the gel by electroelution, subjected to partial acid hydrolysis and trypsin digestion for the phosphoamino acid analysis and phosphopeptide sequence analysis, respectively.

Phosphoamino acid analysis of the protein phosphorylated in hepatocytes showed that only serine was phosphorylated *in vivo* as in the case of *in vitro* phosphorylation (Fig. 4). No phosphothreonine or phosphotyrosine was observed. By measuring the ^{32}P radioactivity of tryptic peptides of GNMT

Table 2 Amino acid sequence of phosphopeptides of GNMT phosphorylated in hepatocytes.

	Phosphopeptide I			Phosphopeptide II		
Cycle	Amino Acid	Yield (pmol)	Residue# on GNMT	Amino Acid	Yield (pmol)	Residue# on GNMT
1	Asn	38	191	Ser	31	196
2	Ile	22	192	Asp	41	197
3	Tyr	13	193	Leu	32	198
4	Tyr	9	194	Thr	49	199
5	?(Lys)	-	195	Lys	12	200
6	?(Ser)	-	196	Asp	38	201
7	?(Asp)	-	197	Ile	26	202
8	?(Leu)	-	198	Thr	32	203
9	?(Thr)	-	199	Thr	25	204
10	?(Lys)	-	200	?(Ser)	9	205
11	?	-	-	Val	16	206
12	?	-	-	?(Leu)	15	207
13	?	-	-	Thr	15	208
14	?	-	-	Val	11	209

separated by reverse phase HPLC, it was found that a peak contained the peptide labeled by ^{32}P (Fig. 5). The amino acid sequencing data showed that the radioactive peak **a** contained a peptide: Asn-Ile-Tyr-Tyr. This peptide is matched to the sequence of liver GNMT from residue 191 to 194. However, phosphoamino acid analysis demonstrated that only serine residues of GNMT were phosphorlyated in hepatocytes. Since the peptide should contain serine as a phosphorylation site, it can be assumed that the tryptide site next to the residue ^{195}lys may not be cleaved, resulting in a longer peptide: Asn-Ile-Tyr-Tyr-Lys -^{196}**Ser**-Asp-Leu-Thr-Lys. A similar experiment was performed to determine whether the residue ^{196}**Ser** is a phosphorylation site. The radioactive peak **a** from second experiment (Fig. 6) contained a peptide : Ser-Asp-Leu-Thr-Lys- Asp-Ile-Thr (phosphopeptide 2 in Table 2). By combining the information from the two experiments, it was assumed that the one overlapping serine (the residue ^{196}**Ser**) seems to be a possible phosphorylation site. Because PKA requires basic residues preceding the phosphorylation site, the observed sequence seems to fit the recognition sequence for the kinase, The position of the peptide is located in the protein as shown in Fig. 3. As shown in the figure, interestingly, the *in vivo* phosphorylation site was different from that of *in vitro* phosphorylation site. *In vitro* phosphorylation site is location to the N-terminal region while GNMT phosphorylated in hepatocytes contained a radioactive peptide which is located in the middle of the entire protein. It is possible that *in vivo* binding of a substrate, AdoMet, or inhibitors such as 5-CH_3-H_4PteGlu$_5$ may cause a conformational change resulting in an exposure of different phosphorylation site. It has been shown that *in vitro* phosphorylation by PKA is inhibited by binding 5-CH_3-H_4PteGlu$_5$ (Wagner et al., 1989). Alternatively, different kinases might be responsible for the *in vivo* phosphorylation of GNMT. Because, in addition to PKA, many protein kinases such as PKC and calcum/calmodulin dependent protein kinases require that basic residues precede the phosphorylation site, it is possible that residue 196 also serve as a phosphorylation site for those protein kinases.

In Vitro Phosphorylation of Liver GNMT by PKC and Identification of Phosphorylated Amino Acyl Residues and Peptides

Since the sequence surrounding serine-^{196}(lys-ser-) represents a preferred PKC phosphorylation site (House et al., 1987), phosphorylation of GNMT by PKC was also tested further. As indicated by the autoradiogram in Fig. 7, purified liver and pancreatic GNMT were readily phosphorylated *in vitro* by PKC in a phospholipid dependent manner. TPA, a potent tumor promoter, has been shown to activate protein kinase C *in vitro* in the presence of both Ca^{2+} and phospholipid (phosphatidylserine and phosphatidylinositol) (Castagna et al., 1982). By adding the mixture of TPA and phosphatidylserine as a lipid micelle, phosphorylation of GNMT was improved. GNMT was almost completely pure as judged by silver-staining of the gel (not shown). However the enzyme revealed a duplex band upon the autoradiogram. The precise nature of the upper band is not known. It might be a highly phosphorylated GNMT or another protein which is a minor contaminant that is not detected by the silver-stain. It can be seen that PKC is also autophosphorylated in the presence of Ca^{2+}, phospholipids and diacylglycerol. [^{32}P]phospho-serine was detected following partial acid hydrolysis (Fig. 8). The trypic peptide of GNMT phosphorylated by protein kinase C were also separated by reverse phase HPLC. There was a peak containing ^{32}P radioactivity which was eluted with 24% acetonitrile at about 40 min (Fig. 9). The radioactive peak **a** was subjected to the amino acid sequence analysis, but the sequencing data showed that the radioactive

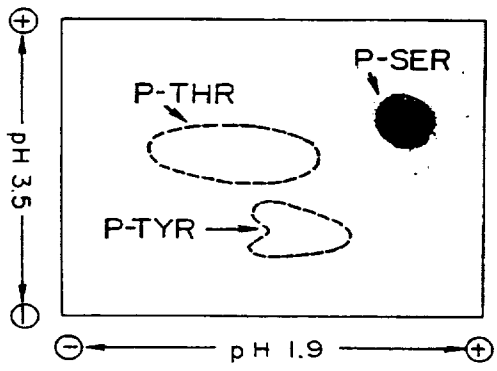

Figure 4. Phosphoamino acid analysis of GNMT isolated from rat hepatocytes. GNMT in hepatocytes was labeled with [^{32}P]phosphate and partially purified by chromatographic methods. The labeled enzyme was further purified by SDS-PAGE and recovered from the gel by electroelution. The [^{32}P]labeled GNMT was hydrolyzed in 6 N HCl and analyzed by two-dimensional thin layer electrophoresis at pH 1.9 and 3.5 as described in Materials and Methods. Autoradiogram of phosphoamino acids from GNMT is shown. Dashed circles indicate the positions of amino acid standards visualized by ninhydrin staining.

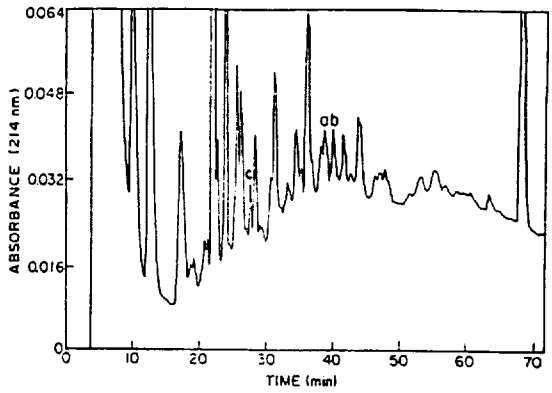

Figure 6. HPLC separation of tryptic digests of liver GNMT phosphorylated in hepatocytes (II). Rat liver GNMT in hepatocytes was labeled with [^{32}P]phosphate and recovered by immunoprecipitation and SDS-PAGE. The protein electroeluted from the gel was further modified by denaturation and carboxymethylation, and it was digested by TPCK-treated trypsin. The tryptic digests were separated by reverse phase HPLC and the radioactivity of each peak was counted. Peak **a**, **b**, and **c** contain [^{32}P]radioactivity, the peptide in peak **a** was further sequenced.

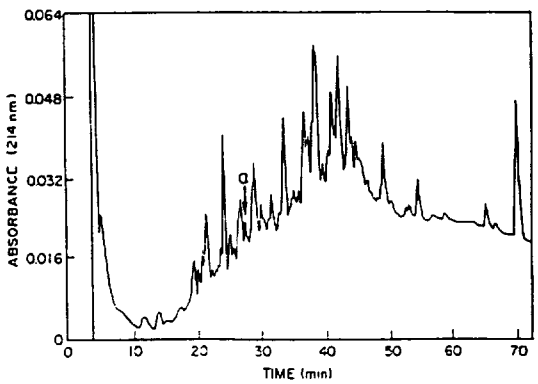

Figure 5. HPLC separation of tryptic digests of liver GNMT phosphorylated in hepatocytes (I). Rat liver GNMT in hepatocytes was labeled with [^{32}P]phosphate and recovered by immunoprecipitation and SDS-PAGE. The eluted protein was further modified by denaturation and carboxymethyl- ation, and digested by TPCK-treated trypsin. The tryptic digests were separated by reverse phase HPLC and the radioactivity of each peak was counted. Peak a contains a phosphopeptide.

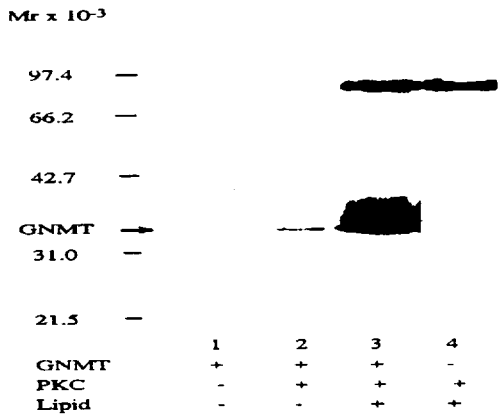

Figure 7. Autoradiogram of phosphorylation of liver GNMT by PKC. PKC (75 ng) was incubated with highly purified liver GNMT (1.5 μg) at 30℃ for 30 min in the absence (lane 2) or presence (lane 3) of lipid mixture. As a control, GNMT preparation was incubated with a reaction mixture not containing kinase and lipids (lane 1). Lane 4 shows the autophosphorylation of PKC. The modified protein was separated by 10% SDS-PAGE and the gel was Coomassie-stained and autoradiographed.

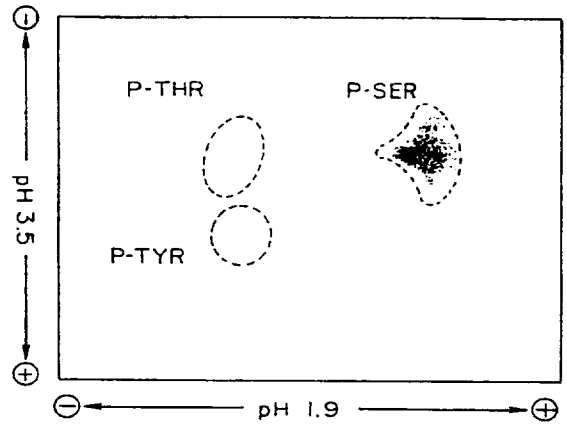

Figure 8. Phosphoamino acid analysis of liver GNMT phosphorylated *in vitro* by PKC. PKC preparation (0.2μg) was incubated with highly purified liver GNMT (20μg) at 30℃ for 30 min in a reaction mixture. The modified protein was separated by 10% SDS-PAGE and the gel was Coomassie-stained except that the protein was not fixed with 12.5% TCA. The stained protein was recovered from the gel by electroelution and dried by speed-vac. The labeled GNMT was hydrolyzed in 6N HCl and analyzed by two-dimensional thin-layer electrophoresis and autoradiography.

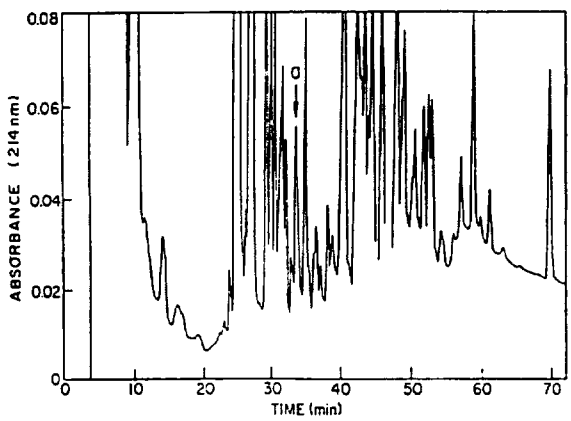

Figure 9. HPLC separation of tryptic digests of liver GNMT phosphorylated by PKC. Purified rat liver GNMT(150 μg) was phosphorylated by partially purified PKC (1.5μg) and the labeled protein was repurified by 10% SDS-PAGE. The eluted protein was modified by denaturation and carboxymethylation, and digested by TPCK-treated trypsin. The digests were applied to a SynChropak RP-P C18 reverse phase HPLC column. Peak **a** contains a phosphopeptide.

peak contains a major tripeptide, Met-Leu-Lys, which is matched to the sequence of liver GNMT from residue 90 to residue 92. Since the peptide does not contain any serine residue which was shown to be phosphorylated *in vitro* by PKC (Fig. 8), it is possible that a minor phosphopeptide containing serine might be present in the radioactive peak. The elution profile of the peptide (Fig.9) was similar to that of the phosphopeptide #1 phosphorylated in hepatocytes (Fig. 5). However the true sequence of the peptide phosphorylated *in vitro* by PKC remains to be elucidated.

Effect of *in vitro* phosphorylation by PKC on GNMT Activity

Recently, it has been shown that the GNMT is phosphorylated *in vitro* by PKA, resulting in a two-fold incerase in GNMT activity (Wagner et al., 1989). Since GNMT is also phosphorylated *in vitro* by PKC, it is of interest to test whether phosphorylation by PKC affects the GNMT activity. GNMT was shown to be partially phosphorylated *in vivo*. In order to enhance the effect of phosphorylation by protein kinase, endogenous phosphates was first removed from purified GNMT by alkaline phosphatase as described under Materials and Methods. Alkaline phosphatase is known to dephophorylate many phosphoproteins. As shown in Table 3, even though GNMT activity was measured following dephosphorylation with alkaline phosphatase and rephosphorylation with PKC in the presence of lipid mixture, the activity was not changed significantly based on the t-test. Addition of the lipid mixture as an activator of PKC did not show any significant differences. Neither ATP nor PKC itself affect the GNMT activity.

Table 3 Effect of *in Vitro* Phosphorylation by Protein Kinase C on GNMT Activity

Sample[a]	GNMT Activity[b]
1. GNMT[c]-Lipid[d]-Protein Kinase[e] +ATP	108.6±1.5
2. GNMT-Lipid+Protein Kinase +ATP	108.0±2.00
3. GNMT+Lipid-Protein Kinase +ATP	106.6±1.64
4. GNMT+Lipid+Protein Kinase +ATP	105.1±3.35
5. GNMT+Lipid+Protein Kinase-ATP	107.6±3.63

a; reactions with various components added to the PKC reaction. The negative (-) and positve (+) symbols represent the absence and presence of each component, respectively.
b; Values are expressed as nmol/mg/min of sarcosine formed during 30 min assay and are the mean±S.E. of triplicate determinations.
c; GNMT dephosphorylated by alkaline phosphatase (1.4 μg, 10 pmol) was used for all reactions.
d; this represents the emulsified lipid mixture containing phosphatidyl serine and TPA. Five $\mu\ell$ of the mixture containing 2.5 μg of phosphatidyl serine and 0.05 μg of TPA was used for each (+) reaction
e; About 75 ng of PKC in 5$\mu\ell$ buffer was used for each (+) reaction.

Discussion

Recently, it has been reported that GNMT is phosphorylated *in vitro* by PKA and freshly isolated rat hepatocytes also incorporate ^{32}P-labeled inorganic phosphate into the enzyme (Wagner *et al.*, 1989). To determine whether GNMT is really a phosphoprotein *in vivo*, we measured phosphate from the native protein purified by column chromatographic methods. At this time, the phosphate content of GNMT was measured by a procedure of Buss and Stull (1983) after all contaminating phosphates were removed from the buffer. The sensitivity of the procedure chosen here is 30 times greater than the standard Fiske-SubbaRow procedure for measuring inorganic phosphate and measures as low as 0.1 nmol phosphate. This procedure incorporates two methods. First, the purified protein sample is ashed to convert protein-bound phosphate to inorganic phosphate. Second, the inorganic phosphate is measured after complexation of phosphomolybdate with the triphenylmethane dye, malachite green. This assay showed that 0.11 mol of phosphate per mol subunit in the preparation of GNMT. Even though the stoichiometry of GNMT- phosphorylation is low, the assay clearly showed that a small portion of GNMT is phosphorylated *in vivo*. It is possible that the molecules containing the phosphorylated residue could represent a functionally important population.

Chock et al. (1980) have proposed that protein phosphorylation is a dynamic process leading to a steady state of phosphorylated and dephosphorylated species. This ratio varies progressively over a wide range of conditions depending on the concentration of effectors which regulate the activities of the protein kinase and phosphatases. So far, the effector system for the phosphorylation of GNMT could not be explained clearly.

Purified GNMT has been shown to be phosphorylated by catalytic subunit of PKA resulting in a two-fold increase in its specific activity (Wagner et al., 1989). Regulation of enzyme activity by protein phosphorylation may result from structural changes occurring near the catalytic site and affect the activity of the enzyme directly (Barford, 1991). Phosphorylation at a site remote from the catalytic site can also regulate the activity by allosteric interactions. The *in vitro* phosphorylation site of GNMT by PKA might be located at or around the catalytic site since phosphorylation is inhibited by binding a competitve inhibiter, 5-CH_3-H_4PteGlu$_5$ (Wagner et al., 1989).

The evidence presented here demonstrated that both liver and pancreatic GNMT were phosphorylated by PKC *in vitro*. However, GNMT activity was not significantly affected as a result of phosphorylation by PKC (Table 3). It is not known yet whether phosphorylation by PKC has a physiological role. To understand further the regulation of GNMT by

phosphorylation, the phosphorylation sites in GNMT were examined after phosphorylation *in vitro* by PKA, PKC and in hepatocytes. Serine[9] has been identified as the major phosphorylation site of GNMT *in vitro* by PKA while serine[196] has shown to be the most likely *in vivo* phosphorylation site of GNMT. The discrepancy between the phosphorylation sites suggests many possiblities. The *in vivo* phosphorylatio of GNMT may be carried out by other kinases or the conformational changes which occur during purification of the protein may alter the pattern of phosphorylation. Since PKC has a strong preference for at least one basic residue which can be located on either the NH2- or COOH- terminal side of the phosphorylatable residue (House et al., 1987), we have tested the possibility that PKC is the enzyme responsible for the *in vivo* phosphorylation. The sites phosphorylated by PKC is still under investigation, but the fact that *in vitro* phosphorylation by PKC results in elution of a tryptic phosphopeptide that is similar in its elution pattern to the *in vivo* phosphopeptide suggests that PKC may be responsible for the *in vivo* phosphorylation.

Similarly it has been demonstrated that glucagon and other conditions which are known to elevate the intracellular levels of cAMP, activites phospholipid methyltransferase in rat hepatocytes by stimulating the phosphorylation of protein at serine residues (Varela et al., 1986). They suggested the ratio of AdoMet/AdoHcy might be an important factor in modulating the phosphorylation of the phospholipid methyl transferase. However the mechanisms by which the ratio of AdoMet/AdoHcy regulates the phosphorylation remains to be elucidated.

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Glycine N-methyltransferase 효소의 인산화와 그 특성 규명

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Glycine N-methyltransferase (GNMT)는 S-adenosylmethione (AdoMet)으로부터 메틸기를 아미노산인 글라이신 (glycine)에 전달하여 sarcosine과 S-adenosylhomocysteine (AdoHcy)의 형성을 촉매하는 효소이다. 특히 간에서 GNMT는 엽산의 유도체인 5-methyltetrahydrofolate pentaglutamates (5-CH_3-H_4PteGlu$_5$)에 의해 그 효소 작용이 억제되기 때문에 이 효소가 메틸기를 포함하는 생체물질들의 합성과 이용을 연결하는 조절 역할을 담당한다고 할 수 있다. GNMT의 생체내에서의 역할과 조절기전을 연구하는 과정중에 이 효소가 간세포내에서 인산화된다는 사실을 발견하였고, 외부에서 주어진 c-AMP-dependent protein kinase (PKA)와 protein kinase C (PKC)에 의해서도 인산화됨을 확인하였다. PKA에 의한 인산화는 이 효소활성을 약 2배 정도 증가시키지만 PKC에 의한 인산화는 이 효소활성에 어떠한 영향도 미치지 않음을 알 수 있다. 이 효소단백질의 구조를 이루는 아미노산 서열중에서 외부에서 인산화된 부위와 간세포 내부에서 인산화된 부위를 비교하였는데, 내부에서 인산화된 부위가 PKA에 의해 인산화된 부위와 일치하지 않고, 오히려 PKC에 의해 인산화된 부위와 일치함을 발견하였다. PKC에 의한 인산화가 생체내에서 어떠한 의미를 갖는 지를 확실히 알 수 없지만, 지금까지의 연구 결과는 인산화 과정도 GNMT의 조절기전중의 하나로 작용할 가능성이 있음을 시사하고 있다.