

Transport of Rat Liver Glycine *N*-Methyltransferase into Rat Liver Nuclei*

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Rat liver cytosolic glycine *N*-methyltransferase (GNMT) catalyzes the *S*-adenosylmethionine-dependent methylation of glycine to sarcosine. It is comprised of four identical 292-amino acid residue subunits. Recently, evidence has been provided to show that GNMT is identical to the cytosolic receptor for benzo[*a*]pyrene, which induces cytochrome P450 1A1 gene expression. In the present study we show that chemical modification of purified rat liver GNMT with fluorescein isothiocyanate (FITC) resulted in dissociation of the tetrameric enzyme and was accompanied by loss of enzyme activity. Amino acid sequence analysis of the FITC-labeled peptides obtained by hydrolysis of the modified protein with *Staphylococcus aureus* V8 protease revealed that lysines 45, 89, 92, 96, 122, and 147 were modified. Lys-122 and Lys-147 were derivatized in tetrameric, dimeric, and monomeric forms of the enzyme. Lysines 45, 89, 92, and 96 were derivatized only in monomeric GNMT, suggesting that modification of these residues resulted in GNMT dissociation. The modified monomeric GNMT was quickly transported into isolated rat liver nuclei. This transport was specific for the GNMT monomer, since neither tetramer nor dimer was able to enter the nuclei. Bovine carbonic anhydrase, similar in size to the GNMT monomer, was labeled with FITC to a similar extent but was not transported into the nuclei. Disruption of the nuclei containing fluorescein-labeled GNMT and subsequent extraction of the nuclear lysate with both high and low salt buffers recovered FITC-GNMT only in the chromatin pellet. Our study supports the suggestion of an additional function for GNMT, probably connected with regulation of cytochrome P450 1A1 gene expression.

cytosol (2). Furthermore, 5-methyltetrahydrofolate polyglutamate was shown to inhibit the enzyme activity (3). It has been suggested that GNMT functions as an alternative route for converting *S*-adenosylmethionine to *S*-adenosylhomocysteine to maintain the *S*-adenosylmethionine/*S*-adenosylhomocysteine ratio, which is believed to regulate cellular methylation (4), whereas inhibition of this reaction by the bound folate links the *de novo* synthesis of methyl groups via the one-carbon folate pool to the availability of methionine in the diet (3).

Early studies of rat liver GNMT revealed low levels of enzyme activity in the nuclei purified from rat livers by a non-aqueous technique (5). Later, immunochemical studies of the tissue distribution of the enzyme also showed its presence in rat liver nuclei (6). However, not all of the nuclei were specifically stained with antibody raised against GNMT (6). Neither the properties of nuclear GNMT nor its function in the nuclei has been studied.

Recently, it was shown that the 4 *S* polyaryl hydrocarbon binding protein, which serves as a transcriptional activator of cytochrome P450 1A1 in response to benzo[*a*]pyrene treatment, is identical to GNMT (7). Introduction of an antisense oligonucleotide to GNMT cDNA into rat hepatoma cells was found to reduce the steady-state level of cytochrome P450 1A1 mRNA and the level of its induction by benzo[*a*]pyrene (8). It has further been shown that binding of the ligand benzo[*a*]pyrene evokes translocation of the 4 *S* protein into the nucleus (9).

In the present study we investigated the interactions of GNMT purified from rat liver cytosol with isolated rat liver nuclei. We used an approach in which GNMT from rat liver cytosol was labeled with fluorescein, the most widely used fluorescent dye (10), to directly visualize GNMT distribution and detect its transport into the nucleus.

EXPERIMENTAL PROCEDURES

Animals—Male rats (200–300 g) were obtained from Harlan Sprague-Dawley and were maintained on a standard laboratory chow (Wayne Lab Blox) *ad libitum*. Animals were anesthetized with sodium pentobarbital (40 mg/kg of body weight) before removal of the livers.

Materials—Me₂SO, trifluoroacetic acid (TFA), and fluorescein isothiocyanate were from Pierce. *Staphylococcus aureus* V₈ protease was obtained from Miles Laboratories, Inc. (Naperville, IL). All electrophoresis reagents were from Bio-Rad. All other chemicals were from Sigma.

Purification of GNMT and Assay of the Enzyme Activity—GNMT was purified from rat liver cytosol as described earlier (2). Purified GNMT gave one band when analyzed by SDS-PAGE (11) with Coomassie staining and showed a specific enzyme activity characteristic for pure GNMT (12). GNMT activity was measured by a rapid assay using acid-washed charcoal for adsorption of unreacted *S*-adenosylmethionine as described earlier (2). Protein concentration was determined by Bradford's protein assay (13).

Preparation and Purification of Anti-GNMT Antibodies—Antiserum against purified rat liver GNMT was obtained from male rabbits as described elsewhere (6). Anti-GNMT antibodies were isolated from the antiserum by affinity chromatography on immobilized GNMT as described earlier (6). Affinity-purified antibody was titered and diluted

Glycine *N*-methyltransferase (GNMT¹; EC 2.1.1.20, *S*-adenosyl-*L*-methionine:glycine *N*-methyltransferase), an abundant protein of rat liver cytosol, catalyzes transfer of a methyl group from *S*-adenosylmethionine to glycine with the formation of *N*-methylglycine (sarcosine) and *S*-adenosylhomocysteine (1, 2). GNMT also binds 5-methyltetrahydrofolate polyglutamate and is one of the two major folate binding proteins in rat liver

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¹ The abbreviations used are: GNMT, glycine *N*-methyltransferase; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; BCA, bovine carbonic anhydrase; HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin; NLS, nuclear localization signal.

1:2,000 to use in Western and dot-blotting techniques.

Chemical Modification of GNMT with FITC—All experiments with FITC and FITC-modified GNMT were performed in the dark. Twenty mg of FITC were dissolved in 0.2 ml of Me_2SO , and 2 μl of the solution were added immediately to 0.5 ml of protein solution (1 mg/ml) in 4 mM HEPES, pH 8.5, containing 1 mM dithiothreitol (molar ratio ~50:1). The reaction mixture was incubated for 2 h at 37 °C followed by 1 h at room temperature. Then 0.1 ml of 2.0 M glycine was added to inactivate excess FITC, and after a 30-min incubation at room temperature, the labeled GNMT was separated from excess fluorescein by gel filtration on a Bio-Gel P-6DG (Bio-Rad) column (0.5 \times 15 cm) equilibrated with 4 mM HEPES, 1 mM dithiothreitol, pH 8.5. Concentration of the labeled protein was estimated by Bradford's method, and concentration of fluorescein was estimated by measuring absorbance at 493 nm, assuming a molar absorption coefficient of $7.45 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (14). Bovine carbonic anhydrase (BCA) was modified using the same procedure.

Size Exclusion Chromatography—Modified or unmodified GNMT was chromatographed on a Sephacryl S-300 (Pharmacia Biotech Inc.) column (2.5 \times 70 cm) in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl and 0.01 M 2-mercaptoethanol. Absorbance at 280 nm was monitored during the chromatography. Fractions of 3.5 ml were collected and tested for GNMT activity. The amount of immunoreactive GNMT in the fractions was determined by dot-blot analysis with specific antibodies against GNMT. The ratio of absorbance at 493/280 nm was measured for the peaks obtained.

Isolation and Purification of Rat Liver Nuclei—Rat liver nuclei were prepared from freshly isolated rat livers as described by Blobel and Potter (15). The isolated nuclear suspension was analyzed by light microscopy and showed no discernible contamination with other subcellular structures. The preparation was stored at 5 °C for no longer than 3 days.

Nuclear Transport of Fluorescein-labeled Proteins—Modified GNMT (about 10 μg) in 0.1 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 25 mM KCl, and 2 mM MgCl_2 (buffer I) was incubated with 0.1 ml of the suspension of purified nuclei at room temperature in a 1.5-ml Eppendorf tube for various periods of time. After the incubation, nuclei were sedimented for 5 s at $17,000 \times g$ in an Eppendorf centrifuge. The supernatant was discarded, and the pellet was washed with 0.1 ml of buffer I three times. Then the pellet was resuspended in 0.1 ml of buffer I, and 0.01 ml of the suspension was immediately examined by both phase-contrast and fluorescence microscopy and photographed.

To quantitate nuclear transport, photographs of the nuclei were scanned with an Arcus II scanner, and the digitized stored images were analyzed to obtain pixel histograms for the areas of individual nuclei using Adobe Photoshop software as described earlier (16). The median rather than the mean value for each fluorescence intensity histogram was used as a measure of fluorescence of an individual nucleus. Since the fluorescence was not evenly distributed within the individual nucleus, the median better reflects the brightness of the nucleus as a whole. At least 12 nuclear images for each incubation time were analyzed, and the mean value of the 12 medians (which did not differ by >10%) was used as a measure of the amount of fluorescent GNMT transported into the nuclei. The mean value of 12 medians at 20 h of incubation was assigned 100% saturation, and mean values for the other incubation times were expressed as percentage of the 20 h value. Percentage of saturation was plotted *versus* the incubation time.

In other experiments, the nuclear preparation was incubated with FITC-modified BCA or with native unmodified GNMT (about 10 μg). After the wash procedure (see above), the nuclei were examined for the presence of fluorescence in the case of BCA or for GNMT enzyme activity and by Western blotting in the experiments with unmodified GNMT.

Transport of Different Oligomeric Forms of Modified GNMT into the Nuclei—Fluorescein-modified GNMT was separated into tetrameric, dimeric, and monomeric forms by size exclusion chromatography on Sephacryl S-300 as described above. Each oligomeric form was concentrated to 0.5 mg/ml of protein using a Centricon-10 concentrator (Amicon, Beverly, MA) and was used for the transport experiments as described above.

Chromatin Isolation from Nuclei Incubated with Modified GNMT—Nuclei were incubated with fluorescein-labeled GNMT and washed three times as described above. Chromatin was isolated from the nuclei by the method of Spelsberg *et al.* (17). This included homogenization in a 1.5-ml Eppendorf tube using an Eppendorf micropestle. Nuclei were homogenized by hand in 0.5 ml of 0.08 M NaCl containing 0.02 M EDTA adjusted to pH 6.3 (disruption of nuclei was monitored by light microscopy) followed by sedimentation at $17,000 \times g$ for 5 min. The pellet was resuspended in 0.5 ml of 0.35 M NaCl, homogenized again, and sedi-

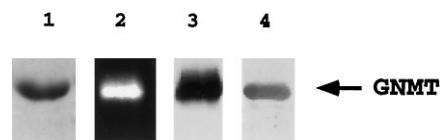


FIG. 1. SDS-PAGE of FITC-modified and unmodified GNMT. FITC-modified GNMT: lane 1, Coomassie-stained; lane 2, fluorescence under UV light; lane 3, immunostained with affinity-purified antibody raised against GNMT and goat anti-rabbit IgG conjugated with alkaline phosphatase. Unmodified GNMT: lane 4, immunostained with the same antibody.

mented. Finally, the pellet was resuspended in 0.5 ml of 2 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA, homogenized, and resedimented. The final pellet was suspended in 0.1 ml of buffer I. The absorbance at 493 nm of the supernatants was used to determine whether any labeled GNMT was released. Aliquots (50 μl) were examined for the presence of GNMT by SDS-PAGE and Western blotting.

Proteolytic Digestion of Modified GNMT—After modified GNMT was separated into tetrameric, dimeric, and monomeric forms by chromatography on Sephacryl S-300, each preparation was concentrated to 0.5 mg/ml protein using a Centricon-10 concentrator (Amicon). One μl of V_8 protease (5 mg/ml) was added to 1 ml of the protein sample (ratio of GNMT/ V_8 was 100/1). The samples were incubated 1 h at 37 °C and then overnight at room temperature. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride to a final concentration of 1 mM. The digested mixture was centrifuged for 5 min at $17,000 \times g$ to remove any insoluble particles.

Purification of Fluorescein-labeled Peptides—The peptides obtained after digestion with V_8 protease were separated by HPLC using a reverse phase Pep-S (C_{18}) column (4 \times 250 mm; Pharmacia) and SP-8700 solvent delivery system (Spectra-Physics, Piscataway, NJ). The column was equilibrated with 0.1% TFA in Milli-Q-treated water (Millipore Corp., Bedford, MA), and peptides were eluted with a linear gradient of 0–80% acetonitrile in 0.1% TFA applied over 80 min at a flow rate of 1 ml/min. Elution profiles were monitored at 224 nm, and peptide peaks were collected manually. The peaks were transferred into 1.5-ml Eppendorf tubes and concentrated to a volume of about 25 μl by evaporation on a SpeedVac Plus concentrator (Savant, Holbrook, NY). Peaks showing a yellow color were further purified on the same column equilibrated with 20 mM ammonium acetate buffer, pH 6.8, and a linear gradient of 0–60% acetonitrile in the same buffer and run for 60 min at a flow rate of 1 ml/min.

Sequencing of the Fluorescein-modified Peptides—Amino acid sequence analysis of the purified fluorescein-labeled peptides was carried out using automated Edman degradation with gas phase analysis on an LF-3000 (Beckman) or Applied Biosystems 470A (Perkin-Elmer) protein sequencer.

RESULTS

Properties of FITC-modified GNMT—We found that modification by FITC resulted in the reproducible incorporation of an average of 2.5 mol of fluorescein/mol of GNMT monomer. BCA showed a similar degree of modification with 1.5 mol of FITC bound/mol of the protein.

The electrophoretic mobility in SDS-PAGE and immunological properties of FITC-modified GNMT were identical to those for unmodified GNMT (Fig. 1). Modified enzyme, however, showed only a trace amount of methyltransferase activity compared with unmodified protein (data not shown).

The oligomeric structure of modified GNMT was studied by chromatography on Sephacryl S-300. In contrast with unmodified GNMT that was eluted from a Sephacryl S-300 column as a single peak with an apparent molecular mass of 130 kDa corresponding to the protein tetramer, the modified protein was eluted mainly (about 70%) as a 35-kDa species corresponding to the protein monomer. Two other peaks observed in these experiments corresponded to GNMT dimer and tetramer forms (Fig. 2A). Only the GNMT tetramer retained enzyme activity (Fig. 2B). Neither dimer nor monomer showed any methyltransferase activity (Fig. 2B). All three oligomeric forms of the modified protein reacted with the antibody raised against GNMT as well as unmodified protein itself (Fig. 2C). Repeated

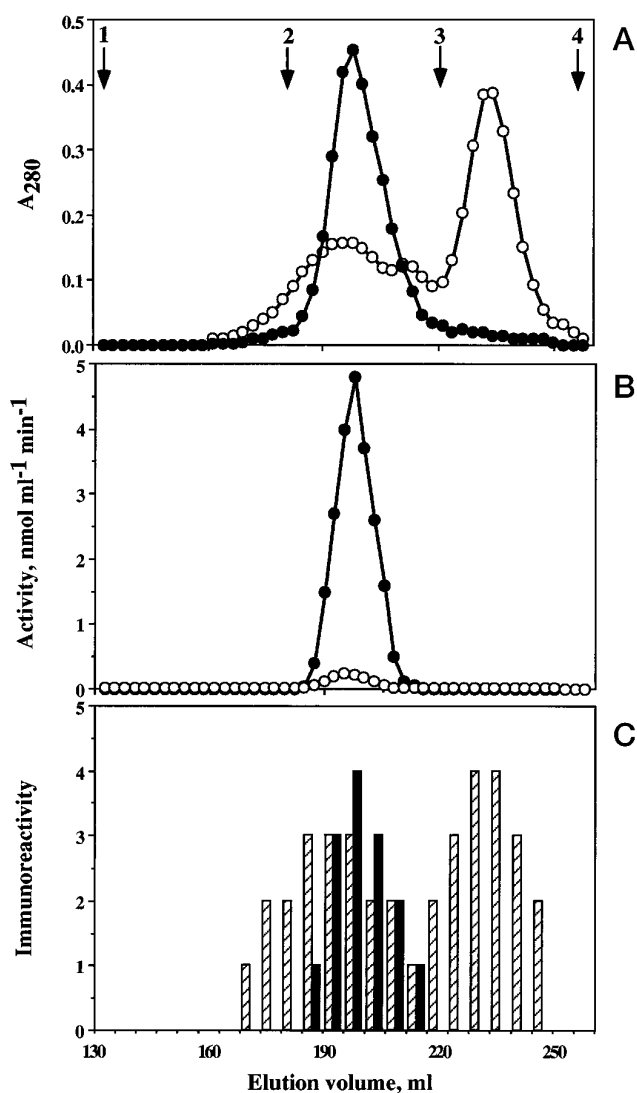


FIG. 2. **Sephacryl S-300 chromatography of FITC-modified and unmodified GNMT.** About 2 ml of modified (open circles or crossed bars) or unmodified (closed circles or bars) GNMT (1 mg/ml) were applied to a Sephacryl S-300 column. Fractions of 3.5 ml were collected and analyzed for absorbance at 280 nm (panel A), enzyme activity (panel B), and immunoreactivity (panel C). Immunoreactivity was expressed in arbitrary units assuming the maximal signal on dot-blot as 4 and the minimal signal as 0. Arrows indicate elution volumes of standards used for calibration of the column: 1, blue dextran 2,000 (2,000 kDa); 2, catalase (232 kDa); 3, bovine serum albumin (67 kDa); 4, chymotrypsinogen A (25 kDa).

chromatography of the fluorescein-modified GNMT monomer on the Sephacryl S-300 column did not reveal any molecular species other than the monomer (data not shown).

We used the A_{493}/A_{280} ratio to estimate the relative extent of modification of different oligomeric forms of GNMT. The ratios were 0.14 for the tetramer, 0.18 for the dimer, and 0.62 for the monomer. This showed that the amount of bound FITC/molecule of the enzyme protomer was 3–4 times higher for the monomer than for the dimer or tetramer.

Study of the Transport of FITC-modified GNMT into Rat Liver Nuclei—Immunoblotting analysis with antibody raised against rat liver GNMT (Fig. 3) and enzyme activity assay (data not shown) revealed that purified rat liver nuclei did not contain GNMT. After 15 min of incubation with FITC-modified GNMT, rat liver nuclei displayed a bright green fluorescence (Fig. 4A), showing accumulation of labeled GNMT inside the nuclei. Washing the nuclei with 0.5% Triton X-100 did not

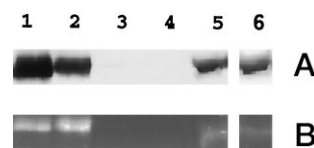


FIG. 3. **SDS-PAGE and Western blot analysis of FITC-modified GNMT before and after incubation with rat liver nuclei.** Where indicated, purified rat liver nuclei (0.1 ml in buffer I) were incubated with 0.1 ml of FITC-modified GNMT (about 10 μ g) in buffer I as described under "Experimental Procedures." After incubation, the nuclei were sedimented, washed three times, and resuspended in 0.1 ml of buffer I. Aliquots (50 μ l) were analyzed by SDS-PAGE and examined by immunoblotting (panel A) or under UV light (panel B). Lane 1, modified GNMT incubated with buffer alone; lane 2, modified GNMT remaining in the supernatant after incubation with nuclei for 30 min and sedimentation; lanes 3 and 4, rat liver nuclei incubated with 0.1 ml of buffer I without modified GNMT; lane 5, sedimented nuclei after a 30-min incubation with modified GNMT; lane 6, chromatin isolated from nuclei after a 30-min incubation with modified GNMT and resuspended in the same volume (0.1 ml) of buffer I as the nuclei.

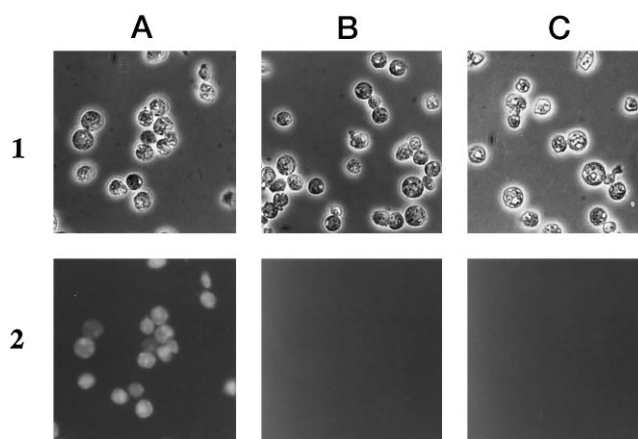


FIG. 4. **Transport of fluorescein-modified GNMT into the nuclei.** Panel 1, a phase-contrast view of the nuclei; panel 2, a view of the same field in UV light. Purified rat liver nuclei were incubated with fluorescein-labeled GNMT for 15 min (A), with buffer I for 15 min (B), with fluorescein-labeled BCA for 20 h (C).

diminish the fluorescence (data not shown). Purified rat liver nuclei showed no fluorescence on their own (Fig. 4B). When isolated tetrameric, dimeric, or monomeric forms of fluorescein-modified GNMT were separately used for transport experiments, only monomeric-modified GNMT accumulated in the nuclei, similar to the results obtained when the mixture of oligomeric forms of the modified protein was used. Neither modified tetramer nor dimer was transported into the nuclei (Fig. 5).

Study of the time course of FITC-modified GNMT transport showed that after a 5-min incubation with the labeled protein the fluorescence of the nuclei was about half of the maximum observed after overnight incubation (Fig. 6). Digital analysis of the brightness of nuclear images gave a $t_{1/2}$ of approximately 4.3 min (Fig. 7). After 2 h of incubation with the FITC-modified GNMT, no significant increase in nuclear fluorescence was noticed (Figs. 6 and 7).

We tried to determine whether native unmodified GNMT could enter the nuclei. After incubating purified liver nuclei with unmodified GNMT, we could detect no GNMT associated with them either by measuring enzyme activity or by immunoblotting with specific antibody (data not shown). To study whether unmodified GNMT can influence the transport of the modified monomer into the nuclei, we preincubated the nuclei with unmodified GNMT or with liver cytosol before addition of the modified protein. In another experiment, the modified GNMT was incubated with the nuclei in the presence of un-

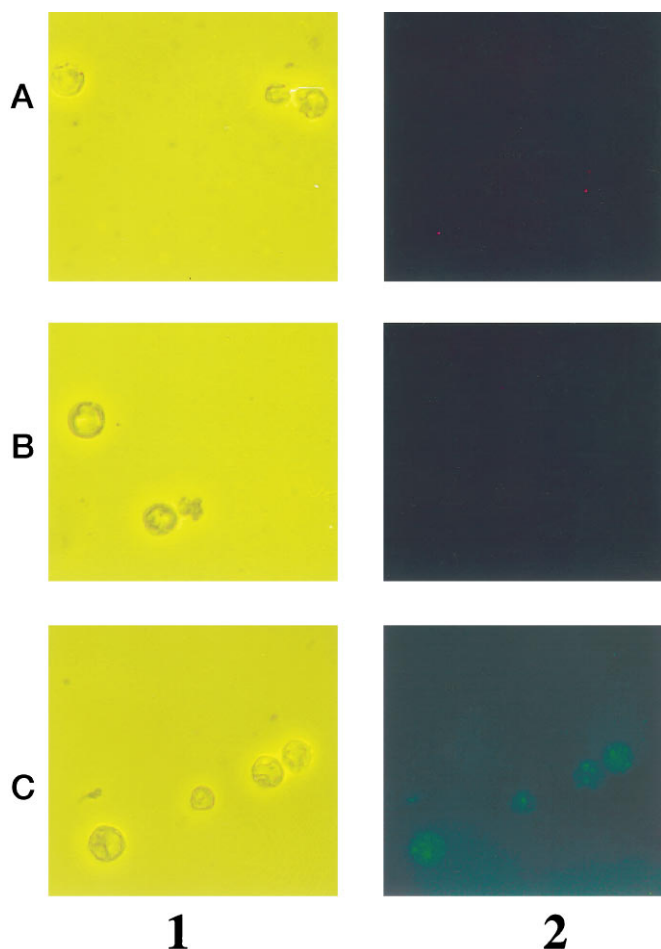


FIG. 5. Transport of fluorescein-labeled oligomeric forms of GNMT into rat liver nuclei. FITC-modified GNMT was separated into tetramer, dimer, and monomer fractions by size exclusion chromatography on Sephacryl S-300, and each fraction was incubated with nuclei for 2 h. A phase-contrast view (1) and a view of the same field in UV light (2) are shown. *Panel A*, nuclei incubated with the tetramer; *panel B*, nuclei incubated with the dimer; *panel C*, nuclei incubated with the monomer.

modified GNMT or rat liver cytosol. No differences in nuclear fluorescence were observed in these experiments, showing that native unmodified protein (which exists as a tetramer) is unable to enter the nuclei and does not prevent the translocation of modified GNMT monomer.

To test the possibility that the fluorescein moiety itself was the factor that targeted FITC-labeled GNMT to the nuclei, we incubated the nuclei with FITC-modified bovine carbonic anhydrase. No fluorescence was observed in the nuclei even after a 20-h incubation (Fig. 4C).

SDS-PAGE followed by Western blotting analysis showed there was no detectable truncation of GNMT upon translocation into the nucleus (Fig. 3, lane 5). We then used a standard procedure (17) involving successive extractions of the nuclei with various buffers to isolate the chromatin. There was no measurable absorbance at 493 nm in the extracts. There was also no fluorescence and no immunochemically detectable GNMT associated with the extracts after SDS-PAGE (data not shown). This indicated that essentially all the fluorescein-labeled protein was associated with the chromatin (Fig. 3, lane 6).

Identification of the FITC-labeled Peptides—To study which lysines might be involved in the reaction with FITC, each of the oligomeric forms of modified GNMT (*i.e.* tetrameric, dimeric, and monomeric) was digested with V_8 protease. Conditions

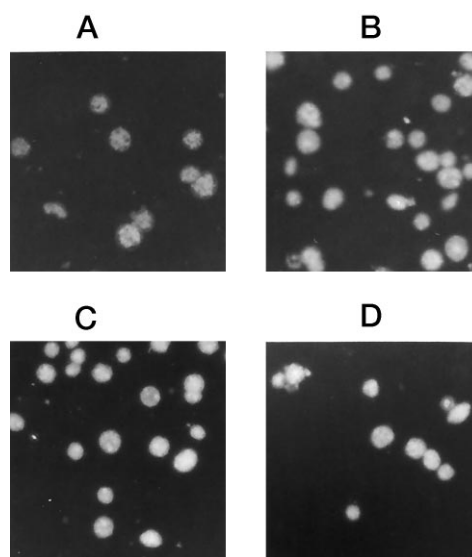


FIG. 6. Fluorescence of rat liver nuclei after different incubation times with FITC-modified GNMT. Rat liver nuclei were incubated with FITC-labeled GNMT for different periods of time. *Panel A*, 5 min; *panel B*, 30 min; *panel C*, 2 h; and *panel D*, 20 h. Only views of the nuclei in UV light are shown. All pictures were taken with the same exposure and developed under the same conditions.

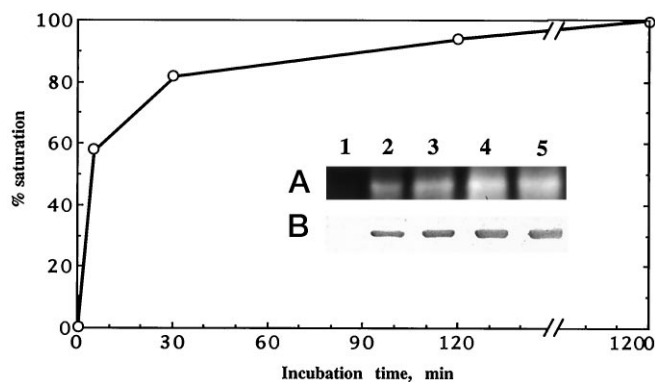


FIG. 7. Time course of FITC-modified GNMT transport into rat liver nuclei. Photographs of the nuclei from Fig. 6 were scanned with the Arcus II scanner (Agfa-Gevaert N.V.) and analyzed using Adobe Photoshop software. Median values of fluorescence intensity histograms were obtained for at least 12 individual nuclei after each incubation time, and the mean value for the 12 medians (differing by not more than 10%) was used as a measure of the amount of fluorescent GNMT transported into the nuclei. The mean value of the medians obtained for the nuclei incubated 20 h was designated as 100% saturation. Mean values for the nuclei at different incubation times were expressed as percentage of the 20 h mean value. *Inset* shows SDS-PAGE in UV light (*panel A*) and Western blot analysis (*panel B*) of nuclei incubated with fluorescein-modified GNMT for 0 min (*lane 1*), 5 min (*lane 2*), 30 min (*lane 3*), 2 h (*lane 4*), and 20 h (*lane 5*). The nuclei were incubated with modified GNMT for different times, sedimented by centrifugation, and washed three times as described under "Experimental Procedures." Aliquots of 50 μ l were applied to SDS-PAGE.

were chosen to provide digestion of polypeptides on the carboxyl sides of both aspartic and glutamic acid residues (18). We purified and analyzed the peptides that bore the fluorescein label (Fig. 8).

Both tetrameric and dimeric forms of GNMT revealed two major labeled peptides, A and B (Fig. 9). The monomeric form of FITC-GNMT, in addition to peptides A and B, yielded four additional peptides containing the fluorescein label (Fig. 9, peptides C–F).

We found during sequencing of these peptides that PTH-(ϵ -PTC)-lysine was produced, although in low yield. This amino acid derivative is usually observed during sequencing of un-

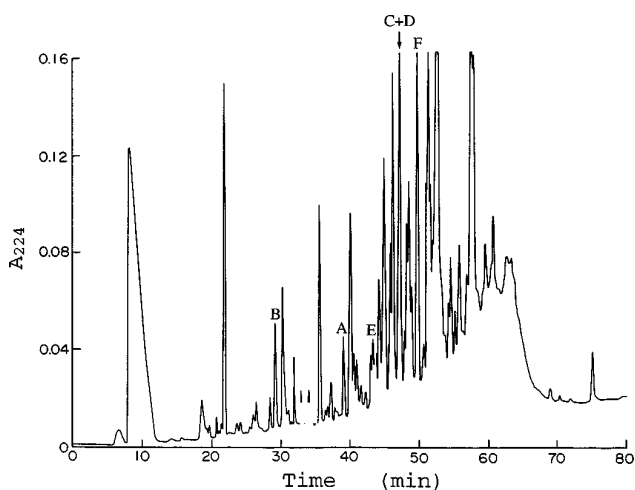


FIG. 8. Reversed-phase HPLC separation of peptides from *S. aureus* V_8 protease digestion of FITC-modified monomeric GNMT. Separation was performed on a Pep-S (C_2/C_{18}) column with a water/acetonitrile/trifluoroacetic acid solvent gradient at a flow rate of 1 ml/min. A linear gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid was applied over 80 min. The effluent was monitored at 224 nm, and peaks were collected manually and concentrated to a volume of 25 μ l. Peaks designated with the letters A through E showed a yellow color upon concentration and were further purified (see “Experimental Procedures” for details) and sequenced. Peaks are designated to correspond to the peptide sequences identified in Fig. 9.

- (A) LysAsp (122-123)
- (B) SerLysGlyAsp (146-149)
- (C) TyrAlaLeuLysGlu (93-97)
- (D) MetLeuLysTyrAlaLeuLysGlu (90-97)
- (E) GlyPheSerValThrSerValAspAlaSerAspLysMetLeu... (78-91)
- (F) TyrLysAlaTrpLeuLeuGlyLeuLeuArgGlnHisGly... (44-56)

FIG. 9. FITC-labeled peptides identified in GNMT. The fluorescein-labeled peptides obtained by proteolysis of FITC-modified GNMT with *S. aureus* V_8 protease were purified by HPLC and sequenced (see “Experimental Procedures” for details). Peptides A and B were obtained from tetrameric, dimeric, and monomeric forms of GNMT. Peptides C–F were purified only from the monomer. Numbers show the position of the peptides in the GNMT sequence.

derivatized peptides. We did not observe any fluorescent product appearing during the lysine cycle. In a separate experiment, we found that treatment of membrane-immobilized fluorescein-labeled GNMT with 100% TFA was able to release the fluorescent label. Therefore, pretreatment of the peptide sample with 100% TFA included in the sequencing protocol could remove the fluorescein label from the ϵ -amino group of lysine and contribute to the formation of the usual PTH-(ϵ -PTC)-lysine derivative. Similar results have been described by others when sequencing a peptide with a fluorescein-modified N-terminal glycine-produced PTH-glycine (19).

Each of the peptide sequences obtained were identified in the amino acid sequence of GNMT (Fig. 9). Peptide F begins with Tyr-44. The previous amino acid is Glu-43, consistent with the Asp, Glu specificity of V_8 protease (18). Although the entire sequence of the peptide could not be determined due to loss of sensitivity with increasing peptide length, the peptide probably ends at Asp-62. Since the only lysine in this sequence is at position 45, it is the one that must be modified. The peptides C, D, and E are parts of an overlapping sequence beginning with Gly-78 and ending with Glu-97 (Fig. 9). Glu is in position 77 of the protein sequence, also consistent with the cleavage specificity of V_8 protease. Since peptide C is complete and contains only one lysine (Lys-96), it is clear that this lysine is modified. Peptide D contains two lysines, Lys-92 and Lys-96, so it is

unclear whether both Lys-92 and Lys-96 are modified or not. The sequence of peptide E is incomplete. It should extend through the site of the next acidic amino acid, Glu-97, so it is difficult to determine which are the derivatized lysines, since it contains Lys-89, Lys-92, and Lys-96. It is apparent, however, that the peptide has been cleaved after Lys-89 and Lys-92, yielding peptides C and D. This may have happened only if both of these lysines have been modified with fluorescein, which altered the specificity of the protease. Thus, it is clear that Lys-45 and Lys-96 are both modified, and it is likely that Lys-89 and Lys-92 are modified also.

DISCUSSION

Several studies have shown (5, 6) that GNMT can be found in liver nuclei, although it is primarily a cytosolic protein and its role in nuclei is unknown. Continuous exchange of macromolecules between nucleoplasm and cytoplasm occurs through nuclear pore complexes. These are large supramolecular structures (molecular mass of about 125×10^6 Da, which contain 100–200 different proteins) accommodating both passive diffusion and active transport (20). Although small proteins (<40 kDa) were believed to be able to enter the nuclei by passive diffusion (21), it has been recently suggested that many nuclear proteins, regardless of their size, may actively enter the nucleus (22). Active transport into the nucleus requires the presence of nuclear localization signals (NLSs), which are both necessary and sufficient for nuclear targeting (23). Changes in the availability or efficiency of the NLSs (*i.e.* by means of phosphorylation and/or binding another protein) were shown to regulate nuclear import of certain transcription factors and protein kinases (24).

We modified GNMT with FITC to directly monitor whether it is translocated into liver nuclei. Indeed, our experiments showed that this does take place. Moreover, the transport of GNMT into nuclei was fast with a $t_{1/2} < 5$ min. It should be noted, however, that this quantitation is not very accurate due to self-quenching of fluorescein at high concentration (25). On the other hand, experiments using detection of GNMT in the nuclei by immunoblotting showed similar results (Fig. 7), consistent with a rapid uptake of the modified monomer. Since treatment with detergents did not reduce the fluorescence of the nuclei, we concluded that the fluorescence reflected accumulation of GNMT inside the nuclei and not binding to their surface. The fact that the fluorescent label remained bound throughout chromatin purification suggested that chromatin was the GNMT target.

We were surprised to find that modification with FITC resulted in dissociation of GNMT from its native tetrameric form to primarily monomers and that this was accompanied by the loss of enzyme activity. This suggested that it was the monomer that was translocated into the nuclei. Indeed, this was found to be the case. Neither modified tetramer nor modified dimer was able to enter the nuclei. Moreover, native unmodified GNMT did not interfere with translocation of the monomer. Fluorescein-labeled bovine carbonic anhydrase, which was modified to a similar extent and had a molecular mass similar to that of the GNMT monomer, was unable to enter the nuclei, indicating that the nuclear transport of the GNMT monomer was not simply the result of conjugation of fluorescein to the protein molecule. This also showed that monomer translocation was characterized by specificity and was not simply due to passive diffusion.

Our results suggest that modification of GNMT with FITC involved amino acid residues that, presumably, participate in the formation of the quaternary structure of the enzyme. Since stable thiourea derivatives of fluorescein are formed only when FITC reacts with primary or secondary amino groups (10) and

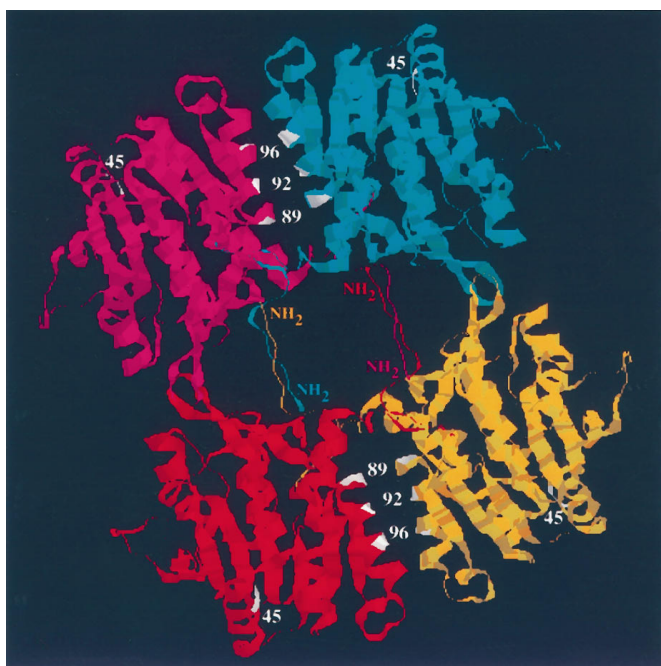


FIG. 10. **Location of the lysine residues essential for GNMT oligomerization in the quaternary structure of the enzyme.** The ribbon diagram of the quaternary structure of GNMT was drawn using the molecular visualization program RasMol Version 2.5 with the molecule coordinate file obtained from the Brookhaven Protein Data Bank. Lysine residues modified only in the monomeric form of GNMT are shown in *white* and numbered 45, 89, 92, and 96 in accord with their position in the polypeptide chain. Lysines 89, 92, and 96 are located in H4 helices of GNMT subunits.

the N termini of GNMT subunits are acetylated (26), amino groups of the lysine side chains must be involved in the interactions between subunits. Experiments showed different modification of tetrameric and monomeric GNMT, the latter having a 3–4 times higher extent of modification. Two theoretical models could explain these differences. In the case of a processive mechanism, the modification of the tetramer is followed by the modification of the monomer. In the case of a distributive mechanism, tetrameric and monomeric GNMT are modified individually at different rates. Purified GNMT, however, exists only in the tetrameric form. The interactions between GNMT subunits are so strong that they do not dissociate to dimers or monomers under normal conditions. We have observed that even in the presence of agents that are usually sufficient to destroy protein-protein interactions (*e.g.* 1 M LiCl, 5% Me₂SO, 1 M LiCl in 5% Me₂SO, 0.5% sodium cholate, 2 or 4 M urea), no dissociation of GNMT tetramer to monomers or dimers could be detected.² These observations support the first model. Apparently, modification of one of the lysine residues essential for subunit interactions may trigger dissociation of the subunits, making other essential lysines more available for further modification and resulting in the higher extent of modification of the monomeric form of GNMT compared with tetramer.

Our conclusion about involvement of lysines in the oligomerization of GNMT protomers is in agreement with the recently resolved crystal structure of GNMT (27). According to the crystallographic data, amino acid residues 87–101 of GNMT subunit form α -helix H4, which together with the H5 helix (residues 104–109), are in close contact with the same helices of the neighboring subunit (Fig. 10). Lys-92 of the first subunit forms a salt bridge with Asp-88 of the second subunit, Asp-88 of the first subunit forms a salt bridge with Lys-92 of the second

subunit, Lys-96 of the first subunit forms a salt bridge with Glu-114 of the second subunit, and Glu-114 of the first subunit, in turn, forms a salt bridge with Lys-96 of the second subunit (27). Modification of these lysines with FITC probably destroys four of the six salt bridges providing close contact of the two subunits, which could be enough for dissociation of the tetramer to monomers. As for Lys-89, it is likely to form hydrogen bonds either with Gly-11 or Asp-19 from the U-shaped N-terminal segment of the neighboring subunit, which corks the active site of the first subunit and apparently is involved in the catalytic reaction (27) (Fig. 10). Its modification also should weaken interaction between the monomers. Apparently, chemical modification of the lysine residues in the H4 helices results in dissociation of GNMT to monomers, whereas the other two lysines available for modification (Lys-122 and Lys-147) are not crucial for maintenance of the tetrameric structure. Since the N terminus of a GNMT subunit is involved in the catalytic center of the neighboring subunit, it seems logical that dissociation of the tetrameric protein results in the loss of enzyme activity. These results are quite consistent with the crystal structure of GNMT.

The fact that only enzymatically inactive GNMT monomers were able to enter the nuclei appears to contradict earlier reports that found GNMT activity in isolated rat liver nuclei (5, 6). In both cases, the amount of the enzyme activity measured was low and could have been due to small amounts of contaminating cytosol. The nonaqueous technique of liver nuclei isolation used by Kerr (5) does not yield sufficiently pure preparations of nuclei (28), whereas the small amount of GNMT activity that was observed using the aqueous technique of nuclei isolation (6) may also have been due to cytosolic contamination. An alternate explanation for the presence of small amounts of GNMT activity associated with the nuclei is that although only GNMT monomers are capable of entering the nuclei, once inside, they may be able to reassociate to form some active tetrameric enzyme. In our experiments, such reassociation was prevented by the irreversible chemical modification of the monomers. We suggest that dissociation of enzymatically active tetrameric GNMT to enzymatically inactive monomeric GNMT exposes elements of the protein structure that include NLSs that are responsible and sufficient for its transport into the nuclei. Although we were unable to find any of the currently established NLS sequences in the primary structure of GNMT, it is possible that there may be an as yet unknown NLS or that a tertiary polypeptide structure may be formed that acts as an NLS.

Transport of the enzymatically inactive monomer of FITC-modified GNMT into the nuclei indicates that the protein may have a different function and oligomeric state in the nucleus. Such a possibility is not unique in the case of GNMT. Several glycolytic enzymes known to be cytoplasmic proteins were recently discovered in nuclei (29). Glyceraldehyde-3-phosphate dehydrogenase was shown to be present in the nucleus and has been characterized as a DNA-binding protein that exhibits the activity of the DNA repair enzyme, uracil DNA glycosylase (30). Another enzyme, phosphoglycerate kinase, was also found in nuclei and was identified as one of the primer recognition proteins of DNA polymerase α in HeLa cells (31). Cytosolic aconitase was shown to have specific RNA binding properties and to participate as an iron regulatory protein in metabolic signaling (32). Moreover, evidence exists that metabolic enzymes need to undergo some cellular modification to be able to enter nuclei and act as DNA-binding proteins (29). Therefore, although the major portion of a protein may have a well established function in the cell, it may also be utilized in smaller amounts for other important functions.

² N. I. Krupenko and C. Wagner, unpublished data.

The present study shows that dissociation of enzymatically active tetrameric GNMT to monomers is accompanied by the loss of enzyme activity and results in a rapid translocation of the monomer into the nucleus where it binds to chromatin. These findings agree with data obtained for the 4 S polycyclic aromatic hydrocarbon receptor that is believed to be identical to GNMT (7). This was shown to be phosphorylated, transported into the nucleus upon binding of its ligand, benzo[a]pyrene (8), and involved in the regulation of cytochrome P450 1A1 gene expression. There is some controversy about this, however, since a more recent study suggests that the 4 S polycyclic aromatic hydrocarbon receptor is not a GNMT subunit (33). Despite such a contradiction, our experiments show directly that monomeric GNMT can penetrate the nucleus and interact with chromatin. Our study leaves open the question about conditions in the cell that could evoke the dissociation of GNMT to monomers. Changes in enzyme conformation resulting in protein dissociation could be induced by modification of amino acid residue(s) and/or binding of a regulatory ligand. Earlier work from our laboratory showed that GNMT can be phosphorylated *in vitro* by the catalytic subunit of cAMP-dependent protein kinase (34). It is also noteworthy that the H4 helix of GNMT involved in tetramer formation contains a tyrosine residue available for phosphorylation. However, whether phosphorylation can alter the oligomeric structure of the protein has not yet been investigated.

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