Identification of phosphorylation sites in glycine N-methyltransferase from rat liver

ZIGMUND LUKA,[1] AMY-JOAN L. HAM,[1,2] JEREMY L. NORRIS,[1,2] EUI-JU YEO,[1,3] VALERY YERMALITSKY,[1] BYRON GLENN,[1] RICHARD M. CAPRIOLI,[1,2] DANIEL C. LIEBLER,[1,2] AND CONRAD WAGNER[1]

[1]Department of Biochemistry and [2]Mass Spectrometry Research Center, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, USA

(RECEIVED October 11, 2005; FINAL REVISION January 11, 2006; ACCEPTED January 13, 2006)

Abstract

Previous studies have shown that rat glycine N-methyltransferase (GNMT) is phosphorylated in vivo, and could be phosphorylated in vitro on serine residues with a significant increase of enzyme activity, but no phosphorylation sites were identified. In this work the identification of the specific phosphorylation sites of rat GNMT is reported. Three different preparations of rat GNMT were analyzed: (1) purified from liver by standard methods of protein purification, (2) prepared from isolated hepatocytes and from liver tissue by immunoprecipitation, and (3) recombinant protein expressed in *Escherichia coli*. We measured the molecular weights of protein isoforms using electrospray mass spectrometry and used liquid chromatography-tandem mass spectrometry (LC-MS/MS) of peptides resulting from tryptic and chymotryptic digests. We also performed chemical analysis of phosphoamino acids and protein sequencing. In all samples, the phosphorylated serine residues 71, 182, and 241 were found. In GNMT prepared from liver tissue and hepatocytes an S9 additional residue was found to be phosphorylated. In hepatocytes and in recombinant GNMT S139 was detected. Serine 9 was also identified as a target for cAMP-dependent protein kinase in vitro. The positions of these phosphorylated residues in the tertiary structure of GNMT indicate their possible effect on enzyme conformation and activity.

Keywords: glycine N-methyltransferase; rat; phosphorylation

Glycine N-methyltransferase is a mammalian enzyme, which catalyzes the transfer of a methyl group from S-adenosylmethionine (SAM) to glycine producing S-adenosylhomocysteine (SAH) and N-methylglycine (sarcosine) (Heady and Kerr 1973; Takusagawa et al. 1999). The biological role of this enzyme was proposed as regulating the ratio of SAM/SAH, which has been considered to be an index of the methylation capacity of the cell (Farrar and Clarke 2002). GNMT is also a major liver folate-binding protein (Cook and Wagner 1984), which binds 5-methyltetrahydrofolate pentaglutamate (5-CH_3-H_4Pte-Glu_5) in vivo and in vitro, and acts as a potent inhibitor of GNMT activity. This serves to link the role of GNMT in the de novo synthesis of methyl groups to the synthesis of preformed methyl groups in the form of methionine (Cook and Wagner 1984; Wagner et al. 1985; Balaghi et al. 1993). It has been pointed out that GNMT is a key enzyme in the degradative conversion of methionine to pyruvate, and as such, can be considered to be involved in gluconeogenesis (Yeo and Wagner 1994; Rowling and Schalinske 2003). The activity of GNMT has been shown to be increased during starvation and diabetes. The activity of cAMP-dependent protein kinase is increased under these conditions (Gorin and Rosenblum 1974; Karasik et al. 1990).

[3]Present address: Department of Biochemistry, Gachon Medical School, Inchon, Republic of Korea.

Reprint requests to: Conrad Wagner, Department of Biochemistry, Medical Center, Vanderbilt University, 620 Light Hall, Nashville, TN 37232, USA; e-mail: Conrad.Wagner@vanderbilt.edu; fax: (615) 343-0407.

Article published online ahead of print. Article and publication date are at http://www.proteinscience.org/cgi/doi/10.1110/ps.051906706.

Although GNMT was first isolated from rabbit, most of the properties of GNMT were obtained using the enzyme from rat, either isolated from rat liver (Ogawa and Fujioka 1982) or recombinant enzyme (Ogawa et al. 1997). It was shown that GNMT is a tetrameric protein with active sites on each identical subunit. The molecular mass of the subunits was found to be 32.5 kDa. Some kinetic data on the GNMTs from other mammals, including human, were obtained and their cDNAs were sequenced (Ogawa et al. 1984, 1993; Aida et al. 1997; Chen et al. 1998). The GNMT proteins were found to be very similar, with identity of amino acid sequences of about 90% (Ogawa et al. 1993). Recombinant rat GNMT was crystallized as the apo-protein (Pattanayek et al. 1998), in complex with SAH and with SAM and acetate (as an analog of glycine) (Fu et al. 1996; Takata et al. 2003). Based on the crystal structures, a mechanism of enzyme action was proposed in which movements of the N-terminal part of the sequence plays a major role in the transfer of the methyl group from SAM to glycine (Takusagawa et al. 1999; Takata et al. 2003).

Study of the rat enzyme showed a difference in the kinetic parameters between recombinant protein and GNMT isolated from liver (Ogawa and Fujioka 1982; Ogawa et al. 1997). Moreover, it was found that the recombinant enzyme was not inhibited by concentrations of 5-CH_3-H_4Pte-Glu_5 that inhibited the native rat liver enzyme (C. Wagner, unpubl.). It was proposed that a possible reason for the difference in kinetics was the N-terminal acetylation of the valine (Ogawa and Fujioka 1982) on the liver enzyme or other post-translational modifications of GNMT (phosphorylation, methylation, etc.) that are not present in the recombinant enzyme. It was found that GNMT could be phosphorylated in vitro by cAMP-dependent protein kinase, resulting in significantly increased enzyme activity (Wagner et al. 1989). In vivo phosphorylation of rat GNMT was recently described using a proteomics approach (Møller et al. 2003). Serine was identified as the site of phosphorylation, but its position was not localized. In this report, we present data on the identification of a number of phosphorylation sites of rat GNMT.

Results

Analysis of rat GNMT by mass spectrometry

Molecular isoforms of intact proteins

Intact GNMT samples prepared both from rat liver and expressed in *Escherichia coli* were analyzed by ESI QqTOF and MS/MS methods. The mass spectra of the proteins greatly depend on the method of sample preparation and storage. When a sample of GNMT, prepared by standard methods of column chromatography and having a high specific activity, was treated with tris(2-carboxyethyl)phosphine (TCEP) to keep cysteine residues fully reduced, the mass spectrum of the protein exhibited only one peak (Fig. 1) with a molecular mass of 32,458.6 Da, which corresponds to a predicted molecular mass for the N-terminal acetylated protein of 32,459.9 Da. As shown in Figure 1, no phosphorylated GNMT was detected as a separate peak.

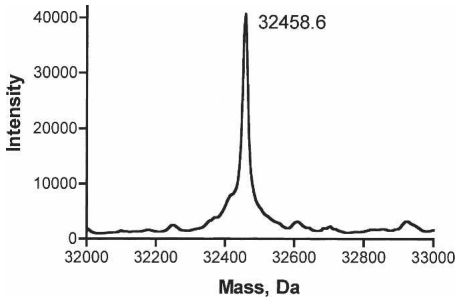

Figure 1. Deconvoluted QqTOF mass spectra of rat GNMT isolated from liver and treated with TCEP. Protein was treated in 25 mM ammonium bicarbonate buffer (pH 7.6). The mass of 32,458.6 Da corresponds to N-terminal acetylated liver enzyme.

Most standard preparations of GNMT, that were routinely kept in the presence of β-mercaptoethanol and DTT as reducing agents, showed multiple peaks in their mass spectra. In recombinant GNMT, in addition to the peak with a molecular mass of 32,420.7 Da, peaks of greater molecular mass (+75–79 Da) or multiples thereof were found probably as a result of β-mercaptoethanol modification of the cysteine residues. In the case of the liver enzyme, multiple peaks were also found, but the lowest molecular mass peak was found to be 32,461.7 Da, which corresponds to full-size protein with an acetylated N-terminal residue.

LC-MS/MS analysis

The conclusion from initial QqTOF analyses of an intact *liver GNMT* sample was that the modified protein was not a significant fraction of the GNMT sample. Because published data (Møller et al. 2003) suggested that rat GNMT is phosphorylated in vivo, a more sensitive approach was used for identification of the phosphorylated site(s). In our work, two mass spectrometry approaches and different samples of GNMT were prepared using inhibitors of kinases and phosphatases.

The first approach used a full-scale LC-MS/MS analysis in order to analyze all possible sites of modification. After the most likely phosphorylated peptides were identified by analysis of Sequest and P-Mod algorithms, specific precursor ions were selected for MS/MS or MS/MS/MS analysis (of neutral loss of phosphoric acid ions) to verify the site of phosphorylation with improved spectral quality.

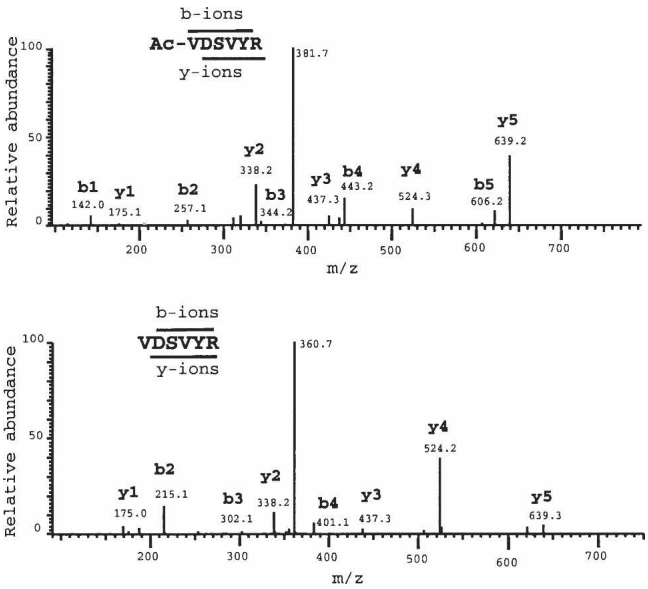

Figure 2. MS/MS spectra of N-terminal peptides of liver and recombinant GNMT. Existence of b- and y-fragments in unmodified peptide in recombinant protein (*bottom*) and in the N-terminal acetylated peptide from liver enzyme (*top*) are clearly detected. The amino acid sequence and expected b- and y-fragments for peptide 1–6 are given at the *top* of each spectra.

Analysis using Sequest showed that trypsin and chymotrypsin digestion of GNMT samples yielded a high coverage of amino acid sequence, not less than 88%. The liver and recombinant enzymes differ in an N-terminal acetylation and the presence of a number of peptides with possible modifications of liver GNMT. This difference in N-terminal acetylation was easily detected by LC-MS/MS analysis as a 42-Da increase of the mass of N-terminal peptide VDSVYR and the presence of all y- and b-ions after fragmentation of the peptide (Fig. 2).

The LC-MS/MS data revealed multiple phosphorylated peptides in all GNMT samples. Additional analysis of the data with another program (P-Mod) revealed phosphorylated tryptic peptides: 9–28, 60–89, 123–147, 176–190, and 240–271, with phosphorylated serine residues of S9, S71, S139, S182, and S241, respectively. A number of chymotryptic peptides containing the same serine residues as above were also detected (Fig. 3; Table 1).

To obtain additional data on the phosphorylated peptides, MS/MS analysis of selected precursor ions from the tryptic digest of GNMT samples was performed. The mass spectrometer was set up to acquire MS/MS spectra specifically of potentially phosphorylated peptides mentioned above by targeting the anticipated ions, rather than acquiring the data in a data-dependent fashion. All spectra that contained a phosphorylated residue were manually inspected for verification.

The most easily detected peptides were two phosphopeptides for which most of the b- and y-ions were found. Peptide 9–28, SLGVAAEGIPDQYADGEAAR, was found in the GNMT prepared by standard chromatography and in GNMT immunoprecipitated from hepatocytes. The phosphorylated peptide 176–190, NYDYILSTGCAPPGK, was found in all GNMT samples. Other peptides that were also detected by either the data-dependent analysis or targeted analysis are listed in Table 1.

Peptide 9–28 contains only one serine residue (S9). The presence of the 9–28 phosphorylated peptide was confirmed by the additional mass of 80 Da, and by the presence of most of the y- and b-ions in the MS/MS spectra, with a correspondingly 80-Da shift to account for the phosphorylation (Fig. 4). In addition, the spectrum contained a neutral loss of phosphoric acid ($M2H^+ – H_3PO_4$), which is an additional characteristic fragmentation that can occur in phosphorylated peptides. Interestingly, phosphorylation of S9 was detected only in GNMT prepared by standard chromatographic methods and in the preparation immunoprecipitated from hepatocytes. Peptide 176–190 was found in all GNMT samples by the presence of additional 80 Da mass units and by the presence of most of the b- and y-ions (Fig. 5).

```
  1 VDSVYRTRSL GVAAEGIPDQ YADGEAARVW QLYIGDTRSR TAEYKAWLLG LLRQHGCHRV

 61 LDVACGTGVD SIMLVEEGFS VTSVDASDKM LKYALKERWN RRKEPAFDKW VIEEANWLTL

121 DKDVPAGDGF DAVICLGNSF AHLPDSKGDQ SEHRLALKNI ASMVRPGGLL VIDHRNYDYI

181 LSTGCAPPGK NIYYKSDLTK DITTSVLTVN NKAHMVTLDY TVQVPGAGRD GAPGFSKFRL

241 SYYPHCLASF TELVQEAFGG RCQHSVLGDF KPYRPGQAYV PCYFIHVLKK TG
```

Figure 3. Position of phosphorylated serines in the sequence of rat GNMT. The phosphorylated tryptic or chymotryptic peptides found by LC-MS/MS analysis are underlined with phosphorylated serine residues marked by an asterisk.

Table 1. *List of phosphorylated tryptic and chymotryptic peptides detected in different GNMT samples by data-dependent and targeted analysis*

Protein	Phosphorylated peptide detected by either data-dependent or targeted analysis
Liver GNMT	9–28 **S**LGVAAEGIPDQYADGEAAR
	60–89 VLDVACGTGVD**S**IMLVEEGFSV TSVDASDK
	62–74 DVACGTGVD**S**IML
	176–190 NYDYIL**S**TGCAPPGK
	178–194 DYIL**S**TGCAPPGKNIYY
	240–261 L**S**YYPHCLASFTELVQEAFGGR
Liver immunoprecipitated GNMT	62–79 DVACGTGVD**S**IMLVEEGF
	60–89 VLDVACGTGVD**S**IMLVEEGFSVT SVDASDK
	119–140 TLDKDVPAGDGFDAVICLGN**S**F
	123–147 DVPAGDGFDAVICLGN**S**FAHL PDSK
	176–190 NYDYIL**S**TGCAPPGK
	178–194 DYIL**S**TGCAPPGKNIYY
	240–261 L**S**YYPHCLASFTELVQEAFGGR
Hepatocytes immunoprecipitated GNMT	1–30 VDSVYRTR**S**LGVAAEGIPDQYAD GEAARVW
	60–89 VLDVACGTGVD**S**IMLVEEGFSVT SVDASDK
	62–74 DVACGTGVD**S**IML
	123–147 DVPAGDGFDAVICLGN**S**FAHL PDSK
	176–190 NYDYIL**S**TGCAPPGK
	240–261 L**S**YYPHCLASFTELVQEAFGGR
Recombinant GNMT	60–89 VLDVACGTGVD**S**IMLVEEGFSVT SVDASDK
	119–140 TLDKDVPAGDGFDAVICLGN**S**F
	123–147 DKDVPAGDGFDAVICLGN**S**FA HLPDSK
	176–190 NYDYIL**S**TGCAPPGK

The phosphorylated serine residues are in bold and underlined.

Of particular interest were the peptides containing phosphorylated S139. The collision-induced fragmentation of this peptide clearly showed the presence of the phosphorylated serine residue (data not shown) in all samples except the standard liver GNMT preparation.

Analysis of rat GNMT by protein chemistry

In addition to mass spectrometric analysis, GNMT phosphorylation was studied using protein chemistry. To identify the phosphorylated residue produced by the isolated hepatocytes they were treated with radiolabeled [^{32}P] sodium phosphate to incorporate [^{32}P] into GNMT. After homogenization, GNMT was isolated by immunoprecipitation and SDS-electrophoresis. It was found that radioactive phosphate was incorporated into GNMT. Phosphoamino acid analysis showed that the only phosphorylated amino acid residue was serine (Fig. 6).

GNMT containing radioactive phosphate was used for determination of phosphopeptides by trypsin digestion and N-terminal sequencing. Identification of the specific phosphorylated serine residue(s) in that GNMT sample by peptide sequencing was complex. Incorporation of the radioactive [^{32}P] into GNMT was low and greatly dependent on the different hepatocytes and crude extract preparations. Our results showed that radioactively labeled peptides were eluted in two or three peaks when the tryptic digest of GNMT was fractionated by reverse-phase chromatography on C18 columns. In one of those peaks a relatively pure peptide was eluted. Its N-terminal sequence was determined as NYDYIL, which corresponded to peptide 176–190, NYDYILSTGCAPPGK. This was confirmed by mass spectrometry. Sequencing of the peptides from another peak revealed the presence of a mixture of peptides, which we were unable to separate.

In vitro phosphorylation of GNMT by cAMP-dependent kinase

We reported earlier that rat GNMT could be phosphorylated in situ (Wagner et al. 1989), but the position of the phosphorylated serine was not determined. It was done later by N-terminal sequencing of the phosphorylated peptide (Yeo 1992). After tryptic digestion of the phosphorylated protein, the peptides were separated by C18 reverse-phase chromatography, and individual fractions of radiolabeled peptide were sequenced. It was found that the sequence of phosphorylated peptide was SLGVAADGIPEQYAGDAAR. This is the sequence of tryptic peptide 9–28, which means that the serine residue modified by cAMP-dependent kinase was S9. This also means that S9 phosphorylation of liver GNMT was a result of action of cAMP-dependent kinase.

Discussion

The main goal of this work was to identify the specific phosphorylation sites of rat GNMT. The phosphorylation of serine in rat glycine N-methyltransferase has been known for more than a decade (Yeo 1992), but the site(s) of phosphorylation remained unknown. Serine phosphorylation in rat GNMT was confirmed recently by Møller et al. (2003) using a proteomics approach for identification of phosphorylated proteins in rat hepatocytes, but the authors did not identify the specific site(s) of phosphorylation.

In the study of protein post-translational phosphorylation two things are critical: protein preparation and

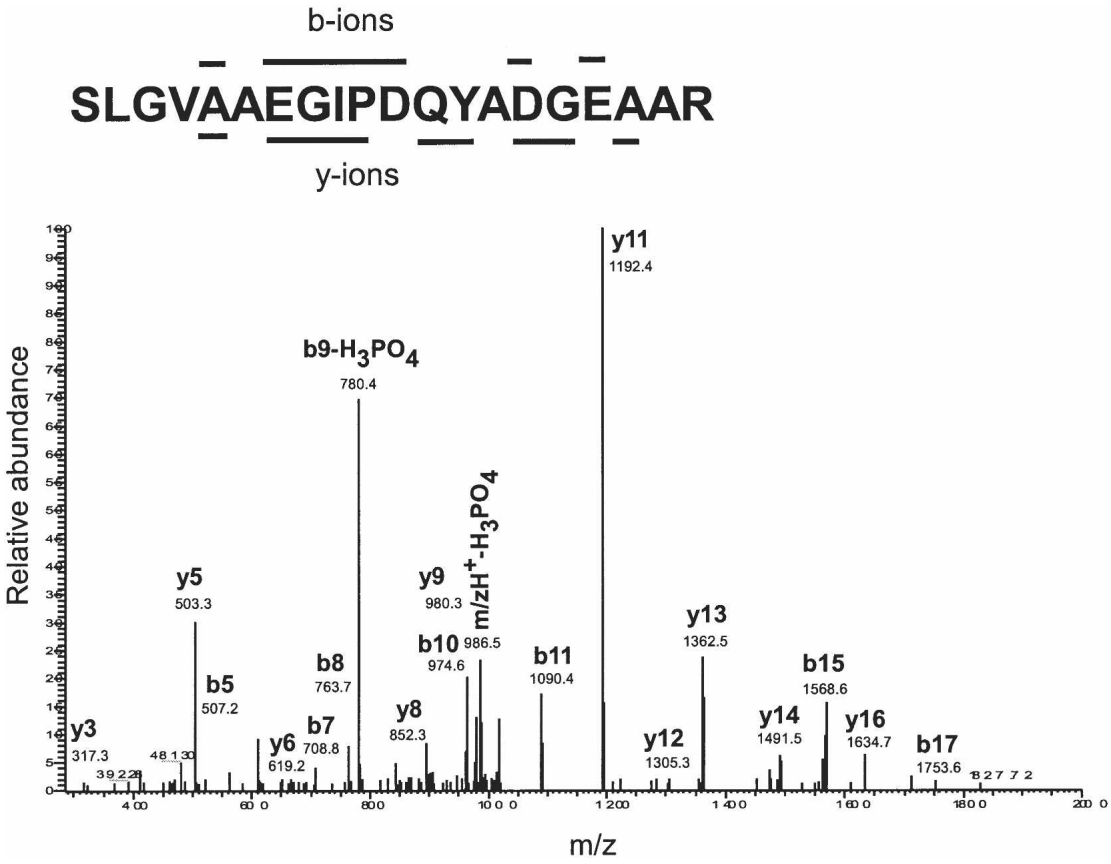

Figure 4. MS/MS spectrum of phosphorylated peptide 9–28 from rat liver GNMT. The trypsin digest of rat liver GNMT was analyzed by MS/MS analysis specifically with a mass/charge ratio of 1035.5 throughout the chromatographic run. This precursor ion corresponds to the presence of one phosphate group in the peptide 9–28. The amino acid sequence and expected b- and y-fragments for peptide 9–28 are given *above* the spectra and the ions found are consistent with the placement of the phosphate group at S9.

sensitivity of the analytical methods. Therefore, we used different methods for GNMT preparation to study effects of different treatments on the location of phosphate groups. Only by using LC-MS/MS mass spectrometry and powerful analytic software were we able to identify the specific serine residues, which are phosphorylated in rat GNMT.

Preparation of liver GNMT by standard chromatographic methods is designed to yield a large amount of purified protein, but takes from two to three weeks since it uses a number of column chromatographic steps. Analysis of a highly purified sample prepared in this way (Fig. 1) showed no major phosphorylated molecular species, but the presence of multiple phosphopeptides was well documented in other preparations (Table 1). Purification using phosphatase inhibitors and immunologic separation was designed to isolate small amounts of protein rapidly.

By using both approaches, we identified the major phosphorylation sites of rat GNMT as serine residues S9 and S182 and minor sites as serines S71 and S241. In all cases, the level of phosphorylation is low and the pattern of phosphorylation depends on the GNMT sample preparation. When precautions were taken to inhibit phosphatase action during GNMT preparation an additional phosphorylation was detected at S139.

The phosphorylated serine residues in rat GNMT are located within different sequence motifs, suggesting that different kinases used GNMT as substrate. A large database for substrate specificity for known kinases (Kennelly and Krebs 1991; Blom et al. 1999) allows us to speculate on the potential kinases that phosphorylated rat GNMT. For S9, located in the sequence YRTRS(9)LGVA, our experimental data showed that this residue is phosphorylated by cAMP-dependent protein kinase (PKA) in situ, for which the consensus motif is R-X(1–2)-S/T-X or R-XX-S/T (Kennelly and Krebs 1991). The most likely kinase that phosphorylates S241 may be a CaM II-dependent kinase for which the consensus sequence site of phosphorylation is R-XX-S/T-X. This is similar to the phosphorylation site of GNMT at Ser241 (FRLSYYPH). The question of whether these

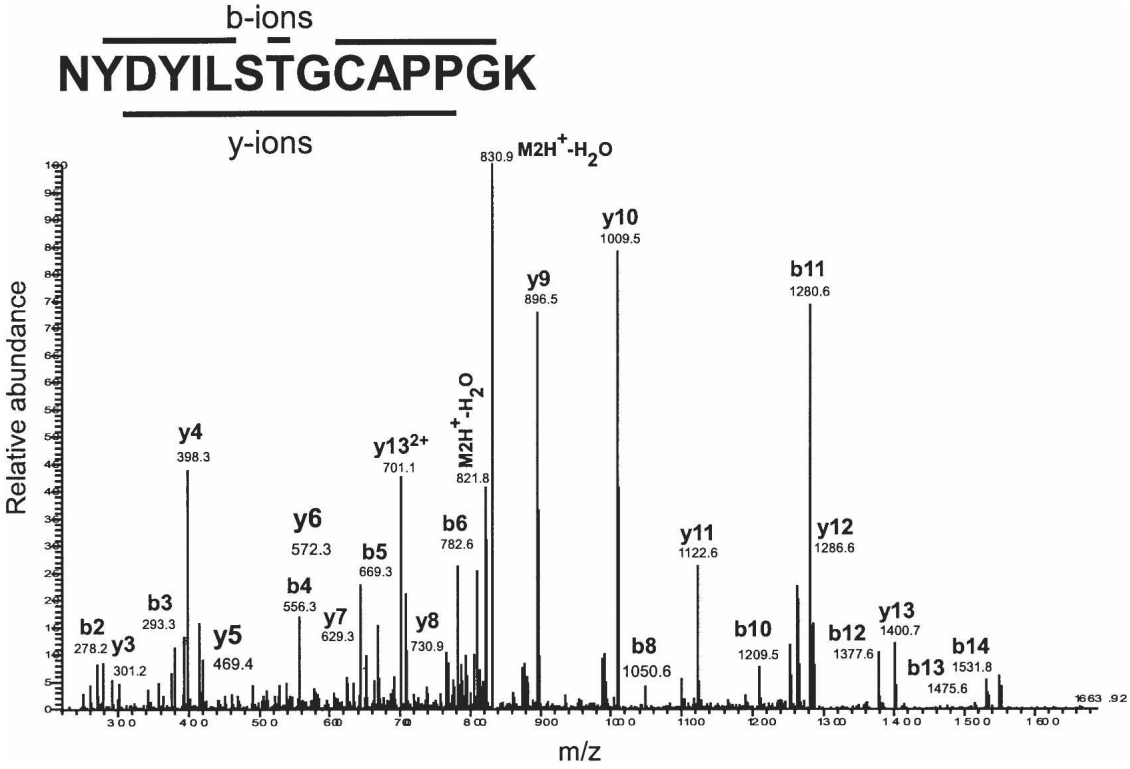

Figure 5. MS/MS spectrum of phosphorylated peptide 176–190 from rat liver immunoprecipitated GNMT. The trypsin digest of rat liver GNMT analyzed by MS/MS in data-dependent mode. The amino acid sequence and expected b- and y-fragments for peptide 176–190 are given *above* the spectra. The ions found are consistent with the placement of the phosphate group at S182.

kinases are responsible for phosphorylation of GNMT in vivo remains to be answered.

A very interesting finding of this work is that almost all the phosphorylated serine residues that were found in the liver enzyme were found also in recombinant GNMT expressed in *E. coli*. The presence of serine/threonine/tyrosine kinases in prokaryotic organisms including *E. coli* is known (Shi et al. 1998). Activity of *E. coli* kinases on expressed mammalian protein is well documented (Du et al. 2005), but how this takes place is unknown.

The identification of the phosphorylation sites of rat GNMT was undertaken to elucidate a potential mechanism of regulation of conformation and activity of GNMT in vivo. Although the level of GNMT phosphorylation is low, it may be higher in vivo, and there is a possibility for regulation of the properties of enzyme by specific serine phosphorylation. GNMT is a tetrameric enzyme, the conformation and stability of which depends on the conformation of each monomer as they are tightly bound by multiple interactions (Fu et al. 1996; Pattanayek et al. 1998; Takata et al. 2003). The phosphorylation is not limited to rat liver, since we have also identified multiple phosphorylation sites in human GNMT expressed in H1299 cells (Z. Luka and C. Wagner, unpubl.), which suggests that it may have broad significance.

The most easily detected phosphorylated residues, serines S9 and S182, are located on the surface of the GNMT molecule. Phosphorylation of these residues could affect conformation and stability of the GNMT tetrameric structure. As shown in Figure 7, S9 and S182 are close enough in the crystal structure to predict a destabilizing effect of the phosphorylation on the

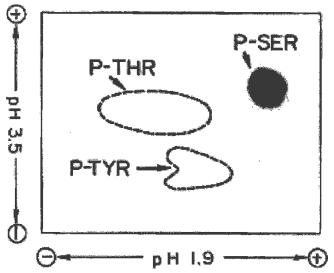

Figure 6. Phosphoamino acid analysis of GNMT isolated from rat hepatocytes. GNMT was labeled in hepatocytes with inorganic [^{32}P]phosphate and purified by chromatographic methods and SDS electrophoresis. The GNMT was eluted from the SDS gel, hydrolyzed in 6 N HCl, and amino acids were separated by two-dimensional thin-layer electrophoresis. Positions of phosphoamino acids are shown with the spot of radioactively labeled amino acid, which is phosphoserine. Figure adapted from Yeo (1992).

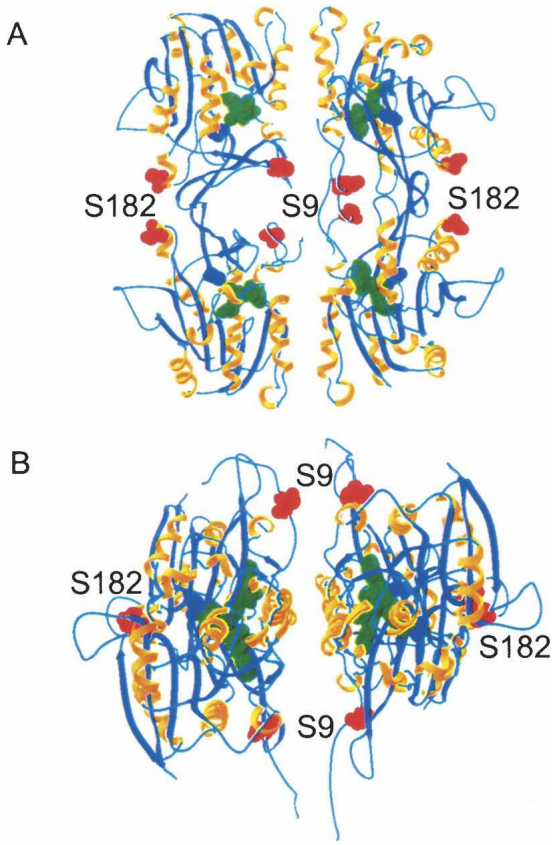

Figure 7. Position of the S9 and 182 residues, which were found phosphorylated in the crystal structure of rat GNMT. The crystal structure of rat GNMT cocrystallized with SAM and acetate was taken from Takata et al. (2003) and is shown in two (*A* and *B*) projections. Serine residues are in red and SAM and acetate are in green.

quaternary structure. We previously have shown that stability of human GNMT tetramer determines activity of the enzyme (Luka and Wagner 2003).

Phosphorylation of S9 may have an impact on GNMT in two ways. This residue is located on the N-terminal part of each monomer, which is proposed to be critical for the enzymatic reaction (Takata et al. 2003). Therefore, phosphorylation of that residue could directly affect enzymatic activity of GNMT. Another possibile way of affecting GNMT action is by modulating folate binding. We have shown that in vitro phosphorylation of S9 by cAMP-dependent protein kinase decreases binding of 5-CH_3-H_4Pte-Glu_5 (Wagner et al. 1989).

The other sites of serine phosphorylation found in this work also suggest regulation of enzyme activity. Serine 71 is located in the middle of α-helix 3 (residues 69–76), which is close to the active center, and it is possible that incorporation of a negative charge into the microenvironment could affect enzyme action. Serine 139 is located within the group of residues that directly participates in rat GNMT enzyme action according to a proposed mechanism (Takata et al. 2003). The G137 makes a hydrogen bond with the substrate glycine, while L136 and H142 form hydrogen bonds with SAM. One could expect a significant effect on the SAM and glycine binding in the active center as a result of the negative charge by phosphorylation of S139. The importance of the microenvironment in this particular portion of the GNMT structure was shown by discovery of a human mutant GNMT with a N140S substitution (Augoustides-Savvopoulou et al. 2003). Substitution of asparagine 140 in human GNMT, which is homologous to N138 in rat GNMT, almost completely inactivated the enzyme. It is important that all proposed mechanisms for regulation of rat GNMT activity by phosphorylation of different serine residues be tested experimentally by in vitro phosphorylation with specific kinases and by site-directed mutagenesis of phosphorylated serines. This work is currently underway.

Materials and methods

Rat GNMT preparation

In several published procedures for purification of rat GNMT no special precautions were taken for inhibition of kinase and phosphatase activities (Suzuki and Wagner 1980; Cook and Wagner 1984). Because most studies were obtained with GNMT purified in this way, it was necessary to analyze phosphorylation of this type of preparation. On the other hand, to study all possible sites of GNMT phosphorylation, phosphatase inhibitors must be included in the homogenization buffer. For this reason four different rat GNMT preparations were studied in this work.

The standard native rat GNMT preparation was isolated from rat liver by a procedure routinely used in our laboratory for many years (Zamierowski and Wagner 1977; Suzuki and Wagner 1980). This included homogenization in isotonic sucrose, preparation of cytosol, and chromatography on gel-exclusion, anion and cation exchange columns, and hydroxylapatite. The homogenization buffer I contained 10 mM K-phosphate buffer (pH 7.0), 0.25 M sucrose, 5 mM EDTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM benzamidine, and 4 μg/mL soybean trypsin inhibitor. No specific inhibitors for phosphatases were used in this procedure. Pure enzyme usually had specific activities over 300 nmol of product/min/mg, but this varied, and specific activities of over 500 have been obtained.

In an attempt to preserve any evidence of native phosphorylation, two other rapid methods of preparing liver GNMT were employed using phosphatase inhibitors and immunoprecipitation with affinity purified anti-rat GNMT polyclonal antibodies. This was prepared by homogenizing rat liver in buffer II containing: 50 mM Tris-HCl (pH 7.5), 50 mM Na-fluoride, 20 mM glycerophosphate, 5 mM Na-pyrophosphate, 1 mM Na-orthovanadate, 2 μM okadaic acid, 2 μM microcystin LR, 1 mM dithiothreitol, 2 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μM leupeptin, 1 μM calpain inhibitor, 1 μM pepstatin A, 1 μM aprotinin, and 10 mM β-mercaptoethanol. The homogenate was centrifugated at 40,000*g* for 1 h and the supernatant was used for GNMT immunoprecipitation. This was done with protein A-Sepharose beads (Sigma). The protein beads (about 10 mg) were first incubated with anti-GNMT antibodies in 20 mM Na-phosphate buffer (pH 8.0) overnight. The liver supernatant (100 μL)

then was added and incubated on ice for 30 min. Unbound proteins were removed with 50 mM Na-phosphate buffer (pH 8.0), and all bound proteins were removed from the Sepharose beads by boiling in the SDS sample buffer. The protein sample was fractionated by SDS electrophoresis, and the GNMT protein band was excised from the gel and subjected to in-gel digestion as described below.

We also purified GNMT from rat hepatocytes using immunoprecipitation. Rat liver hepatocytes were isolated by a modification of the procedure described by Horne et al. (1978). After the required surgical preparation, the liver was perfused first without recirculation at 14 mL/min for 7 min with Ca-free Krebs-Ringer HEPES (pH 7.4) equilibrated at 37°C with 100% oxygen. Then ~80 mL was recirculated and 20 mg of collagenase and 5 mg of soybean trypsin inhibitor added. The resulting suspension was shaken for 10 min at 37°C in a plastic Erlenmeyer flask aerated with a stream of 100% oxygen. The suspension was passed through nylon mesh and the viable (more dense) cells were collected by centrifugation at 50*g* for 1 min. The pellet was washed in incubation medium (Krebs-Ringer HEPES at pH 7.4, containing 2% gelatin and MEM-essential and nonessential amino acids, vitamins, and glutamine) and resuspended in homogenization buffer II of the same composition as used for preparation of liver GNMT by immunoprecipitation. GNMT was then prepared by the procedure used for preparation of immunoprecipitated liver GNMT.

Recombinant GNMT was prepared by expression of rat GNMT in *E. coli* using the recombinant plasmid prepared in Dr. Ogawa's laboratory according to a published protocol (Ogawa et al. 1997) with minor modifications.

Phosphorylation of GNMT in hepatocytes

The rat hepatocytes prepared as above (3 mL) were suspended in 3 volumes of modified Krebs-Ringer HEPES, containing no phosphate, 16 mM lactate, and 4 mM sodium pyruvate as an energy source, and 1.5% gelatin plus MEM essential and nonessential amino acids, vitamins, and 2 mM glutamine. GNMT may be considered as an enzyme involved in gluconeogenesis, since it is one of the principal enzymes in the pathway from methionine to pyruvate. Its activity is induced by starvation or by diabetes (Gorin and Rosenblum 1974), and unpublished experiments (C. Wagner and R.J. Cook) have shown that glucagon administration to intact rats results in elevated GNMT activity after 2 h. For this reason glucagon was included during incubation of the hepatocytes.

To label the proteins, 2 mCi of [^{32}P] phosphate was added and the reaction was incubated for 1 h at 37°C. Then 100 nM glucagon and 10 μM 8-para-chloro-phenyl-thio-cAMP (cAMP analog) were added to enhance the in situ phosphorylation reaction. After 5 min incubation at 37°C the cells were washed and homogenized in three volumes of homogenization buffer. The homogenate was centrifugated at 100,000*g* for 1 h. GNMT was isolated from the supernatant by immunoprecipitation and SDS-electrophoresis. The GNMT band was visualized by radioautography, excised, and protein was extracted by electroelution. GNMT was hydrolyzed in 6 N HCl for the phosphoamino acid analysis and was digested with trypsin for the phosphopeptide sequence analysis.

Phosphopeptide sequence analysis

For phosphopeptide sequence analysis the cysteine residues of GNMT were carboxyamidomethylated in 8 M urea with iodoacetamide, and the modified protein was digested with trypsin in 2 M urea at 37°C for 36 h. The tryptic digest of GNMT containing [^{32}P] was fractionated by reverse-phase HPLC on a SynChropak RP-P C-18 column or Rexchrom S5-100-ODS (25 cm×4.6 mm) column (Regis Technologies) with monitoring at 214 nm. Radioactive peptides were sequenced at the Protein Analysis Core Laboratory of Vanderbilt University School of Medicine.

Identification of phosphorylated amino acid residues

For phosphoamino acid analysis carboxyamidomethylated GNMT was hydrolyzed in 6 N HCl at 110°C for 2 h under nitrogen. The hydrolysate was mixed with phosphorylated amino acid standards and analyzed by two-dimensional thin-layer electrophoresis on thin-layer cellulose plates 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 run at 1000 V in acetic acid:pyridine:water (50:5:945, v/v; pH 3.5) for 35 min. The internal standards were located by ninhydrin staining, and radiolabeled amino acid was visualized by autoradiography.

In vitro phosphorylation of GNMT by cAMP-dependent protein kinase

Rat liver GNMT was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase as described (Wagner et al. 1989). The reaction mixture contained 5 μmol of HEPES buffer (pH 7.4), 1 μmol of DTT, 0.5 μmol of $MgCl_2$, 10 μmol of cold ATP, 10 μCi of [γ-^{32}P]ATP, 0.3 μg of catalytic subunit of protein kinase, and 10 μg of purified GNMT in a final volume of 100 μL. After incubation at 25°C for 1 h, the reaction was stopped by adding 100 μL of SDS sample buffer. After SDS electrophoresis the protein (GNMT) from the radioactive band was electroeluted and analyzed for phosphoamino acids and for phosphopeptides as above.

In-gel trypsin and chymotrypsin digestion for mass spectrometry analysis

The GNMT band in the gel after SDS electrophoresis and Coomassie staining was digested by trypsin or chymotrypsin according to a standard procedure for in-gel digestion (Ham 2005). Briefly, the gel fragment was washed in 50 μL of 50 mM ammonium bicarbonate, then in 50 μL of 50% acetonitrile–25 mM ammonium bicarbonate. After drying, the gel was placed into 50 mM ammonium bicarbonate–10 mM DTT and protein was reduced at 56°C for 15 min. Protein was carboxyamidomethylated in 20 mM iodoacetamide for 15 min and iodoacetamide was washed out.

Tryptic digestion was done with Promega Porcine trypsin in 50 mM ammonium bicarbonate at 37°C overnight. Peptides were extracted by 25 mM ammonium bicarbonate and one additional step with 50 μL of 5% formic acid–50% acetonitrile. Chymotryptic digestion was similar to tryptic digestion, with the exception that the digestion was performed for 5 h at room temperature. Sequencing grade chymotrypsin from Princeton Separations was used.

Intact protein analysis by electrospray mass spectrometry

The molecular mass of intact GNMT was measured using a QSTAR mass spectrometer (Applied Biosystems), having QqTOF geometry, equipped with an ESI source and a Finnigan TSQ700 (Thermo Electron) with an ESI source. The protein sample for QqTOF measurement was prepared in 25 mM ammonium bicarbonate (pH 7.9) and treated with TCEP. Protein samples without treatment with TCEP were analyzed in 20 mM Tris (pH 8.5), 1 mM EDTA, 1 mM sodium azide diluted 1:10 with 100/100/10 (v/v/v) of methanol/water/acetic acid.

GNMT analysis by LC-MS/MS

The LC-MS/MS analyses were performed on a ThermoFinnigan LTQ linear ion trap mass spectrometer equipped with a ThermoFinnigan Surveyor LC pump, NanoSpray source (Thermo Electron), and Xcalibur 1.4 instrument control and data analysis software. HPLC separation of the chymotryptic peptides was achieved with 100 μm×11 cm C-18 capillary column (Monitor C18, 5 micron, 100 Å, Column Engineering), at 0.7 μL/min flow rate. Solvent A was H_2O with 0.1% formic acid, and solvent B was acetonitrile containing 0.1% formic acid. The gradient program was: 0–3 min, linear gradient from 0%–5% B; 3–5 min, 5% B; 5–50 min, linear gradient to 50% B; 50–52 min, linear gradient to 80% B; 52–55 min, linear gradient to 90% B; 55–56 min, 90% B in solvent A. MS/MS scans were acquired using an isolation width of 3 m/z, an activation time of 30 msec, and activation Q of 0.250 and 30% normalized collision energy using 1 microscan and ion time of 100 for each MS/MS scan. The mass spectrometer was tuned prior to analysis using the synthetic peptide TpepK (AVAGKAGAR), so some parameters may have varied slightly from experiment to experiment, but typically the tune parameters were as follows: spray voltage of 1.8 kV, a capillary temperature of 150°C, a capillary voltage of 50 V, and tube lens 100 V. Initial analysis was performed using data-dependent scanning in which one full MS spectra, using a full mass range of 400–2000 amu, was followed by three MS/MS spectra. In the subsequent analysis, several specific precursor masses were selected for MS/MS analysis in a targeted fashion.

Protein potential modification sites were identified using the SEQUEST algorithm (Eng et al. 1994) and the SEQUEST Browser software (Thermo Electron). A list of peptides was created and each peptide was run through P-Mod software to check for possible chemical modifications (Hansen et al. 2005). The candidate modifications found by software were verified by visual inspection of corresponding spectra. Theoretical protein, peptide, and peptide fragment ion masses were generated using General Protein Mass Analysis for Windows version 6.01.1 (Lighthouse Data, Odense, Denmark) and Protein Prospector (http://prospector.ucsf.edu).

Protein analysis

The concentration of protein samples was determined by the BCA method (BCA Protein Assay Kit, Pierce) using bovine serum albumin as a standard. SDS electrophoresis was performed according to standard protocol in 10%–12% gels. Gels were stained with Bio-Safe Coomassie (Bio-Rad).

Acknowledgments

We thank Hans-Rudolf Aerni for technical assistance in Qq-TOF analysis and Salisha Hill for technical assistance in the phosphopeptide analysis by LC/MS. We thank Vanderbilt University for institutional support of the Proteomics Laboratory in the Mass Spectrometry Research Center through the Academic Venture Capital Fund. This work was supported by grants DK15289 and 5P30DK58404 from the U.S. Public Health Service and The Research Service of the Department of Veterans Affairs to C.W.

References

Aida, K., Tawata, M., Negishi, M., and Onaya, T. 1997. Mouse glycine N-methyltransferase is sexually dimorphic and regulated by growth hormone. *Horm. Metab. Res.* **29:** 646–649.

Augoustides-Savvopoulou, P., Luka, Z., Karyda, S., Stabler, S.P., Allen, R.H., Patsiaoura, K., Wagner, C., and Mudd, S.H. 2003. Glycine N-methyltransferase deficiency: A new patient with a novel mutation. *J. Inherit. Metab. Dis.* **26:** 745–759.

Balaghi, M., Horne, D.W., and Wagner, C. 1993. Hepatic one-carbon metabolism in early folate deficiency in rats. *Biochem. J.* **291:** 145–149.

Blom, N., Gammeltoft, S., and Brunak, S. 1999. Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. *J. Mol. Biol.* **294:** 1351–1362.

Chen, Y.M., Shiu, J.Y., Tzeng, S.J., Shih, L.S., Chen, Y.J., Lui, W.Y., and Chen, P.H. 1998. Characterization of glycine-N-methyltransferase-gene expression in human hepatocellular carcinoma. *Int. J. Cancer* **75:** 787–793.

Cook, R.J. and Wagner, C. 1984. Glycine N-methyltransferase is a folate binding protein of rat liver cytosol. *Proc. Natl. Acad. Sci.* **81:** 3631–3634.

Cooper, J.A., Sefton, B.M., and Hunter, T. 1983. Detection and quantification of phosphotyrosine in proteins. *Methods Enzymol.* **99:** 387–402.

Du, P., Loulakis, P., Xie, Z., Simons, S.P., and Geoghegan, K.F. 2005. Tandem mass spectrometry of multiply phosphorylated forms of a "histidine-tag" derived from a recombinant protein kinase expressed in bacteria. *Rapid Commun. Mass Spectrom.* **19:** 547–551.

Eng, J.K., McCormack, A.L., and Yates III, J.R. 1994. An approach to correlate tandem mass-spectral data of peptides with amino-acid-sequences in a protein database. *J. Am. Soc. Mass Spectrom.* **5:** 976–998.

Farrar, C. and Clarke, S. 2002. Altered levels of S-adenosylmethionine and S-adenosylhomocysteine in the brains of L-isoaspartyl (D-Aspartyl) O-methyltransferase-deficient mice. *J. Biol. Chem.* **277:** 27856–27863.

Fu, Z., Hu, Y., Konishi, K., Takata, Y., Ogawa, H., Gomi, T., Fujioka, M., and Takusagawa, F. 1996. Crystal structure of glycine N-methyltransferase from rat liver. *Biochemistry* **35:** 11985–11993.

Gorin, E. and Rosenblum, S. 1974. Effect of triamcinolone treatment, starvation and diabetes on rat liver protein kinase activity. *Biochim. Biophys. Acta* **343:** 510–518.

Ham, A.-J. 2005 Proteolytic digestion protocols. In *The encyclopedia of mass spectrometry, Volume 2. Biological applications Part A: Peptides and proteins* (eds. R.M. Caprioli and M.L. Gross), pp. 10–17. Elsevier Ltd., Kidlington, Oxford, UK.

Hansen, B.T., Davey, S.W., Ham, A.-J.L., and Liebler, D.C. 2005. P-Mod: An algorithm and software to map modifications to peptide sequences using tandem MS data. *J. Proteome Res.* **4:** 358–368.

Heady, J.E. and Kerr, S.J. 1973. Purification and characterization of glycine N- methyltransferase. *J. Biol. Chem.* **248:** 69–72.

Horne, D.W., Briggs, W.T., and Wagner, C. 1978. Transport of 5-methyltetrahydrofolic acid and folic acid in freshly isolated hepatocytes. *J. Biol. Chem.* **253:** 3529–3535.

Karasik, A., Rothenberg, P.L., Yamada, K., White, M.F., and Kahn, C.R. 1990. Increased protein kinase C activity is linked to reduced insulin receptor autophosphorylation in liver of starved rats. *J. Biol. Chem.* **265:** 10226–10231.

Kennelly, P.J. and Krebs, E.G. 1991. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **266:** 15555–15558.

Luka, Z. and Wagner, C. 2003. Human glycine N-methyltransferase is unfolded by urea through compact monomer state. *Arch. Biochem. Biophys.* **420:** 153–160.

Møller, M.T.N., Samari, H.R., Fengsrud, M., Strømhaug, P.E., Østvold, C., and Seglen, P.O. 2003. Okadaic acid-induced, naringin-sensitive phosphorylation of glycine N-methyltransferase in isolated rat hepatocytes. *Biochem. J.* **373:** 505–513.

Ogawa, H. and Fujioka, M. 1982. Purification and properties of glycine N-methyltransferase from rat liver. *J. Biol. Chem.* **257:** 3447–3452.

Ogawa, H., Gomi, T., Hori, T., and Fujioka, M. 1984. Molecular cloning of cDNA for rat glycine methyltransferase. *Biochem. Biophys. Res. Commun.* **124:** 44–50.

Ogawa, H., Gomi, T., and Fujioka, M. 1993. Mammalian glycine N-methyltransferases. Comparative kinetic and structural properties of the enzymes from human, rat, rabbit and pig livers. *Comp. Biochem. Physiol. B* **106:** 601–611.

Ogawa, H., Gomi, T., Takata, Y., Date, T., and Fujioka, M. 1997. Recombinant expression of rat glycine N-methyltransferase and evidence for contribution of N-terminal acetylation to co-operative binding of S-adenosylmethionine. *Biochem. J.* **327:** 407–412.

Pattanayek, R., Newcomer, M.E., and Wagner, C. 1998. Crystal structure of apo- glycine N-methyltransferase (GNMT). *Protein Sci.* **7:** 1326–1331.

Rowling, M.J. and Schalinske, K.L. 2003. Retinoic acid and glucocorticoid treatment induce hepatic glycine N-methyltransferase and lower plasma homocysteine concentrations in rats and rat hepatoma cells. *J. Nutr.* **133:** 3392–3398.

Shi, L., Potts, M., and Kennelly, P.J. 1998. The serine, threonine, and/or tyrosine- specific protein kinases and protein phosphatases of prokaryotic organisms: A family portrait. *FEMS Microbiol. Rev.* **22:** 229–253.

Suzuki, N. and Wagner, C. 1980. Purification and characterization of a folate binding protein from rat liver cytosol. *Arch. Biochem. Biophys.* **199:** 236–248.

Takata, Y., Huang, Y., Komoto, J., Yamada, T., Konishi, K., Ogawa, H., Gomi, T., Fujioka, M., and Takusagawa, F. 2003. Catalytic mechanism of glycine N-methyltransferase. *Biochemistry* **42:** 8394–8402.

Takusagawa, F., Ogawa, H., and Fujioka, M. 1999. Glycine N-methyltransferase, a tetrameric enzyme. In *S-Adenosylmethionine-dependent methyltransferases: Structure and functions* (eds. X. Cheng and R.M. Blumental), pp. 93–122. World Scientific Publishing, Singapore.

Wagner, C., Briggs, W.T., and Cook, R.J. 1985. Inhibition of glycine N-methyltransferase activity by folate derivatives: Implications for regulation of methyl group metabolism. *Biochem. Biophys. Res. Commun.* **127:** 746–752.

Wagner, C., Decha-Umphai, W., and Corbin, J. 1989. Phosphorylation modulates the activity of glycine N-methyltransferase, a folate binding protein. In vitro phosphorylation is inhibited by the natural folate ligand. *J. Biol. Chem.* **264:** 9638–9642.

Yeo, E.-J. 1992. "Studies on a cytosolic folate-binding protein: Glycine N-methyltransferase." Ph.D. thesis, Vanderbilt University, Nashville, TN.

Yeo, E.J. and Wagner, C. 1994. Tissue distribution of glycine N-methyltransferase, a major folate-binding protein of liver. *Proc. Natl. Acad. Sci.* **91:** 210–214.

Zamierowski, M.M. and Wagner, C. 1977. Identification of folate binding proteins of rat liver. *J. Biol. Chem.* **252:** 933–938.