Phosphorylation Modulates the Activity of Glycine N-Methyltransferase, a Folate Binding Protein

**IN VITRO PHOSPHORYLATION IS INHIBITED BY THE NATURAL FOLATE LIGAND***

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Glycine N-methyltransferase (EC 2.1.1.20) was recently identified as a major folate binding protein of rat liver cytosol (Wagner, C., and Cook, R. J. (1984) *Proc. Natl. Acad. Sci. U. S. A.*, 81, 3631–3634). Activity of the enzyme is inhibited when the natural folate ligand, 5-methyltetrahydropteroylpentaglutamate (5-CH$_3$-H$_4$PteGlu$_5$), is bound. It has been suggested that glycine N-methyltransferase plays a role in regulating the availability of methyl groups in the liver. Purified transferase was phosphorylated in vitro by the catalytic subunit of cAMP-dependent protein kinase protein. If 5-CH$_3$-H$_4$PteGlu$_5$ was first bound to the transferase, phosphorylation was inhibited. Phosphorylation of glycine N-methyltransferase in vitro increased its activity approximately 2-fold. 5-CH$_3$-H$_4$PteGlu$_5$ inhibited the activity of newly phosphorylated enzyme as well as native enzyme. Freshly isolated rat hepatocytes incorporated $^{32}$P-labeled inorganic phosphate into this folate binding protein. Chemical analysis of purified enzyme showed about 0.55 mol of phosphate present per mol of glycine N-methyltransferase subunit. These results indicate that phosphorylation of glycine N-methyltransferase may provide a mechanism for modulating the activity of this enzyme and support its role in regulating the availability of methyl groups.

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**Experimental Procedures**

Materials—GNMT was prepared by a minor modification of the method described previously (3). The material containing the enzyme activity following Sephadex G-150 chromatography was concentrated by ammonium sulfate precipitation (0–40% saturation) prior to chromatography on TEAE-cellulose (Bio-Rad). This was followed by chromatography on CM-cellulose (Bio-Rad) and fast protein liquid chromatography using first a mono-S then a mono-Q column (Pharmacia LKB Biotechnology Inc.). The final preparation gave a single band on SDS-PAGE and had a specific activity of 150 units/mg. The catalytic subunit of cAMP-dependent protein kinase was obtained as previously described (9) and was >99% pure as determined by SDS-PAGE. Synthetic (6-amino)-5-CH$_3$-H$_4$PteGlu$_5$ was prepared from synthetic PteGlu$_5$ (kindly provided by Dr. Carlos Krumdieck, University of Alabama) and purified as described previously (10). Radioactive $^{32}$P-labeled ATP and inorganic phosphate were obtained from Du Pont-New England Nuclear. Synthetic heptapeptide substrate for protein kinase assays (Kemptide) was obtained from Sigma. Male rats were obtained from Harlan Sprague-Dawley.

Measurement of GNMT Phosphorylation—Reaction mixtures were prepared containing 2.5 pmol of pH 7.4 HEPES buffer, 0.5 pmol of dithiothreitol, 0.5 pmol of purified GNMT (Bio-Rad) and varying amounts of protein kinase in GNTM in 50 µL reactions. The reaction mixtures were then placed on ice and at 37 °C for 30 min. The reaction mixtures were stopped by adding 50 µL of a solution containing 40% SDS, 0.1% bromphenol blue, and 20% glycerol in 0.125 M Tris buffer, pH 6.8, and heating to 100 °C for 5 min. The solutions were then subjected to SDS-PAGE (11) in 1.5-mm gels. The gels were silver-stained (12), dried, and autoradiographed carried out using Kodak X-Omat AR film.

Inhibition of In Vitro Phosphorylation by 5-CH$_3$-H$_4$PteGlu$_5$—Reaction mixtures containing 2.5 pmol of pH 7.4 HEPES buffer, 0.5 pmol of dithiothreitol, 500 nmol of MgCl$_2$, 5 pmol of [γ-$^{32}$P]ATP (1 µCi), and 250 nmol of MgCl$_2$ for 30 min at 25 °C. A second incubation was carried out after the addition of 10 ng of protein kinase, 5 pmol of ATP (1.4 µCi), and 250 nmol of MgCl$_2$ for 30 min at 25 °C. The final volume was 51 µL. Reactions were terminated as described above and subjected to SDS-PAGE, silver staining, and autoradiography as described above.

Measurement of GNMT Activity—This was carried out as described previously (3). When the effects of phosphorylation on GNMT activity were measured, GNMT activity was determined in a single reaction mixture. The protein kinase reaction was carried out first using unlabeled ATP. The reaction mixtures were then placed on ice and in the pH adjusted by the addition of 20 µmol of pH 9.0 Tris buffer. This was followed by the addition of 0.8 µmol of glycine and 0.02 µmol of [methyl-$^{32}$H]AdoMet (80,000 dpm). The final volume of incubation was 100 µL. After incubation at 37 °C for 30 min the reaction was stopped by the addition of 50 µL of trichloroacetic acid and...
250 µl of a suspension of acid-washed charcoal (76 mg/ml) in 0.1 N acetic acid. This was placed on ice for 15 min, centrifuged, and a portion of the supernatant counted. Blanks were prepared without glycine and ranged from 5 to 20% of the complete reaction mixture. If protein kinase or ATP was omitted from the initial incubation, they were added at the end of the reaction following the addition of trichloroacetic acid and prior to the addition of charcoal to prevent variations in the binding capacity of the charcoal.

**Measurement of Protein Kinase Activity**—This was a modification of the filter paper assay of Roskoski (13). Reaction mixtures contained 2.5 µmol HEPES buffer, pH 7.4, 0.5 µmol of dithiothreitol, 5 µg of Kemptide substrate, 5 nmol of ATP (1 µCi), 250 nmol of MgCl₂, and purified protein kinase in a total volume of 52 µl. The reaction mixture was incubated for 10 min at 25 °C and stopped by spotting 30 µl on a 2-cm square of phosphocellulose paper and dropping immediately into a beaker of 75 mM phosphoric acid. The beaker was shaken gently for 1 min and the phosphoric acid decanted. The paper squares were washed four times with phosphoric acid, once with ethanol, and dried with hot air. They were counted in a liquid scintillation counter using a toluene-based mixture.

**Phosphate Measurement**—This was carried out after digestion with H₂SO₄ and H₂O₂ as described by Bartlett (14).

**Antiserum**—Antiserum to GNMT was prepared in rabbits and checked for its ability to inhibit enzyme activity as described previously (3).

**Protein Measurement**—This was carried out using the method of Bradford (15) which was calibrated against purified GNMT. The amount of GNMT was established by amino acid analysis (16).

**Labeling of GNMT in Hepatocytes**—Rat liver hepatocytes were isolated as described previously (17). One and a half ml of packed cells was suspended in 4.5 ml of Krebs-Ringer HEPES containing low phosphate (0.1 mM) (18) and 1.5% gelatin (17) plus minimal essential medium essential and nonessential amino acids and vitamins and 2 mM glutamine. Two µCi of [³²P]phosphate was added, and the cells were incubated at 37 °C for 4 h. The cells were then centrifuged at 1,000 × g for 1 min, resuspended in 10 volumes of 0.25 M sucrose containing 10 mM potassium phosphate buffer, pH 7.0, 1 mM sodium azide, 0.1 mM phenylmethylsulfonyl fluoride, 100 mM sodium fluoride, 15 mM EDTA, and 2 mM EGTA. The cells were washed once and then resuspended in 5 volumes of the same buffer. They were homogenized with 3 strokes of a small glass-Teflon homogenizer. The homogenate was centrifuged for 60 min at 100,000 × g. Pure GNMT (480 µg) was then added to the homogenate and repurified monitoring both GNMT activity and [³²P]-radioactivity. The sample was first chromatographed on a Sephadex G-150 column (2.4 × 30 cm) equilibrated with 10 mM phosphate buffer, pH 7.0, containing 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM sodium azide, and 0.1 mM phenylmethylsulfonyl fluoride. The tubes containing GNMT activity were collected and chromatographed on a TEAE-cellulose column equilibrated with 10 mM Tris buffer, pH 8.5, containing 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM sodium azide, and 0.1 mM phenylmethylsulfonyl fluoride. The enzyme activity was eluted with a gradient of NaCl from 0–0.5 M. The fraction containing the peak of GNMT activity coincided with a peak of radioactivity. One hundred µl of this fraction was placed on a Laemmli gel, electrophoresed (11), and stained with rapid Coomassie stain (Diversified Biotech). The gel was dried and exposed to Kodak X-Omat AR film with one intensifying screen for 8 days.

**RESULTS**

**In Vitro Phosphorylation of Glycine N-Methyltransferase**—Purified GNMT was phosphorylated when incubated with purified catalytic subunit of protein kinase. Fig. 1 shows that phosphorylation of as little as 10 µg/ml GNMT in the reaction mixture was easily detected. When GNMT was preincubated with 5-CH₃-H₃PteGlu₉, the natural folate ligand, subsequent phosphorylation by protein kinase was inhibited. This is shown in Fig. 2 which also illustrates the well known auto-phosphorylation of protein kinase.

**Effect of 5-CH₃-H₃PteGlu₉ on Kinase Activity**—Although the concentration of 5-CH₃-H₃PteGlu₉ used for the experiment described in Fig. 2 was only 37.3 × 10⁻⁶ M, it was possible that the activity of the protein kinase itself was inhibited by this agent. In order to examine this, the effect of the folate polyglutamate on the activity of the kinase toward

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**FIG. 1. Phosphorylation of GNMT in vitro.** Reactions were carried out as described under "Experimental Procedures" using 21 ng of protein kinase and different amounts of purified GNMT. Incubation was for 30 min at 25 °C. Lanes A, B, and C contained 0, 0.5, and 1.0 µg of GNMT. Gels were exposed to Kodak X-Omat AR film for 3 days with one intensifying screen. A synthetic substrate was examined. As shown in Table I, the presence of (6-arnbo)-5-CH₃-H₃PteGlu₉, at a final concentration of 36.5 × 10⁻⁶ M had no effect on the activity of the kinase. This indicates that binding of the ligand to GNMT decreases its effectiveness as a substrate for the kinase.

**Effect of in Vitro Phosphorylation on GNMT Activity**—When GNMT activity was measured following phosphorylation with protein kinase, the activity was increased approximately 2-fold (Table II). Because 5-CH₃-H₃PteGlu₉ is an inhibitor of GNMT activity as well as an inhibitor of *in vitro* phosphorylation we sought to determine whether 5-CH₃-H₃PteGlu₉ would inhibit the activity of the enzyme after it had been phosphorylated. A series of reactions were set up in which GNMT was first incubated with 5-CH₃-H₃PteGlu₉ or with the *in vitro* phosphorylating system. A second incubation was then carried out during which the sample which had been incubated with the folate polyglutamate was then phosphorylated and the sample which had first been phosphorylated was incubated with the folate polyglutamate. The results are presented in Table III. Sample 2 demonstrates the inhibition of GNMT activity by 5-CH₃-H₃PteGlu₉ and sample 3 shows the increase of GNMT activity by *in vitro* phosphorylation. When GNMT was first phosphorylated, however, and then incubated with 5-CH₃-H₃PteGlu₉, the activity of the enzyme was inhibited as much as when it had not been phosphorylated. This indicates that the phosphorylated enzyme is still susceptible to inhibition by the folate polyglutamate.

**Phosphorylation of GNMT in Isolated Hepatocytes**—Freshly isolated hepatocytes were isolated from rat liver and incubated for 2 h in pH 7.4 Krebs-Ringer buffer containing vitamins and a complete amino acid mixture plus 0.5 mCi of [³²P] inorganic phosphate. The cells were centrifuged, washed, and
Phosphorylation of a Glycine N-Methyltransferase

**TABLE I**

*Effect of 5-CH$_3$H$_4$PteGlu$_5$ on protein kinase activity*

<table>
<thead>
<tr>
<th>Conditions*</th>
<th>Protein kinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 ng kinase</td>
</tr>
<tr>
<td>No addition</td>
<td>7,520</td>
</tr>
<tr>
<td>Plus 1.9 nmol of 5-CH$_3$H$_4$PteGlu$_5$</td>
<td>7,507</td>
</tr>
</tbody>
</table>

*Reactions were carried out as described under "Experimental Procedures" and contained two levels of protein kinase. In addition, half the tubes contained 1.9 nmol of 5-CH$_3$H$_4$PteGlu$_5$.

*Values are the means of duplicate determinations. Values ranged from 2 to 15% of the mean.

**TABLE II**

*Effect of phosphorylation on glycine N-methyltransferase activity*

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Glycine N-Methyltransferase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1*</td>
</tr>
<tr>
<td>Complete</td>
<td>21,130 ± 1,527</td>
</tr>
<tr>
<td>Minus protein kinase</td>
<td>10,022 ± 583</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>15,122 ± 272</td>
</tr>
</tbody>
</table>

*Reaction mixtures contained 2.5 μmol of pH 7.4 Hepes buffer, 0.5 μmol of dithiothreitol, 5 nmol of ATP, 250 nmol of MgCl$_2$, 800 ng of GNMT, and 0.5 ng of protein kinase in half the samples. The total volume was 50 μl. Incubation was carried out at 25°C for 30 min. Glycine, [methyl-$^3$H]AdoMet, and pH 9.0 Tris buffer was added, and activity was assayed as described under "Experimental Procedures."

*Conditions were the same as for experiment 1 except that reaction mixtures contained 290 ng of GNMT and 21 ng of protein kinase. The incubation was carried out for 45 min at 25°C. Either protein kinase or ATP was omitted from some samples. Incubation for the GNMT assay was increased to 60 min.

*Values are expressed as disintegrations/min of sarcosine product formed during the 30-min assay and are the mean ± S.E. of 2–4 determinations. Formation of product was significantly higher in the complete systems than in the systems minus protein kinase or ATP (p < 0.006). In experiment 2 there was no significant difference between the systems minus protein kinase or ATP.

**TABLE III**

*Effects of folate polyglutamate and phosphorylation on glycine N-methyltransferase activity*

<table>
<thead>
<tr>
<th>Sample</th>
<th>First incubation*</th>
<th>Second incubation*</th>
<th>GNMT activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 GNMT</td>
<td>9,376 ± 994</td>
<td>9,376 ± 994</td>
<td></td>
</tr>
<tr>
<td>2 GNMT + 5-CH$_3$H$_4$PteGlu$_5$</td>
<td>1,722 ± 127</td>
<td>1,722 ± 127</td>
<td></td>
</tr>
<tr>
<td>3 GNMT + ATP-Mg, kinase</td>
<td>15,818 ± 726</td>
<td>15,818 ± 726</td>
<td></td>
</tr>
<tr>
<td>4 GNMT + 5-CH$_3$H$_4$PteGlu$_5$, ATP-Mg, kinase</td>
<td>1,698 ± 501</td>
<td>1,698 ± 501</td>
<td></td>
</tr>
<tr>
<td>5 GNMT + ATP-Mg, kinase 5-CH$_3$H$_4$PteGlu$_5$</td>
<td>976 ± 246</td>
<td>976 ± 246</td>
<td></td>
</tr>
</tbody>
</table>

*For the first incubation period reaction mixtures contained 2.5 μmol of Hepes buffer, pH 7.4, 0.5 μmol of dithiothreitol, and 278 ng of GNMT. Sample 2 also contained 5 nmol of 5-CH$_3$H$_4$PteGlu$_5$. Sample 3 also contained 5 nmol of ATP, 250 nmol of MgCl$_2$, and 84 ng of protein kinase in a total volume of 49 μl. Samples were incubated for 30 min at 25°C.

*For the second incubation period, sample 4 received 5 nmol of ATP, 250 nmol of MgCl$_2$, and 84 ng of protein kinase. Sample 5 received 5 nmol of 5-CH$_3$H$_4$PteGlu$_5$. The total volume of all samples for the second incubation period was 95 μl. Samples were then incubated an additional 30 min at 25°C.

*GNMT activity was measured by the addition of glycine, [methyl-$^3$H]AdoMet, and pH 9.0 Tris buffer as described under "Experimental Procedures" for 30 min at 37°C. Values are expressed as disintegrations/min of sarcosine product formed during the 30-min assay and are the mean ± S.E. of triplicate determinations.

Homogenized. The homogenate was centrifuged at 100,000 × g for 60 min. Purified GNMT (480 μg) was added as carrier, and GNMT was partially repurified by chromatography on Sephadex G-150 and TEAE-cellulose. The peak of enzyme activity eluting from the TEAE column (not shown) coincided with a peak of radioactivity. Material from the peak tube was subjected to SDS-PAGE, stained for protein, and then autoradiographed. The results are shown in Fig. 3. Several protein bands were seen in this partially purified sample and several radioactive bands were seen on the autoradiograph. One of the radioactive bands corresponded exactly to a protein band which migrated to the same position as purified GNMT.

**DISCUSSION**

The role of glycine N-methyltransferase in liver has been a matter of speculation since its discovery by Kerr in rabbit liver (1). It is extremely abundant comprising about 3% of the soluble protein in rabbit liver (1) and about 1% in rat liver (6, 7). The product of the reaction, sarcosine, has no known metabolic function and is rapidly oxidized back to glycine in liver mitochondria by sarcosine dehydrogenase (20). This led Kerr to suggest that the true function of GNMT might be to remove excess methyl groups by methylation of a
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Nonessential metabolite. Our discovery (3) that GNMT from rat liver was identical to a cystolic folate binding protein we had purified several years earlier (4) led to the observation that 5-CH₃-H₄PteGlu₅, the natural ligand of this folate binding protein, was a potent inhibitor of the enzyme. We have proposed a metabolic scheme which provides a role for 5-CH₃-H₄PteGlu₅ to control the rate of GNMT activity and thereby regulate the ratio of AdoMet to AdoHyc (5). This is consistent with the hypothesis that the ratio of AdoMet to AdoHyc is a critical factor in intracellular methylation reactions (21).

The observations that GNMT is a phosphoprotein when isolated from rat liver, that it can be further phosphorylated by protein kinase and ATP, that in vitro phosphorylation leads to an increase in enzyme activity, and that this is blocked by the specific folate ligand, 5-CH₃-H₄PteGlu₅, suggest that phosphorylation is not a trivial property of the enzyme. The effects of phosphorylation on activity of the enzyme are shown in schematic fashion in Fig. 4. This depicts the enzyme as consisting of four identical subunits (4, 6, 19) with approximately half of the subunits bearing 1 mol of phosphate as isolated. Upon treatment with protein kinase and Mg-ATP the phosphorylation increases and the enzyme activity increases about 2-fold. Failure to achieve a greater stimulation may be due to the fact that about half the subunits are phosphorylated to begin with. Binding 5-CH₃-H₄PteGlu₅ to the native enzyme results in an inactive enzyme. The scheme depicts 1 mol of the folate ligand binding to the tetrameric protein since binding studies from our laboratory have indicated this. It is suggested that the inactive form of the enzyme containing the bound ligand is in an altered configuration and that this may restrict access of the protein kinase to the site of phosphorylation. It is apparent that phosphorylation of the enzyme does not prevent binding of the ligand since activities of both the native, partially phosphorylated form of the enzyme and the more highly phosphorylated form obtained in vitro are inhibited by the ligand.

We have observed two separate mechanisms of regulating GNMT activity. The first is the allosteric inhibition of activity by 5-methyltetrahydrofolate polyglutamates. Factors which affect the cellular concentration of 5-methyltetrahydrofolates as well as the polyglutamate chain length will play a role here (5). The second mechanism of regulating GNMT activity is the stimulation by covalent phosphorylation. In vitro phosphorylation of GNMT occurs at a concentration of the cAMP-dependent protein kinase (5–10 nM) which is well below the concentration (100–1000 nM) normally found in cells (22). Although we have demonstrated that this can be accomplished in vitro by the catalytic subunit of the cAMP-dependent protein kinase, other kinases may be responsible in vivo. In this respect, however, it is interesting to note that the activity of GNMT has been shown to increase markedly during diabetes. Xue and Snoswell (23) have shown the activity of the enzyme in sheep liver was increased 65-fold as a result of alloxan-induced diabetes. The activity of GNMT was increased 2-fold in the alloxan diabetic rat. This elevated activity may be due to the increased turnover of proteins which takes place during diabetes to provide amino acids for gluconeogenesis. It is known that levels of cAMP in the liver are elevated by alloxan diabetes (24). This should result in activation of cAMP-dependent kinases which is consistent with the observations reported here.

Utilization of methyl groups is an important metabolic

Fig. 4. Modulation of glycine N-methyltransferase.
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process with AdoMet as the donor for methylation of macromolecules (proteins and nucleic acids) as well as small molecules. The resulting homocysteine may be remethylated to methionine or undergo transsulfuration to form cystathionine and eventually cysteine. The distribution of homocysteine between the competing pathways of remethylation and transsulfuration is highly regulated (25). The utilization of S-adenosylmethionine for the various methylation reactions has been thought to be under control of the ratio of AdoMet to AdoHyc because most methyltransferases are product inhibited by AdoHyc (21). The observations presented here that GNMT activity is affected by a posttranslational phosphorylation lead one to speculate that other regulatory sites of the methionine-homocysteine metabolic complex may also be influenced by a phosphorylation mechanism.

The complete amino acid sequence of rat liver glycine N-methyltransferase has been derived from the cDNA sequence by Ogawa et al. (19). Several regions within the molecule may be identified as possible sites for phosphorylation by CAMP-dependent protein kinase.

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REFERENCES