Function and Reactivity of Sulfhydryl Groups of Rat Liver Glycine Methyltransferase†

Motoji Fujioka,* Yoshimi Takata, Kiyoshi Konishi, and Hirofumi Ogawa
Department of Biochemistry, Toyama Medical and Pharmaceutical University Faculty of Medicine, Sugitani, Toyama 930-01, Japan
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ABSTRACT: Rat liver glycine methyltransferase is completely inactivated by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Treatment of the inactivated enzyme with KCN results in a reactivated enzyme having values of V_{max} and $S_{0.5}$ for *S*-adenosyl-L-methionine comparable to those of the native enzyme and about a 4-fold greater K_m value for glycine. Kinetics of inactivation and reactivation show that one cysteine residue is involved in this process. Reaction of the methyltransferase with iodoacetate leads to partial inactivation of the enzyme; about 22% of the initial activity is retained in the modified enzyme. The relationship between the loss of enzyme activity and the number of iodoacetate molecules incorporated and the sequence analysis of peptides containing the modified residues indicate that carboxymethylation of Cys-282 is responsible for loss of activity. The observations that the activity of the cyanylated glycine methyltransferase shows no decrease upon incubation with iodoacetate and, conversely, the residual activity associated with the iodoacetate-modified enzyme is not abolished by DTNB suggest that Cys-282 is also involved in the inactivation by DTNB. Besides this residue, Cys-185, Cys-246, and Cys-262 are modified upon prolonged incubation with iodoacetate. 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine (FSBA) inactivates glycine methyltransferase by forming 1 disulfide/subunit [Fujioka, M., & Ishiguro, Y. (1986) *J. Biol. Chem. 261*, 6346–6351]. Despite this stoichiometry, treatment of the FSBA-inactivated enzyme with unlabeled iodoacetate and then with iodo[^{14}C]acetate after reduction with 2-mercaptoethanol and subsequent peptide analysis show that the incorporated radioactivity is distributed equally among Cys-185, Cys-246, Cys-262, and Cys-282. It is suggested that FSBA reacts with two of these residues in a mutually exclusive manner, leading in each case to formation of a disulfide. Although these residues are not essential for catalytic activity of the enzyme, the disulfide formation leads to complete loss of activity.

Glycine methyltransferase (EC 2.1.1.20) catalyzes the *S*-adenosyl-L-methionine (AdoMet)[1] dependent methylation of glycine to form sarcosine. The enzyme from rat liver is a simple protein consisting of four identical subunits (Ogawa & Fujioka, 1982), the amino acid sequence of which has been deduced from the nucleotide sequence of a cDNA clone (H. Ogawa, K. Konishi, Y. Takata, H. Nakashima, and M. Fujioka, unpublished results). In spite of its simple structure, the enzyme shows rather complex catalytic properties. A sigmoidal rate behavior with respect to AdoMet and hyperbolic kinetics with respect to glycine are observed (Ogawa & Fujioka, 1982). It is also reported that the enzyme activity is strongly inhibited by 5-methyltetrahydropteroylpentaglutamate, a compound structurally not related to the substrates (Wagner et al., 1985).

A previous report from this laboratory has shown that rat liver glycine methyltransferase is inactivated by FSBA (Fujioka & Ishiguro, 1986). The inactivation involves two types of reactions: the sulfonylbenzoyladenosylation of a tyrosine residue and the loss of SH groups. The kinetics of inactivation are consistent with the contention that the two reactions proceed independent of each other and either type of reaction is sufficient to eliminate all enzyme activity. The loss of SH groups is not accompanied by covalent attachment of the reagent to the enzyme, and a stoichiometry of two SH groups lost per mole of enzyme subunit is obtained for complete inactivation. While these observations suggest that the inactivation is due to the formation of a disulfide between two cysteine residues that occur in close proximity, it is not known whether the disulfide formation is between two specific residues residing on each identical subunit or not. The present investigation was undertaken to answer this question and further to gain an insight into the role of these and other SH groups in the catalytic activity of glycine methyltransferase. We report here the results obtained by using DTNB, iodoacetate, and FSBA as the cysteine modification reagents.

Experimental Procedures

Materials. Rat liver glycine methyltransferase was purified by the procedure of Ogawa and Fujioka (1982). The enzyme gave a single band on electrophoresis on sodium dodecyl sulfate–polyacrylamide gels. Molar concentrations of the enzyme were calculated on the basis of a subunit M_r of 32 400. 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).

AdoMet (grade II) obtained from Sigma was purified by reverse-phase chromatography on a C_{18} cartridge (Sep-Pak, Waters Associates) as described previously (Fujioka & Ishiguro, 1986). Iodoacetic acid (Merck) was recrystallized from hot chloroform. Iodo[2-^{14}C]acetic acid (55 mCi/mmol) was purchased from Amersham. FSBA hydrochloride, α-

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* Address correspondence to this author.

[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 2-nitro-5-mercaptobenzoic acid; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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chymotrypsin, and adenosine deaminase (type VI) were supplied by Sigma, and L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin was from Worthington. The amino acid calibration mixture, standard PTH-amino acids, and phenyl isothiocyanate were from Wako Pure Chemical Industries, Osaka, Japan. Other chemicals were of the highest purity available from commercial sources.

Assay of Glycine Methyltransferase Activity. The glycine methyltransferase activity was determined spectrophotometrically by a coupled assay with *S*-adenosylhomocysteinase and adenosine deaminase (Fujioka & Ishiguro, 1986). The assay was performed at 25 °C in a reaction mixture containing 0.12 mM AdoMet, 2.5 mM glycine, and sufficient amounts of *S*-adenosylhomocysteinase and adenosine deaminase in 2.0 mL of 20 mM potassium phosphate, pH 7.5. The decrease in absorbance at 265 nm due to the conversion of adenosine to inosine was continuously followed in a Hitachi Model 320 spectrophotometer by using expanded scales.

Reaction of Glycine Methyltransferase with DTNB. All solutions of DTNB in appropriate buffers were made fresh and used within 10 min of dissolution. Glycine methyltransferase was incubated with DTNB in 0.1 M Tris-HCl, pH 8.0, containing 0.4 M ammonium sulfate and 1 mM EDTA. The reaction was followed spectrophotometrically at 412 nm. A value of 14 150 was used as the molar extinction coefficient for the TNB anion (Riddles et al., 1983). The extent of inactivation was determined by measuring the residual enzyme activity. An aliquot (≤10 μL) of the reaction mixture was directly added to the assay mixture (2.0 mL), and the decrease in absorbance at 265 nm was followed. The reagent carried over to the assay mixture did not interfere with the assay.

Reaction of Glycine Methyltransferase–TNB with Potassium Cyanide. Glycine methyltransferase (0.5 mg/mL) was incubated with 0.2 mM DTNB under the same conditions as above, and the reaction was allowed to go to completion. Following exhaustive dialysis of the reaction mixture against 50 mM potassium phosphate, pH 7.5/1 mM EDTA, the glycine methyltransferase–TNB derivative was treated at 25 °C with 20 mM potassium cyanide in the same buffer. The reaction was followed spectrophotometrically at 412 nm.

Reaction of Glycine Methyltransferase with Iodoacetate. Solutions of iodoacetate were prepared before each experiment by dissolving the solid in water and neutralizing with NaOH. The reaction of glycine methyltransferase with iodoacetate was carried out in the dark in 0.1 M Tris-HCl, pH 9.0 at 25 °C. The inactivation was monitored by periodic assay of aliquots of the reaction mixture as described above. The concentration of iodoacetate present during the assay (≤0.2 mM) had no effect on enzyme activity.

Incorporation of Radioactive Iodoacetate into Glycine Methyltransferase as a Function of Inactivation. Glycine methyltransferase (0.65 mg/mL) was incubated with 5.0 mM iodo[2-^{14}C]acetate (1×10^3 cpm/nmol) in 0.1 M Tris-HCl, pH 9.0. At time intervals aliquots were removed for measurements of residual enzyme activity, and radioactivity fixed. The radioactivity incorporated into the protein was determined by the filter paper disk method of Bollum (1968). An aliquot (25 μL) of the reaction mixture was placed on a Toyo Roshi No. 51 filter paper disk (2.5 cm in diameter), and 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. The radioactivity was determined in a scintillation counter by directly immersing the disk in a toluene scintillation liquid.

Labeling of Cysteine Residues Modified by Treatment with FSBA. Glycine methyltransferase (4 mg) was incubated with 0.5 mM FSBA in 4.0 mL of 20 mM potassium phosphate, pH 7.5, until no enzyme activity was detected. The reaction mixture was then chilled on ice, and the unreacted reagent was removed by dialysis at 4 °C against 20 mM Tris-HCl, pH 8.0, and finally against water. The modified enzyme, after lyophilization, was treated under N_2 with 70 mM iodoacetate in the dark in 1.0 mL of 0.5 M Tris-HCl, pH 8.6, containing 6 M guanidine hydrochloride and 1 mM EDTA for 1.5 h at 37 °C. Following extensive dialysis against 20 mM Tris-HCl, pH 8.0, and water, the carboxymethylated enzyme was treated with 70 mM 2-mercaptoethanol in 1 mL of 0.5 M Tris-HCl, pH 8.6, containing 6 M guanidine hydrochloride and 1 mM EDTA at 50 °C, to cleave the disulfide formed. After 4 h, the mixture was added to 70 mM iodo[^{14}C]acetate and further incubated for 1.5 h at 37 °C. The reaction mixture was dialyzed at 4 °C against 20 mM Tris-HCl, pH 8.0, containing 2 M guanidine hydrochloride, and then against water. The dialyzed enzyme was lyophilized.

Proteolytic Digestion. Digestion with trypsin was carried out in 0.1 M NH_4HCO_3, pH 7.9, by adding the protease in a 1:100 ratio (w/w) to glycine methyltransferase. After incubation for 3 h at 37 °C a second addition of the same amount of trypsin was made, and the digestion was continued for an additional 5 h. The digest was lyophilized and stored at −80 °C. Subdigestion of the isolated peptide with chymotrypsin was made in the same buffer at an enzyme to substrate ratio of 1:50 (w/w).

Separation of Peptides by HPLC. Peptides were separated by HPLC on a Toyo Soda CCP 8000 liquid chromatograph with a TSK ODS 120T reverse-phase column (0.46 × 25 cm) (Toyo Soda).

Amino Acid Analysis. Samples were hydrolyzed in vacuo in 5.7 M HCl containing 1% phenol and 1% 2-mercaptoethanol for 24 h at 108 °C. Amino acid compositions were determined by the precolumn derivatization method of Heinrikson and Meredith (1984) with slight modification (Gomi et al., 1986).

Sequence Analysis. Amino acid sequences of the isolated peptides were determined by either manual or automated Edman degradation. Manual Edman degradation was carried out according to the method of Tarr (1977), identifying PTH-amino acids by reverse-phase HPLC as described by Tsunazawa et al. (1985). Automated sequence analysis was performed on an Applied Biosystems 470A gas-phase sequenator equipped with a 120A high-performance liquid chromatograph system. Under the chromatographic conditions used, the retention times of PTH-Cys(Cm) and PTH-Asp were identical, and they were identified by analyzing aliquots of the PTH derivatives by the method of Tsunazawa et al. (1985).

RESULTS

Reaction with 5,5'-Dithiobis(2-nitrobenzoate). Incubation of glycine methyltransferase with DTNB at pH 8.0 and 25 °C resulted in a rapid release of TNB from the reagent. The addition of DTNB (200 μM) to glycine methyltransferase (13.7 μM subunit) caused an almost instantaneous increase in absorbance at 412 nm, followed by a slower reaction which was complete within 10 min (Figure 1A). The fast phase of the reaction corresponded to the release of 2.06 mol of TNB/mol of enzyme subunit, and a total of 2.92 mol/mol of subunit was released under these conditions. The slow phase of the reaction followed first-order kinetics with a rate constant of 0.79 min^{-1} (Figure 1B). Glycine methyltransferase appears to have SH groups in close proximity in the three-dimensional structure (Fujioka & Ishiguro, 1986). Since the TNB half

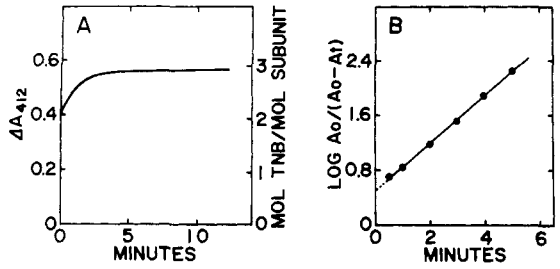

FIGURE 1: Reaction of glycine methyltransferase with DTNB. Glycine methyltransferase (13.7 μM subunit) was incubated with 0.2 mM DTNB at pH 8.0 as described under Experimental Procedures. (A) Tracing of a spectrophotometer chart; (B) semilogarithmic plot. A_t and A_0 represent changes in absorbance at 412 nm at a given time and at 100% reaction, respectively.

of the protein–TNB mixed disulfide is a better leaving group than glycine methyltransferase itself, it is possible that DTNB reacts only with two SH groups and one of the mixed disulfides undergoes an attack by a neighboring SH, releasing a total of 3 mol of TNB/mol of subunit. The absorption spectrum of the mofidied enzyme (after exhaustive dialysis against potassium phosphate, pH 7.5/1 mM EDTA), however, showed that this was not the case. The glycine methyltransferase–TNB derivative exhibited an absorption maximum at 330 nm, slightly shifted from that of DTNB in aqueous solution (324 nm). By use of a value of 8890 for the molar absorptivity of one-half DTNB (Riddles et al., 1983), the derivative was calculated to contain 3.37 TNB/subunit.[2] Thus, the reaction of DTNB occurs on three cysteine residues/subunit under these conditions.

The catalytic activity of glycine methyltransferase was totally abolished by the reaction with DTNB. Under the conditions of Figure 1, the inactivation was complete within the time of mixing, indicating that modification of one or both of the residues that react rapidly with DTNB eliminates all enzyme activity. To assess the difference in reactivity of these two residues and to determine which of these is responsible for the loss of activity, glycine methyltransferase was treated successively with limited amounts of DTNB. The rate of increase in absorbance at 412 nm following the addition of DTNB equivalent in amount to the glycine methyltransferase subunit was slightly greater than that observed after the addition of a second equivalent. Consistent with the data of Figure 1, a much slower rate was seen with a third equivalent (data not shown). Measurement of the enzyme activity remaining after each addition (measured when the change in absorbance at 412 nm reached a maximal value) showed that a large portion of activity was lost by the reaction with the second equivalent; the remaining activities were 81% and 25% of the initial activity after the addition of the first and second equivalents, respectively. Thus, modification of the SH group having intermediate reactivity appears to be responsible for inactivation. That only one residue is critical for activity is seen more clearly in the experiment to be described below, which shows that enzyme activity is recovered concomitant with the conversion of one of TNB mixed disulfides to the thiocyano derivative (cf. Figure 2).

The DTNB inactivation of glycine methyltransferase was fully reversed by treatment with dithiothreitol; the original enzyme activity was regained within 10 min upon incubation with 20 mM dithiothreitol.

[2] The shift of absorption maximum of the enzyme–TNB to a longer wavelength suggests that the environment of the SH groups is less polar than the solvent, and a higher value may be expected for the molar absorptivity.

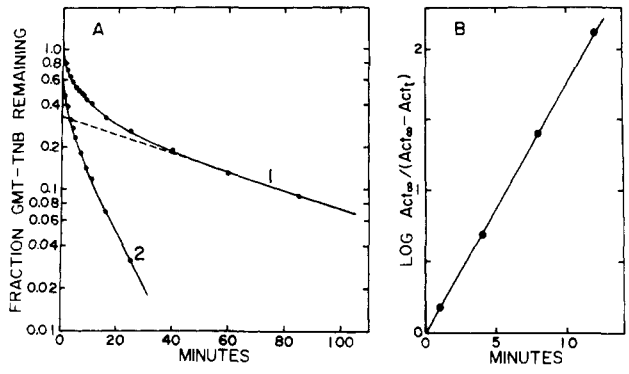

FIGURE 2: Time course of release of TNB (A) and regeneration of activity (B) from the glycine methyltransferase–TNB derivative (GMT–TNB) by KCN. GMT–TNB (6.4 μM subunit) was incubated with 20 mM KCN as described under Experimental Procedures. Activity measurements were made under standard assay conditions except that the glycine concentration was increased to 7.5 mM. Act_t is the activity at time t and Act_∞ is the maximum activity. See text for details.

Conversion of the Glycine Methyltransferase–TNB Mixed Disulfide to the Thiocyano Derivative. (A) Reaction of Glycine Methyltransferase–TNB with Cyanide. Incubation of glycine methyltransferase containing approximately 3 mol of bound TNB/mol of subunit with excess potassium cyanide (Vanaman & Stark, 1970) resulted in the quantitative release of the bound TNB (2.83 mol/mol of subunit) as measured by the increase in absorbance at 412 nm. A semilogarithmic plot of the fraction of the protein–TNB remaining against time was nonlinear, indicating different reactivities of the mixed disulfides toward cyanide (Figure 2A, line 1). Extrapolation of the slow phase of reaction, which had a pseudo-first-order rate constant of 0.015 min^{-1}, to the zero time axis yielded a value of 0.34, showing that this represents the reaction of one disulfide (Ray & Koshland, 1961). Subtraction of the values along the extrapolated slope from the observed values again generated a curved plot (Figure 2A, line 2). Thus, each of the three protein–TNB mixed disulfides reacts with cyanide at a different rate. Apparent first-order rate constants of 0.093 and 0.47 min^{-1} were obtained for the two rapidly reacting species by the same subtraction method.

The cyanide treatment caused a recovery of enzyme activity from the inactive glycine methyltransferase–TNB, concomitant with the release of 1 equiv of TNB. The regeneration of activity was rapid and followed pseudo-first-order kinetics with a rate constant of 0.39 min^{-1} (Figure 2B). Since this value is comparable to the rate constant of TNB release from the most reactive mixed disulfide (see above), it is reasonable to conclude that the recovery of activity arises from the displacement of TNB by cyanide in this disulfide. To show that the thiocyanates are indeed formed by this treatment, the enzyme–TNB was allowed to react to completion with cyanide, and the behavior of the resulting enzyme toward DTNB was examined. The addition of excess DTNB to the cyanylated enzyme (after removal of excess cyanide by dialysis) caused no change in absorbance at 412 nm nor resulted in any loss of the regained activity.

(B) Kinetic Properties of the Cyanylated Glycine Methyltransferase. The kinetic properties of the native and cyanylated glycine methyltransferases were compared at pH 7.5. Like the native enzyme (Ogawa & Fujioka, 1982), the cyanylated enzyme showed positive cooperativity with respect to AdoMet and a hyperbolic rate behavior toward glycine. From Hill plots (Hill, 1913) of the initial velocity data with AdoMet as the variable substrate, maximum Hill coefficients of 2.40 and 2.08 were obtained for the native and cyanylated enzymes,

Table I: Kinetic Parameters of Native and Cyanylated Glycine Methyltransferases[a]

enzyme	K_m(Gly) (mM)	$S_{0.5}$(AdoMet) (μM)	V_{max} (μmol min^{-1} mg^{-1})
native	0.18	45.7	1.30
cyanylated	0.69	56.2	1.21

[a] Initial velocity measurements were made in 20 mM potassium phosphate, pH 7.5, at 25 °C, by the coupled assay as described under Experimental Procedures. Values of kinetic parameters are apparent values. $S_{0.5}$(AdoMet) was determined at a glycine concentration of 5.0 mM, and K_m(Gly) and V_{max} were determined at 0.15 mM AdoMet.

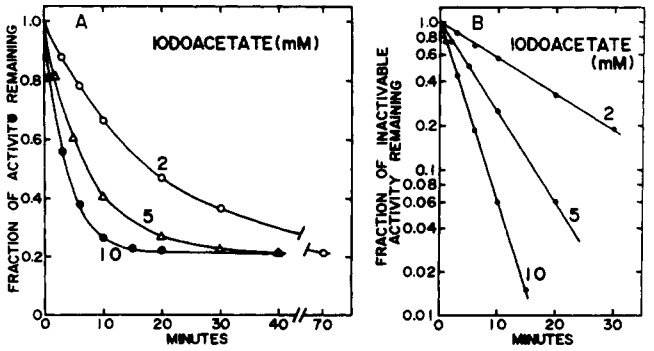

FIGURE 3: Inactivation of glycine methyltransferase by iodoacetate. Glycine methyltransferase (11.3 μM subunit) was incubated at pH 9.0 with iodoacetate at the concentrations indicated. The right panel (B) shows semilogarithmic plots of the fraction of inactivatable activity remaining $[(Act_t - Act_\infty)/(Act_0 - Act_\infty)]$ vs. time. Act_0 and Act_t represent enzyme activities at time 0 and t, and Act_∞ is the remaining activity.

respectively (plots not shown). Whereas the kinetic properties with respect to AdoMet as well as V_{max} were not very different between the two enzymes, there was a 4-fold increase in the K_m for glycine in the cyanylated enzyme (Table I).

Reaction with Iodoacetate. (*A*) *Inactivation of Glycine Methyltransferase by Iodoacetate.* Upon incubation with iodoacetate at pH 9.0, glycine methyltransferase was inactivated in a time-dependent manner. The inactivation, however, was not complete; 21–23% of the original activity remained after prolonged incubation irrespective of the concentration of iodoacetate used (Figure 3A). An additional iodoacetate added at a point where the enzyme activity was maximally reduced did not cause further inactivation. These results indicate that the remaining activity observed is an inherent property of the modified enzyme. A semilogarithmic plot of the fraction of inactivatable activity remaining vs. time yielded a straight line (Figure 3B), indicating that the decrease in enzyme activity occurs in an all-or-none fashion. The value of the apparent first-order rate constant was directly proportional to the iodoacetate concentration, and a second-order rate constant of 0.028 mM^{-1} min^{-1} was calculated at pH 9.0 and 25 °C.

(*B*) *Amino Acid Residue Modified by Iodoacetate.* Incubation of glycine methyltransferase with 5 mM iodo[^{14}C]-acetate at pH 9.0 for 8 h at 25 °C resulted in the incorporation of radioactivity equivalent to 3.4 mol of the reagent/mol of subunit. Amino acid analysis of the modified enzyme revealed the presence of 3.2 mol of *S*-(carboxymethyl)cysteine/mol of subunit. Except for (carboxymethyl)cysteine, no peak other than those corresponding to standard amino acids was detected in the chromatogram. Furthermore, greater than 90% of the radioactivity applied to the column was eluted at the position of (carboxymethyl)cysteine. Thus, the reaction of iodoacetate is restricted to cysteine residues under these conditions even though the reagent potentially reacts with several types of

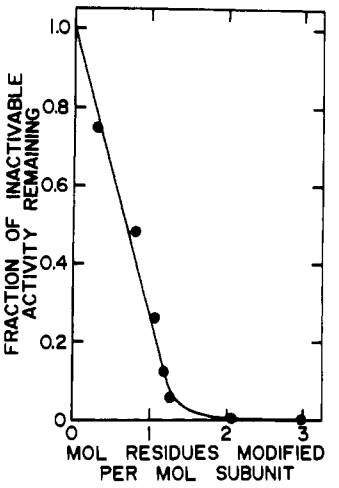

FIGURE 4: Relationship between the fraction of inactivatable activity remaining and the number of residues modified. The number of residues modified was determined by incorporation of iodo[^{14}C]acetate as described under Experimental Procedures.

amino acid side chains in proteins (Grundlach et al., 1959a,b).

(*C*) *Relationship between Loss of Activity and Number of Residues Modified.* The relationship between the loss of activity and the number of cysteine residues modified was examined by incorporation of the radiolabel from iodo[^{14}C]-acetate into the protein. A plot of the fraction of inactivatable activity remaining against moles of the reagent incorporated per enzyme subunit is approximately linear. Extrapolation of this line to the abscissa gave a value of 1.3 mol of reagent incorporated/mol of subunit (Figure 4), suggesting that the loss of activity is correlated directly with the modification of a single cysteine residue and that this residue reacts with iodoacetate much more rapidly than the other modifiable residues.

To examine whether the inactivation by DTNB is due to the modification of the cysteine residue that is responsible for loss of activity by iodoacetate, the following experiment was carried out. Glycine methyltransferase was incubated with iodoacetate at pH 9.0. Immediately after the enzyme had been maximally inactivated, excess iodoacetate was removed by gel filtration over a column of Sephadex G-25 equilibrated with 50 mM potassium phosphate, pH 7.5/1 mM EDTA, and the modified enzyme was treated with excess DTNB. No significant decrease of the remaining activity was observed by this treatment, although slightly less than 2 equiv of TNB/subunit was released. If a cysteine other than that involved in the iodoacetate inactivation reacts with DTNB to cause complete loss of activity, this treatment would result in a further decrease in activity eventually causing complete inactivation. Conversely, the activity of the cyanylated enzyme was not affected by incubation with iodoacetate. Thus, it is likely that the same residue is involved in the inactivation by the two reagents. The residues titratable with DTNB disappeared when the incubation with iodoacetate was carried out for a prolonged time.

(*D*) *Identification of Cysteine Residues Modified by Iodoacetate.* To identify the cysteine residues reactive toward iodoacetate in the primary structure of glycine methyltransferase, the enzyme was modified with iodo[^{14}C]acetate, and the amino acid sequences of radioactive tryptic peptides were determined. To facilitate analysis, the iodo[^{14}C]acetate-modified enzyme (after removal of the radioactive reagent) was treated with unlabeled iodoacetate under denaturing conditions prior to tryptic digestion. Figure 5 shows the elution pattern of tryptic peptides from a reverse-phase HPLC column,

Table II: Amino Acid Compositions of Isolated Peptides

amino acid	residues/peptide							
	I	II	II1	II2	II3	II4	II5	III
Asx	2.18	0.84	0.07	0.02	0.02	0.60	0.03	0.04
Glx	0.04	1.69	0.69	0.01	0.79	0.07	0.06	2.59
Cys(Cm)	1.12	1.61	0.00	0.00	0.75	0.00	0.66	0.81
Ser	1.16	0.95	0.04	0.03	0.98	0.07	0.05	1.79
Gly	2.14	1.94	1.01	0.01	0.08	1.16	0.09	1.88
His	0.00	1.61	0.02	1.04	0.95	0.06	0.02	0.84
Arg	0.02	1.00	1.00	0.05	0.04	0.02	0.02	1.00
Thr	1.38	0.00	0.05	0.04	0.05	0.07	0.05	0.91
Ala	1.08	0.95	1.03	0.03	0.03	0.04	0.03	1.84
Pro	1.58	2.54	0.70	0.02	0.02	0.82	0.63	0.61
Tyr	2.04	2.59	1.05	0.01	0.01	1.10	1.16	1.69
Val	0.19	2.84	0.01	1.21	1.06	0.07	1.22	0.95
Met	0.05	0.16	0.01	0.00	0.00	0.00	0.00	0.00
Ile	1.16	0.93	0.02	1.25	0.00	0.02	0.03	0.13
Leu	1.11	1.82	0.03	1.29	0.85	0.03	0.03	2.73
Phe	0.01	1.70	0.01	0.01	0.01	0.93	1.00	1.67
Lys	1.00	1.82	0.08	1.00	0.06	1.00	0.07	0.05
corresponding residue no. from sequence	176–190	262–289	274–279	285–289	262–267	268–273	280–284	240–261

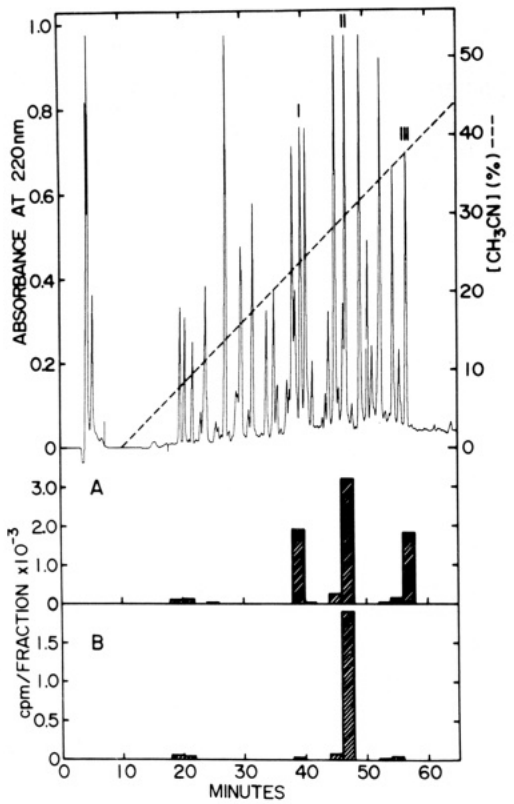

FIGURE 5: HPLC peptide mapping of tryptic peptides from glycine methyltransferase treated with iodo[^{14}C]acetate. The iodo[^{14}C]-acetate-modified enzyme was treated with unlabeled iodoacetate under denaturing conditions before tryptic digestion. About 30 nmol of tryptic peptides was fractionated by reverse-phase HPLC on a TSK ODS 120T column (0.46 × 25 cm) with a linear gradient from 0.05% trifluoroacetic acid to 0.05% trifluoroacetic acid containing 80% acetonitrile. The flow rate was 0.8 mL/min. (A) and (B) show the distribution of radioactivity after incubation with 5.0 mM iodo-[^{14}C]acetate at 25 °C for 8 h and 25 min, respectively.

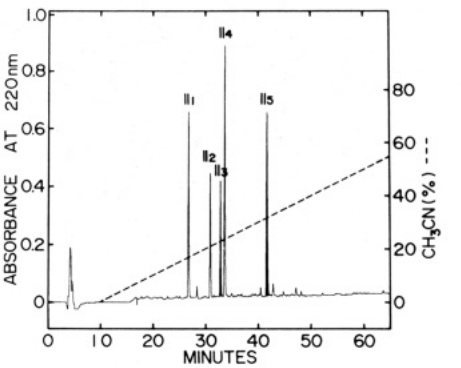

FIGURE 6: Separation of chymotryptic peptides derived from peptide II. Peptide II of Figure 5A was digested with chymotrypsin for 2 h at 37 °C as described under Experimental Procedures, and the digest was fractionated under the same conditions as in Figure 5. Shadowed peaks represent radioactive peaks.

together with the radioactivity profiles. Analysis of the enzyme treated with the reagent for a prolonged time (8 h) revealed the presence of three major radioactive peaks, of which peak II had a radioactivity higher than that of the other two (Figure 5A). Each of the radioactive fractions was purified by re-chromatography using a different solvent system (a linear gradient between 5 mM ammonium acetate, pH 6.8, and the same buffer containing 80% acetonitrile over a period of 80 min). Although peaks I and II were resolved into several peaks on rechromatography, the radioactivity was associated with a single peptide in each case (data not shown).

Amino acid analysis showed that the purified radioactive peptides I and III each contained 1 mol of (carboxymethyl)cysteine/mol of peptide, whereas 2 mol of (carboxymethyl)cysteine was present in peptide II (Table II). No "abnormal" peak was detected in amino acid analyses. To determine whether the radioactivity in peptide II is confined to one (carboxymethyl)cysteine or is distributed between the two residues, the peptide was further cleaved with chymotrypsin. The digestion yielded five peptides, of which two (peptides II3 and II5) contained 1 mol of (carboxymethyl)-cysteine/mol of peptide (Figure 6 and Table II). Radioactivity was found in both of these (carboxymethyl)cysteine-containing peptides, the specific radioactivity of peptide II3 being about half that of peptide II5.

The tryptic digest from glycine methyltransferase that had been treated with iodo[^{14}C]acetate for a time period sufficient to maximally reduce the enzyme activity contained only one radioactive peak (Figure 5B), which was identical with peak II of Figure 5A as seen from its retention time and amino acid composition. Analysis of chymotryptic peptides derived from this peptide showed that only the peptide corresponding to peptide II5 in Figure 6 was radioactive, consistent with the result of Figure 4.

Edman degradation (Table III) established the following sequences for the (carboxymethyl)cysteine-containing peptides: I, Asn-Tyr-Asp-Tyr-Ile-Leu-Ser-Thr-Gly-Cys(Cm)-Ala-Pro-

Table III: Summary of Edman Degradations of Isolated Peptides

	I[a]		II3[b]		II5[b]		III[a]	
cycle	residue	pmol	residue	pmol	residue	pmol	residue	pmol
1	Asn	3091	Cys(Cm)	697	Val	5290	Leu	1394
2	Tyr	2693	Gln	408	Pro	2266	Ser	711[c]
3	Asp	1870	His	116	Cys(Cm)	911	Tyr	722
4	Tyr	2654	Ser	385[c]	Tyr	94	Tyr	922
5	Ile	2150	Val	264	Phe	23	Pro	656
6	Leu	1853	Leu	143			His	88
7	Ser	151[c]					Cys(Cm)	*d*
8	Thr	145[c]					Leu	884
9	Gly	140					Ala	731
10	Cys(Cm)	*d*					Ser	141[c]
11	Ala	461					Phe	554
12	Pro	280					Thr	113[c]
13	Pro	275					Glu	169
14	Gly	232					Leu	384
15	Lys	620					Val	326
16							Gln	209
17							Glu	162
18							Ala	253
19							Phe	364
20							Gly	189
21							Gly	270
22							Arg	89

[a] Determined by automated Edman degradation. [b] Determined by manual Edman degradation. [c] These values do not include degradation products of PTH-Ser or PTH-THR. [d] Identified by the method of Tsunazawa et al. (1985).

Pro-Gly-Lys; II3, Cys(Cm)-Gln-His-Ser-Val-Leu; II5, Val-Pro-Cys(Cm)-Tyr-Phe; III, Leu-Ser-Tyr-Tyr-Pro-His-Cys-(Cm)-Leu-Ala-Ser-Phe-Thr-Glu-Leu-Val-Gln-Glu-Ala-Phe-Gly-Gly-Arg. From comparison of the sequence data with the deduced sequence of rat liver glycine methyltransferase, the reactive cysteine residues are assigned to Cys-185 (peptide I), Cys-246 (peptide III), Cys-262 (peptide II3), and Cys-282 (peptide II5). Modification of Cys-282 clearly leads to a loss of enzyme activity.

Identification of Cysteine Residues Modified by Treatment with FSBA. It has been shown previously that glycine methyltransferase is inactivated by FSBA concomitant with the loss of two SH groups/subunit (Fujioka & Ishiguro, 1986, and confirmed in the present study), probably as the result of formation of a disulfide. The native glycine methyltransferase is devoid of disulfide bonds since the amount of radioactivity incorporated into the protein by the reaction with iodo[^{14}C]-acetate under denaturing conditions does not differ in the presence or absence of 2-mercaptoethanol. Radioactivity equivalent to approximately 6 mol of iodo[^{14}C]acetate was incorporated per mole of subunit under both sets of conditions. This number also agrees with that of SH groups titratable with DTNB in the presence of 0.2% sodium dodecyl sulfate (Fujioka & Ishiguro, 1986) or 6 M guanidine hydrochloride.[3] Therefore, treatment of the FSBA-modified glycine methyltransferase under denaturing conditions with unlabeled iodoacetate in the absence of reducing agent, followed by reduction with 2-mercaptoethanol and alkylation with iodo[^{14}C]acetate, would be expected to label the cysteines forming a disulfide. The FSBA-modified glycine methyltransferase was treated with unlabeled and radioactive iodoacetate as described under Experimental Procedures. The resulting enzyme containing radioactivity equivalent to 2.4 mol of radioactive iodoacetate/mol of subunit was digested with trypsin, and the peptides were analyzed under the same conditions as in Figure 5. The analysis showed the presence of three major radioactive peptides that were identical with peptides I, II, and III of Figure 5A as evidenced by their retention times and amino acid compositions (not shown). Peptide II contained twice as much radioactivity as peptides I and III, and analysis of the chymotryptic peptides derived from peptide II revealed that the radioactivity was distributed equally between Cys-262 and Cys-282.

Discussion

In its native state, the rat liver glycine methyltransferase subunit possesses four cysteine residues reactive toward iodoacetate. Amino acid sequence analysis of tryptic and chymotryptic peptides from the modified enzyme shows that Cys-185, Cys-246, Cys-262, and Cys-282 are the sites of modification, Cys-282 being the most reactive and Cys-262 the least reactive residue. DTNB rapidly reacts with three cysteine residues/subunit. Because of the instability of TNB mixed disulfides during peptide purification and because of the potential cleavage of peptide bonds at cyanylated cysteine residues under denaturing conditions (Vanaman & Stark, 1970), no attempt has been made to isolate and characterize peptides containing cysteines modifiable by the DTNB/KCN treatment. However, the fact that the glycine methyltransferase that has been previously carboxymethylated fails to react with DTNB suggests that cysteine residues modifiable by iodoacetate include those that react with DTNB.

The iodoacetate-modified glycine methyltransferase retains about 22% of the original activity. This partial inactivation is apparently the consequence of carboxymethylation of Cys-282, which reacts with the reagent more rapidly than the rest (Figures 4 and 5). Reaction of the enzyme with DTNB, on the other hand, leads to complete loss of activity. While the extents of inactivation by the two reagents are different, the observation that DTNB fails to further decrease the remaining activity associated with the enzyme that has been maximally inactivated by iodoacetate but still possesses approximately two cysteine residues available for reaction with DTNB suggests that modification of the same residue is responsible for inactivation. The nonessential nature of the residue modified by DTNB is demonstrated by the conversion of the TNB mixed disulfide to the thiocyano derivative.

[3] The amino acid sequence of the glycine methyltransferase subunit derived from the nucleotide sequence of cloned cDNA contains seven cysteine residues. The reason for this discrepancy is not known at present.

Reaction of KCN with the enzyme containing three TNB mixed disulfides/subunit causes regeneration of activity concomitant with the release of 1 equiv of TNB. The cyanylated enzyme has values of $S_{0.5}$ for AdoMet and V_{max} comparable to those of the native enzyme and only a 4-fold increased K_m value for glycine. Treatment of glycine methyltransferase–TNB with sodium sulfite also regenerates enzyme activity comparable to that of the native enzyme when measured under standard assay conditions (result not shown).

Despite the stoichiometry of disappearance of two SH groups/subunit observed for the inactivation by FSBA (Fujioka & Ishiguro, 1986), the peptide analysis of the FSBA-inactivated enzyme that has been treated with unlabeled iodoacetate followed by reduction and alkylation with iodo-[^{14}C]acetate shows that the radioactivity is distributed equally among Cys-185, Cys-246, Cys-262, and Cys-282. If SH groups other than those forming a disulfide are susceptible to autoxidation and become oxidized during dialysis to remove the unreacted FSBA, a labeling pattern such as this may be obtained. This possibility, however, is unlikely since (1) the enzyme treated similarly in the absence of FSBA shows no significant incorporation of radioactivity, (2) the same radioactivity profile is seen in HPLC of peptides from the enzyme at the early stage of modification, and (3) the amount of radioactivity incorporated is close to the stoichiometry of two residues modified/subunit. Thus, the result is consistent with the idea that FSBA can react in a mutually exclusive manner with at least two of these residues, leading in each case to formation of a disulfide with a cysteine residue located in close proximity in the three-dimensional structure. The fact that the specific radioactivities of the four residues are about the same indicates that the FSBA-reactive residues have a similar, if not identical, reactivity toward the reagent. Which of these residues are the sites of attack of FSBA and which residues are contained in each disulfide are not known at present. Mutually exclusive reactions of FSBA with different residues within a single FSBA-binding site are reported in the inactivation of rabbit muscle pyruvate kinase (DeCamp & Colman, 1986) and of bovine mitochondrial F_1-ATPase (Bullough & Allison, 1986).

The results described in this paper indicate that none of the reactive cysteine residues of glycine methyltransferase, Cys-185, Cys-246, Cys-262, or Cys-282, has a direct functional role in catalysis, but attachment of a bulky group to Cys-282 exerts a profound effect on the catalytic activity. It may be conceivable that Cys-282 is located near the active site and introduction of a bulky group to this residue blocks the active site to result in inactivation. This possibility, however, appears unlikely because the enzyme having this residue cross-linked with another cysteine and thus having no bulky substituent is also devoid of activity. While modification of Cys-185, Cys-246, and Cys-262 apparently has no effect on the catalytic activity, the formation of a disulfide involving these residues leads to inactivation. The mutually exclusive nature of the reaction of FSBA and the facile formation of disulfides suggest that these cysteine residues occur spatially close together, and this portion of the enzyme may be important in the maintenance of the conformation required for catalysis.

Registry No. Cys, 52-90-4; Gly, 56-40-6; AdoMet, 29908-03-0; DTNB, 69-78-3; FSBA, 57454-44-1; iodoacetate, 64-69-7; glycine methyltransferase, 37228-72-1.

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