# Chemical Modification of a Functional Arginine Residue of Rat Liver Glycine Methyltransferase†

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ABSTRACT: Rat liver glycine methyltransferase is inactivated irreversibly by phenylglyoxal in potassium phosphate buffer. The inactivation obeys pseudo-first-order kinetics, and the apparent first-order rate constant for inactivation is linearly related to the reagent concentration. A second-order rate constant of 10.54 ± 0.44 $M^{-1}$ $min^{-1}$ is obtained at pH 8.2 and 25 °C. Amino acid analysis shows that only arginine is modified upon treatment with phenylglyoxal. Sodium acetate, a competitive inhibitor with respect to glycine, affords complete protection in the presence of *S*-adenosylmethionine. Acetate alone has no effect on the rate of inactivation. The value of the dissociation constant for acetate determined from the protection experiment is in good agreement with that obtained by kinetic analysis. Comparison of the amount of [$^{14}$C]phenylglyoxal incorporated into the protein and the number of arginine residues modified in the presence and absence of protecting ligands indicates that modification of one arginine residue per enzyme subunit eliminates the enzyme activity, and this residue is identified as Arg-175 by peptide analysis. The arginine-modified glycine methyltransferase appears to bind *S*-adenosylmethionine as the native enzyme does, as seen from quenching of the protein fluorescence by *S*-adenosylmethionine. These results suggest the requirement of Arg-175 in binding the carboxyl group of the substrate glycine.

Rat liver glycine methyltransferase (EC 2.1.1.20), which catalyzes the *S*-adenosylmethionine (AdoMet)[1]-dependent methylation of glycine to form sarcosine, is a tetrameric protein consisting of identical subunits (Ogawa & Fujioka, 1982a). The enzyme exhibits a sigmoidal rate behavior with respect to AdoMet and hyperbolic kinetics with respect to glycine and is strongly inhibited by the product AdoHcy (Ogawa & Fujioka, 1982a) and by 5-methyltetrahydropteroylpentaglutamate (Wagner et al., 1985). The amount of the enzyme is significantly increased in the liver when rats are fed on a high methionine diet, along with the increase in activities of methionine adenosyltransferase and *S*-adenosylhomocysteinase (Ogawa & Fujioka, 1982b; Matsumoto et al., 1984; Finkelstein & Martin, 1986). These properties of the methyltransferase suggest its importance in the regulation of hepatic levels of AdoMet and AdoHcy as well as of metabolism of sulfur amino acids by the transsulfuration pathway. The complete amino acid sequence of the rat liver enzyme has recently been deduced from the nucleotide sequence of cloned cDNA and the structure of the gene characterized (Ogawa et al., 1987). However, almost nothing is known about which residues in the active site participate in binding and in catalysis. Thus far, the only amino acid residue that is thought to occur at or near the active site is a tyrosine (Fujioka & Ishiguro, 1986), but its functional role remains to be elucidated. In a number of proteins, arginine residues are found to be engaged in the binding of carboxyl or phosphoryl groups of substrates or cofactors (Riordan et al., 1977; Schneider, 1978). As the substrates of glycine methyltransferase contain carboxyl groups, we attempted to explore the involvement of arginine residues in the function of the enzyme by using phenylglyoxal as the arginine modification reagent. In this paper, evidence is presented indicating that glycine methyltransferase with blocked Arg-175 is catalytically inactive.

## Experimental Procedures

*Materials.* Rat liver glycine methyltransferase was purified by the method of Ogawa and Fujioka (1982a) except that 0.5 mM dithiothreitol was included in the buffer for hydroxylapatite chromatography. The dithiothreitol was removed from the final preparation by exhaustive dialysis against 20 mM potassium phosphate buffer, pH 7.2/1 mM EDTA. The enzyme gave a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Molar concentrations of the enzyme were calculated on the basis of a subunit $M_r$ of 32 400 (Ogawa et al., 1987). Protein content was determined by the method of Lowry et al. (1951) with glycine methyltransferase as the standard. *S*-Adenosylhomocysteinase was purified from rat liver as described previously (Fujioka & Takata, 1981). Phenylglyoxal, AdoMet (grade II), AdoHcy, calf intestinal mucosa adenosine deaminase (type VI), and α-chymotrypsin were obtained from Sigma. [7-$^{14}$C]Phenylglyoxal (25 mCi/mmol) was purchased from Amersham. The amino acid calibration mixture was from Wako Pure Chemical Industries, Osaka, Japan. AdoMet was purified by $C_{18}$ reverse-phase chromatography before use as described previously (Fujioka & Ishiguro, 1986). Other chemicals were of the highest purity available from commercial sources and were used without further purification.

*Reaction of Glycine Methyltransferase with Phenylglyoxal.* The reaction of glycine methyltransferase with phenylglyoxal and the assay of enzyme activity were carried out in potassium phosphate buffer, unless otherwise indicated. The modification reaction was started by adding an appropriate amount of phenylglyoxal in water to an enzyme solution in 20 mM potassium phosphate buffer, pH 8.2. The extent of inactivation

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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was monitored by measuring the residual enzyme activity. The enzyme activity was determined by coupling the methyltransferase reaction with *S*-adenosylhomocysteinase and adenosine deaminase (Fujioka & Ishiguro, 1986). An aliquot (5–10 μL) of the reaction mixture was added to 2.0 mL of the assay mixture containing 0.12 mM AdoMet, 2.0 mM glycine, and sufficient amounts of *S*-adenosylhomocysteinase and adenosine deaminase in 20 mM potassium phosphate buffer, pH 7.5, and the decrease in absorbance at 265 nm due to the conversion of adenosine to inosine was followed. The reagent carried over to the assay mixture (≤10 μM) did not interfere with the assay.

*Incorporation of Radioactive Phenylglyoxal into Glycine Methyltransferase.* Preliminary study indicated that the radioactivity incorporated into the protein from [$^{14}$C]phenylglyoxal was stable in 10% trichloroacetic acid. Therefore, radioactivity incorporation as a function of time was determined by the filter paper disk method of Bollum (1968). Glycine methyltransferase was incubated with [7-$^{14}$C]-phenylglyoxal ($1.52 \times 10^3$ cpm/nmol), and a 25-μL aliquot removed from the reaction mixture was placed on a Toyo Roshi No. 51 filter paper disk (2.5 cm in diameter). The disk was immediately immersed into 10% trichloroacetic acid. It was then washed 5 times with 50-mL portions of 10% trichloroacetic acid, twice with 50-mL portions of ethanol, and finally with 50 mL of ethyl ether. After drying, the disk was placed in a scintillation vial and the radioactivity was determined in a toluene scintillation fluid.

*Proteolytic Digestion of [$^{14}$C]Phenylglyoxal-Labeled Glycine Methyltransferase.* Glycine methyltransferase treated with [7-$^{14}$C]phenylglyoxal in 20 mM potassium phosphate buffer, pH 8.2, in the presence or absence of protecting ligands was freed from excess reagent and other components by gel filtration over Sephadex G-25 equilibrated and eluted with 50 mM potassium phosphate buffer, pH 5.8. After dialysis against 10 mM HCl and evaporation of the HCl, the phenylglyoxal-treated enzyme was carboxymethylated with iodoacetate (Gurd, 1967). The carboxymethylated, phenylglyoxal-modified enzyme was dialyzed against 20 mM potassium phosphate buffer, pH 7.2, and water and then lyophilized. The lyophilized sample was suspended in 0.2 M $NH_4HCO_3$ and digested with α-chymotrypsin at a protease to substrate ratio of 1:100 (w/w). After incubation for 4 h at 37 °C, a second addition of the same amount of chymotrypsin was made, and the digestion was continued for an additional 4 h.

*Separation of Peptides by High-Performance Liquid Chromatography.* The lyophilized chymotryptic digest was dissolved in 6 M guanidine hydrochloride, and the peptides were separated by HPLC on a Toyo Soda CCP 8000 liquid chromatograph using a TSK ODS 120T reverse-phase column (0.46 × 25 cm) (Toyo Soda). Elution was carried out by a gradient from 0.05% trifluoroacetic acid to 0.05% trifluoroacetic acid containing 80% acetonitrile at a flow rate of 0.8 mL/min. The effluent was monitored by absorbance at 220 nm and collected in 1.6-mL fractions (2 min). Aliquots from each fraction were determined for radioactivity. Purification of peptides was performed on the same column using a 0–80% acetonitrile gradient in 5 mM ammonium acetate, pH 6.8.

*Amino Acid Analysis.* Glycine methyltransferase was incubated with 2 mM phenylglyoxal in 20 mM potassium phosphate buffer, pH 8.2, at 25 °C for 8 h. The reaction mixture was then acidified with HCl to prevent regeneration of free arginine (Takahashi, 1968) and immediately dialyzed against 0.1 M HCl. After evaporation of the HCl, the residue was hydrolyzed in 5.7 M HCl for 24 h at 110 °C in a sealed evacuated tube. Amino acid analysis was performed in an LKB 4400 amino acid analyzer. The enzyme treated similarly but in the absence of phenylglyoxal served as the control. Amino acid compositions of the isolated peptides were determined by the precolumn derivatization method of Heinrikson and Meredith (1984) with slight modification (Gomi et al., 1986).

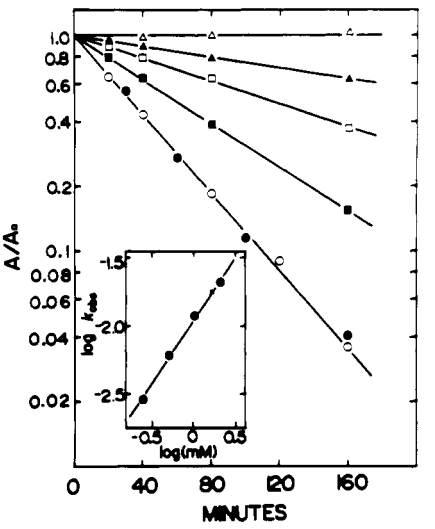


FIGURE 1: Time course of inactivation of glycine methyltransferase by phenylglyoxal. Glycine methyltransferase (0.3 mg/mL) was incubated with 0 (△), 0.25 (▲), 0.5 (□), 1.0 (■), and 2.0 mM (○) phenylglyoxal in 20 mM potassium phosphate buffer, pH 8.2, at 25 °C. At the times indicated, aliquots were removed for measurements of the residual enzyme activity as described under Experimental Procedures. Closed circles indicate the results obtained by incubating the enzyme in 20 mM sodium borate buffer, pH 8.2, and assaying the enzyme activity in 20 mM potassium phosphate/20 mM sodium borate buffer, pH 7.5.

*Sequence Analysis.* Amino acid sequence determination of the isolated peptides was carried out by automated Edman degradation on an Applied Biosystems 470A gas-phase sequencer equipped with a 120A high-performance liquid chromatograph system.

*Determination of Total Sulfhydryl Groups.* Glycine methyltransferase was inactivated by phenylglyoxal to <5% of the initial activity. After exhaustive dialysis against 0.1 M Tris-HCl buffer, pH 8.0, sulfhydryl content for the modified enzyme was determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of 0.2% sodium dodecyl sulfate. The number of sulfhydryl groups was calculated with a value of 13700 $M^{-1}$ $cm^{-1}$ as the molar absorptivity of the thionitrobenzoate anion (Riddles et al., 1983).

*Other Analytical Procedures.* Absorbance measurements were made with a Hitachi 320 recording spectrophotometer and fluorescence measurements with a Farrand spectrofluorometer, MK-2. Circular dichroism spectra were recorded with a JASCO spectropolarimeter, J-500A. Radioactivity was determined in an Aloka liquid scintillation spectrometer, LSC 903.

## RESULTS

*Inactivation of Glycine Methyltransferase by Phenylglyoxal.* When glycine methyltransferase was incubated with phenylglyoxal in 20 mM sodium borate buffer, pH 8.2, and aliquots (10 μL) periodically removed from the reaction mixture were assayed for enzyme activity in 2.0 mL of an assay mixture containing 20 mM borate, a time-dependent loss of enzyme activity was observed. The inactivation followed pseudo-first-order kinetics as shown in Figure 1 (closed circles). In contrast to a linear assay in the presence of borate, the

enzyme activity gradually increased during the assay period when borate was omitted from the assay mixture, suggesting that the phenylglyoxal inactivation was reversible. The reversibility of inactivation was demonstrated by the following experiment. Glycine methyltransferase was inactivated to 40% residual activity by incubation with 2 mM phenylglyoxal in 20 mM borate buffer, pH 8.2, and the reaction mixture was subjected to gel filtration over Sephadex G-25 in 20 mM potassium phosphate buffer, pH 7.5, to remove excess phenylglyoxal and borate. A recovery of enzyme activity to about 80% of the original activity was observed within 30 min at 25 °C. This value did not increase further over a period of 12 h.

Treatment of glycine methyltransferase with phenylglyoxal in potassium phosphate buffer, on the other hand, resulted in an irreversible inactivation. The assay was linear with respect to time in the presence and absence of borate, and furthermore, no recovery of enzyme activity was observed after removal of phenylglyoxal by gel filtration. The rate of inactivation was about the same whether the reaction with phenylglyoxal was conducted in borate or phosphate buffer (open circles, Figure 1). In all subsequent experiments, phosphate buffer was used to avoid the reversal of inactivation.

The phenylglyoxal inactivation of the methyltransferase in phosphate buffer obeyed pseudo-first-order kinetics (Figure 1), indicating that the inactivation occurred in an all-or-none fashion. A plot of log (apparent first-order rate constant) vs log [phenylglyoxal] (Figure 1, inset) yielded a straight line with a slope of 1.05. The second-order rate constant for inactivation was calculated to be 10.54 ± 0.44 $M^{-1}$ $min^{-1}$ from the same data.

The rate of inactivation was dependent on pH. The apparent first-order rate constant for inactivation increased progressively with increasing pH up to pH 10, the highest pH studied (data not shown). Because of the instability of glycine methyltransferase above pH 10.5, experiments at higher pH were not feasible and it was not possible to determine the p$K$ value of the residue responsible for inactivation.

*Effect of Ligands on Inactivation.* It has been shown that rat liver glycine methyltransferase exhibits stringent substrate specificity. Glycine is the only compound so far known to act as the methyl acceptor substrate. The following compounds are reported to be inert as substrates: common L amino acids, L-homoserine, L-ornithine, glycylglycine, glycine ethyl ester, glycinamide, and ethanolamine. The product AdoHcy strongly inhibits the reaction, but *S*-adenosyl-L-cysteine and adenosine are not inhibitors (Ogawa & Fujioka, 1982a). In order to obtain further information about the features of the glycine-binding site, several additional compounds were tested for their ability to act as a competitive inhibitor with respect to glycine. Acetate was found to be a good competitive inhibitor with a $K_i$ value of 0.20 ± 0.01 mM at pH 8.2 and 25 °C (in the presence of 0.1 mM AdoMet). The $K_m$ value for glycine determined under the same conditions was 0.21 ± 0.01 mM. A much weaker inhibition was obtained with glycolate ($K_i$ = 1.25 mM). Propionate, β-alanine, L-alanine, D-alanine, and dimethylglycine were totally ineffective at 2 mM concentrations. D-Alanine and β-alanine also did not serve as substrates.

Table I lists the effect of added ligands on the rate of inactivation of glycine methyltransferase by phenylglyoxal. In the presence of a nearly saturating concentration of AdoMet (0.1 mM), there was a 2-fold increase in the rate of inactivation. AdoHcy had no significant effect. When added singly, glycine and sodium acetate were virtually without effect, but they produced protective effects in the presence of AdoHcy

Table I: Effect of Ligands on Inactivation[a]

| compound added | $k_{obsd} \times 10^3$ ($min^{-1}$) |
|---|---|
| none | 15.9 |
| AdoMet (0.1 mM) | 31.2 |
| AdoHcy (0.2 mM) | 16.9 |
| sodium acetate (5.0 mM) | 15.8 |
| glycine (10.0 mM) | 15.1 |
| AdoMet (0.1 mM) + sodium acetate (5.0 mM) | 1.3 |
| AdoHcy (0.1 mM) + glycine (10.0 mM) | 11.2 |

[a] Glycine methyltransferase (0.3 mg/mL) was incubated with 1.5 mM phenylglyoxal in the presence of compounds at the concentrations indicated. The rate constants were determined from a semilogarithmic plot of residual activity vs time.

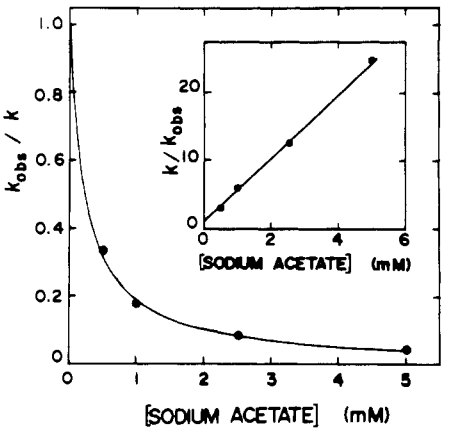


FIGURE 2: Effect of AdoMet plus acetate on the pseudo-first-order rate constant for inactivation. Glycine methyltransferase (0.3 mg/mL) was incubated with 1.6 mM phenylglyoxal in the presence of sodium acetate at the concentrations indicated. All incubation mixtures contained 0.1 mM AdoMet in addition. The solid curve is calculated from eq 1.

or AdoMet. At high concentrations of acetate (>10 mM) and in the presence of 0.1 mM AdoMet, no measurable inactivation was observed over a period of 80 min, at which time the control lacking acetate lost more than 90% of activity. To demonstrate that the protection afforded by acetate is caused by its specific interaction at the glycine-binding site, we performed a titration experiment. Figure 2 shows a plot of apparent first-order rate constants for inactivation obtained at different concentrations of acetate and a constant concentration of AdoMet (0.1 mM) against acetate concentrations. The data can be fitted to eq 1 (where $k_{obsd}$ and $k$ are apparent first-order rate constants

$$k_{obsd} = k/(1 + [\text{acetate}]/K_d) \quad (1)$$

for inactivation in the presence and absence of acetate, respectively, and $K_d$ is its dissociation constant), which is derived under the assumption that the enzyme bound with AdoMet and acetate is incapable of reacting with phenylglyoxal to produce inactivated enzyme. A value of 0.23 ± 0.01 mM was found for $K_d$, in excellent agreement with that obtained by kinetic analysis under the same conditions (see above).

*Amino Acid Residue Modified by Phenylglyoxal.* Phenylglyoxal reacts most rapidly with the guanido group of arginine in proteins and peptides, and the reaction can be followed as a loss of arginine in amino acid analysis after acid hydrolysis. Among other residues, only the sulfhydryl group and α- and ε-amino groups are known to react with the reagent (Takahashi, 1968, 1977). The reaction of phenylglyoxal with the amino group would lead to deamination (Takahashi, 1968). The nature of the reaction with the cysteine sulfhydryl group is not well characterized. Amino acid analysis of a completely

Table II: Amino Acid Composition of Native and Phenylglyoxal-Treated Glycine Methyltransferases[a]

| amino acid | mol/mol of enzyme monomer: native enzyme | mol/mol of enzyme monomer: phenylglyoxal-treated enzyme |
|---|---|---|
| aspartic acid[b] | 29.2 | 28.6 |
| threonine | 13.3 | 12.8 |
| serine | 14.8 | 14.2 |
| glutamic acid[b] | 22.7 | 21.5 |
| proline | 15.3 | 15.1 |
| glycine | 27.4 | 26.6 |
| alanine | 24.0 | 24.0 |
| valine | 23.1 | 22.3 |
| methionine | 4.0 | 4.2 |
| isoleucine | 9.8 | 9.6 |
| leucine | 27.5 | 27.1 |
| tyrosine | 14.2 | 13.7 |
| phenylalanine | 10.0 | 9.3 |
| histidine | 9.9 | 8.9 |
| lysine | 17.2 | 16.8 |
| arginine | 17.0 | 11.6 |

[a] Samples for amino acid analysis were prepared as described under Experimental Procedures. The numbers of residues are calculated on the basis of 24 alanine residues per enzyme monomer. [b] These values include both free and amidated residues.

inactivated enzyme (prepared by incubation with 2 mM phenylglyoxal in 20 mM potassium phosphate buffer, pH 8.2, for 8 h) showed that an average of 5.4 out of a total of 17 arginines per subunit were lost (nine analyses from three experiments). Arginine was the only type of amino acid lost by the treatment, and no significant change was noted in the contents of other amino acids including lysine (Table II). A separate experiment showed that the number of sulfhydryl groups also was not altered by phenylglyoxal treatment. Values of 5.85 and 5.97 sulfhydryl groups per subunit were obtained for the native and inactivated enzymes, respectively, by reaction with 5,5′-dithiobis(2-nitrobenzoate) under denaturing conditions (see Experimental Procedures). The $NH_2$-terminal valine of rat liver glycine methyltransferase is acetylated (Ogawa et al., 1987), and therefore, reaction at this site is not possible.

Upon incubation of glycine methyltransferase with [7-$^{14}$C]phenylglyoxal, incorporation of radioactivity into the protein was observed. When the labeled enzyme isolated by gel filtration (in 20 mM potassium phosphate buffer, pH 7.5) was immediately treated with 10% trichloroacetic acid, the bound radioactivity was not released, indicating that the reagent was covalently attached to the protein. Prolonged treatment with trichloroacetic acid (5 h) did not diminish the amount of radioactivity fixed. Thus, the bound radioactivity is acid stable, and we used in the following experiments the filter paper disk method of Bollum (1968), which uses trichloroacetic acid, to remove the unreacted reagent (see Experimental Procedures).

Table III shows the relationship between the loss of enzyme activity and the number of [$^{14}$C]phenylglyoxal molecules incorporated in the presence and absence of 5 mM sodium acetate. (Both reaction mixtures contain 0.1 mM AdoMet.) In the presence of acetate, no significant loss of enzyme activity was observed, and the difference in moles of the reagent incorporated per mole of subunit under two sets of conditions was close to the fraction of enzyme activity lost, as shown in the last column of Table III.

To correlate the number of phenylglyoxal molecules incorporated with that of arginine residues lost, the two samples from 160-min incubation (last line, Table III) were analyzed for their amino acid compositions. A difference of 0.8 arginine per subunit (14.3 vs 15.1 residues) was obtained. Thus, the inactivation of glycine methyltransferase by phenylglyoxal is due to the modification of 1 arginine per subunit and this arginine reacts with the reagent in a 1:1 stoichiometry.

While the bound radiolabel is acid stable, some of the radioactivity was released at pH 7.5. Glycine methyltransferase with 35% residual activity and 1.2 mol of [$^{14}$C]phenylglyoxal incorporated/mol of subunit was isolated by gel filtration and incubated in potassium phosphate buffer, pH 7.5 at 25 °C. At time intervals aliquots were determined for bound radioactivity by the filter paper disk method. Radioactivity equivalent to about 0.5 mol of [$^{14}$C]phenylglyoxal/mol of subunit was lost in about 1 h, after which time no further loss of radioactivity was observed. No increase in enzyme activity was observed in this experiment, indicating that the radiolabel bound to the arginine residue whose modification leads to loss of enzyme activity is stable even at slightly alkaline pH.

*Identification of Differentially Labeled Arginine Residue.* Glycine methyltransferase was incubated with 1.6 mM [$^{14}$C]phenylglyoxal in potassium phosphate buffer, pH 8.2, containing 0.1 mM AdoMet for 60 min in the presence and absence of 10 mM sodium acetate. In the absence of acetate, the enzyme lost 85% of its activity while greater than 95% of activity was retained in the enzyme treated in the presence of acetate. After carboxymethylation and chymotrypsin treatment as described under Experimental Procedures, the two enzyme samples showed a difference in radioactivity corresponding to 0.7 mol of [$^{14}$C]phenylglyoxal/mol of subunit. (The molar concentration of subunit was determined by amino acid analysis of the digest.) Figure 3 shows the HPLC peptide mapping analyses of chymotryptic peptides derived from the two incubations. As seen in the figure, the two samples exhibit a similar radioactivity profile except that a larger amount of radioactivity is contained in the fraction collected between 32 and 34 min (fraction 17) in the unprotected sample. Greater than 90% of the applied radioactivity was recovered in the effluent in both cases.

Examination of the elution profiles of peptides revealed that a peak appearing at 33.4 min in the enzyme modified in the

Table III: Incorporation of [7-$^{14}$C]Phenylglyoxal in the Absence and Presence of Acetate[a]

| incubation time (min) | AdoMet: change in enzyme act. (%) | AdoMet: no. of phenylglyoxal incorpd (mol/mol of subunit) | AdoMet + acetate: change in enzyme act. (%) | AdoMet + acetate: no. of phenylglyoxal incorpd (mol/mol of subunit) | Δ incorpn |
|---|---|---|---|---|---|
| 20 | 31 | 0.69 | 0 | 0.41 | 0.28 |
| 40 | 51 | 1.21 | 2 | 0.75 | 0.46 |
| 60 | 62 | 1.73 | 3 | 1.08 | 0.65 |
| 100 | 79 | 2.59 | 5 | 1.80 | 0.79 |
| 160 | 92 | 3.47 | 7 | 2.57 | 0.90 |

[a] Glycine methyltransferase (0.3 mg/mL) was incubated with 0.8 mM [7-$^{14}$C]phenylglyoxal ($1.52 \times 10^3$ cpm/nmol) in 20 mM potassium phosphate buffer, pH 8.2, containing 0.1 mM AdoMet in the absence and presence of 5.0 mM sodium acetate. The radioactivity incorporation was determined as described under Experimental Procedures.

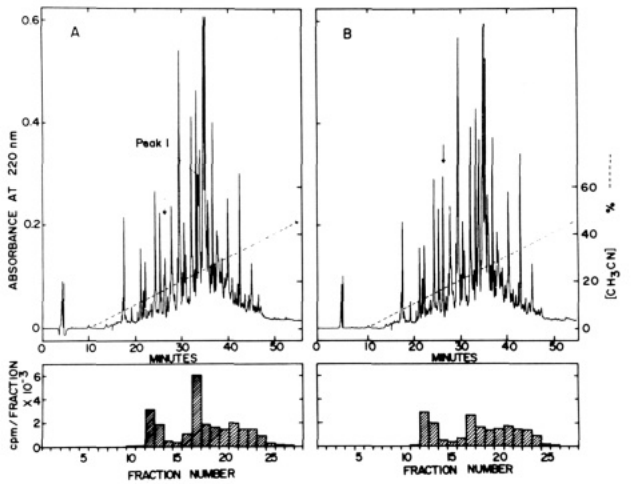


FIGURE 3: HPLC peptide mapping of chymotryptic peptides derived from glycine methyltransferase modified by [$^{14}$C]phenylglyoxal in the absence (A) or presence (B) of acetate. Glycine methyltransferase (40 $\mu$M subunit) was incubated with 1.6 mM [7-$^{14}$C]phenylglyoxal ($3.36 \times 10^3$ cpm/nmol) in 20 mM potassium phosphate buffer, pH 8.2, containing 0.1 mM AdoMet in the absence or presence of 10 mM sodium acetate for 60 min. Preparation of the chymotryptic digest and chromatographic conditions are described under Experimental Procedures. Chymotryptic peptides from each sample (2.5 nmol) were separated by chromatography on a TSK ODS 120T column using an acetonitrile gradient in 0.05% trifluoroacetic acid.

absence of acetate (peak I, Figure 3A) was not present in the enzyme modified under acetate protection. To examine whether the difference in radioactivity contained in fraction 17 is due to the presence of peak I, fraction 17 of Figure 3A was rechromatographed under the same conditions, and radioactivity associated with each peak was determined. About 60% of radioactivity was found in peak I, and the remainder was distributed in two peaks that were eluted before peak I (data not shown). Similar analysis on fraction 17 of Figure 3B showed that about the same amounts of radioactivity were associated with the corresponding two peaks. Thus, the difference in radioactivity in fraction 17 from the two samples can be accounted for by the presence of radioactive peak I.

On rechromatography in a different solvent system peak I was resolved into two major peaks that were eluted closely (peaks IA and IB) and one minor peak (Figure 4). Peptides IA and IB were radioactive. Amino acid analysis of peptides IA and IB showed that they had the same amino acid composition ($Asp_2$, Val, Ile, His, Tyr). Automated Edman degradation of peptides IA and IB revealed that the amino acid sequences were also identical except for the sixth residues, which was Asn in peptide IA and Asp in peptide IB. This suggests that peptide IB arises from peptide IA through deamidation during isolation and purification. In cycle 5 of Edman degradation, the product did not correspond to any of standard phenylthiohydantoin amino acids, and the majority of radioactivity (79 and 70% of the sum of radioactivity released at each cycle) was associated with this step. The sequence uniquely fits the segment $^{171}$Val-Ile-Asp-His-Arg-Asn-Tyr$^{177}$ in the primary structure of rat liver glycine methyltransferase (Ogawa et al., 1987). Thus, it is reasonable to conclude that residue 5 of the isolated peptides is modified Arg-175. In the unprotected sample (Figure 3A), a peak around 26 min (fraction 14) appears to be smaller than that in the acetate-protected sample (Figure 3B) (shown by arrows in the figure). Rechromatography of fraction 14 of Figure 3B showed that it contained five peptides (data not shown). One of the peptides, which was isolated in an overall yield of 58%, had an amino acid composition $Asp_2$, Val, Ile, His, Tyr,

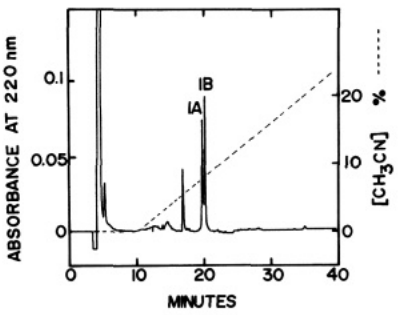


FIGURE 4: Rechromatography of peak I. Peak I of Figure 3 was rechromatographed on a TSK ODS 120T column with an acetonitrile gradient in 5 mM ammonium acetate, pH 6.8.

Arg. The corresponding peptide was found only in a negligible amount in the rechromatogram of fraction 14 of Figure 3A. Therefore, this peptide is considered to be the peptide from residue 171–177 with unmodified Arg-175.

In order to confirm that modification of Arg-175 is protected by acetate, we performed the following experiment. Glycine methyltransferase was incubated with unlabeled phenylglyoxal in the presence and absence of acetate as described above. When the enzyme incubated in the absence of acetate was inactivated to 15% residual activity, both reaction mixtures were subjected to gel filtration over Sephadex G-25 to remove excess phenylglyoxal and acetate. The resulting enzymes were then treated with [$^{14}$C]phenylglyoxal in the absence of acetate for a time period sufficient to destroy more than 90% of the activity associated with the acetate-protected sample. HPLC of chymotryptic peptides from the two samples each showed an elution profile (monitored by absorbance at 220 nm) very similar to that shown in Figure 3A. As expected, a much larger amount of radioactivity was present in the fraction containing peak I (corresponding to fraction 17 of Figure 3) of the acetate-protected sample. The distribution of radioactivity in other fractions was comparable for both samples (data not shown).

*Properties of Phenylglyoxal-Modified Glycine Methyltransferase.* Upon excitation at 280–310 nm, the native glycine methyltransferase exhibited a fluorescence with an emission maximum at 330 nm, characteristic of a tryptophan-containing protein. Addition of AdoMet caused a concentration-dependent decrease in the fluorescence intensity without affecting the shape of spectrum. The maximum quenching was found to be 51%. As observed in steady-state kinetics (Ogawa & Fujioka, 1982a), a plot of changes in fluorescence ($\Delta F$) vs AdoMet concentrations showed a sigmoidal curve (Figure 5). From a Hill plot of the same data, an $S_{0.5}$ value of 50.1 $\mu$M and a maximum Hill coefficient of 2.33 were obtained. The former value is only slightly larger than the limiting value obtained kinetically (Ogawa & Fujioka, 1982a). Glycine had no effect on the protein fluorescence.

The fluorescence emission maximum as well as the intensity was not changed by the phenylglyoxal treatment. Furthermore, titration of the modified enzyme with AdoMet showed the same fluorescence quenching as with the native enzyme (Figure 5). Thus, the modification of arginines exerts no influence on the environment of the fluorescing tryptophan residue(s), and the modified enzyme appears to normally bind the substrate AdoMet.

The phenylglyoxal modification did not result in change in the quaternary structure of the enzyme as evidenced by identical behavior of the native and modified enzyme on a gel filtration column (TSK gel 3000SW, Toyo Soda). Comparison of the circular dichroism spectra between 190 and 280 nm of the native and the phenylglyoxal-treated enzymes also revealed

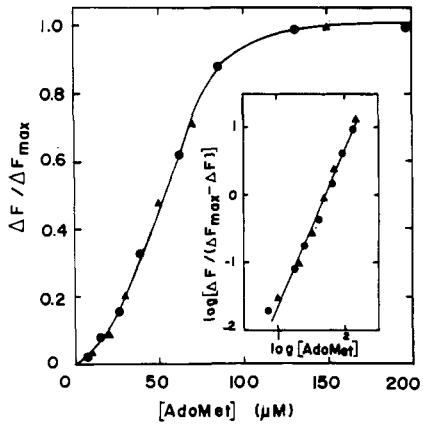


FIGURE 5: Fluorometric titration of the native and phenylglyoxal-modified glycine methyltransferase AdoMet. The native (●) and phenylglyoxal-modified glycine methyltransferases (▲) were titrated with AdoMet in 50 mM potassium phosphate buffer, pH 7.5/1 mM EDTA, at 25 °C, at the protein concentration of 0.1 mg/mL. An excitation wavelength of 310 nm was used to avoid inner filter effect. The inset shows Hill plots. The modified enzyme (residual activity <5%) was prepared by incubating glycine methyltransferase with 1.5 mM phenylglyoxal, followed by dialysis against 50 mM potassium phosphate, pH 7.5/1 mM EDTA.

no significant difference (not shown).

## Discussion

Rat liver glycine methyltransferase is inactivated by the arginine-selective reagent phenylglyoxal. In addition to arginine residue, phenylglyoxal potentially reacts with sulfhydryl and $\alpha$- and $\epsilon$-amino groups in proteins. Obviously the phenylglyoxal inactivation of glycine methyltransferase is not due to the modification of the sulfhydryl group or $\alpha$-amino group. Reaction of phenylglyoxal with amino group would involve Schiff base formation and transamination, the latter transformation being irreversible (Takahashi, 1968). Since the reagent is not covalently incorporated into the protein in this modification reaction and lysine residues occur in abundance in glycine methyltransferase (17 residues per subunit), it appears difficult to exclude the possibility that the inactivation is caused by modification of a lysine rather than arginine. However, repeated amino acid analyses show that the content of lysine does not change even after prolonged incubation (8 h) (Table II). Glycine methyltransferase is inactivated by phenylglyoxal in borate buffer at the same rate as in phosphate buffer (Figure 1). However, when the enzyme inactivated in borate buffer is freed from borate by gel filtration, a partial but rapid restoration of enzyme activity is observed. Reaction of guanido group with a dicarbonyl reagent would form a reversible *cis*-diol adduct, which could be stabilized by complex formation with borate. Dissociation of borate from the complex might regenerate the reactants or might lead to rearrangement of the adduct to a more stable product (Werber et al., 1975). Thus, the reversibility of inactivation in borate buffer also argues against the idea that modification of a lysine residue is responsible for the inactivation. In phosphate buffer, phenylglyoxal leads to an irreversible loss of enzyme activity. Probably the *cis*-diol adduct in this case is rapidly converted to a stable rearrangement product in the absence of borate.

Since AdoMet is known to bind to the free enzyme (Figure 5 and Fujioka & Ishiguro, 1986), the rate enhancement of phenylglyoxal inactivation in the presence of AdoMet suggests that the binding of AdoMet induces a conformational change of the enzyme so as to make the pertinent arginine more susceptible to modification. The finding that the inactivation is protected by acetate, a competitive inhibitor with respect to glycine, only in the presence of AdoMet also suggests that the conformational change caused by the binding of AdoMet generates the binding site for glycine. The value of the dissociation constant for acetate determined from the protection experiment is in close agreement with that obtained kinetically. This indicates that the complete protection is achieved by its specific interaction at the glycine site. In view of the propensity of the guanidium group to form a complex with a carboxyl group (Adams & Small, 1974) and since acetate has no functional group other than carboxyl, it is highly likely that the modifiable arginine residue is involved in binding the glycine carboxyl group, although the possibility of inactivation through steric blockage or conformational change cannot be excluded. The lack of reaction of phenylglyoxal at the AdoMet/AdoHcy site is supported by the fact that the inactivated enzyme normally binds AdoMet (Figure 5).

Comparison of the amount of phenylglyoxal incorporated into the protein in the absence of ligands and that obtained under the condition of maximum protection shows that incorporation of 1 mol of phenylglyoxal/mol of subunit is sufficient to eliminate enzyme activity (Table III). This value agrees with the difference in the number of arginine residues lost per subunit as determined by amino acid analysis. Thus, a stoichiometry of one phenylglyoxal to one arginine residue is obtained for this residue modified in phosphate buffer. Although the stoichiometry of 2 mol of phenylglyoxal/mol of arginine is found in model systems (Takahashi, 1968) and in some proteins (Schloss et al., 1978; Daemen & Riordan, 1974; Lange et al., 1974; Takata & Fujioka, 1983), there are several examples in which 1:1 stoichiometry is found even in nonborate buffers (Borders & Riordan, 1975; Berghauser, 1977; Philips et al., 1979; Koland et al., 1982). It might be conceivable that the condensation of a second molecule of phenylglyoxal to the *cis*-diol adduct is prevented by very rapid rearrangement of the adduct or by steric reason. The bis-(phenylglyoxal)-arginine complex is reported to be unstable at neutral or alkaline pH and decomposes slowly to regenerate the original arginine (Takahashi, 1968). This would contradict the irreversibility of inactivation of glycine methyltransferase in phosphate buffer. The reaction order of 1.05 found for the inactivation (Figure 1, inset) is also consistent with the observed stoichiometry.

That the inactivation of glycine methyltransferase by phenylglyoxal is caused by the modification of a single specific arginine residue is demonstrated by analysis of chymotryptic peptides. The stability of the reagent attached to this arginine when the enzyme is modified in phosphate buffer allowed us to identify the residue readily. The difference in radioactivity in the enzymes modified by [$^{14}$C]phenylglyoxal in the absence and presence of protecting ligands can be accounted for by the presence of radiolabel in a single residue, Arg-175. Although a few arginine residues other than Arg-175 are modifiable by phenylglyoxal, the reaction at these sites apparently does not affect the enzyme activity as seen in the protection experiment (Table III) and by the kinetics of inactivation (Figure 1). If a partial inactivation occurs, the semilogarthmic plot should not be linear (Ray & Koshland, 1961).

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