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Recombinant Rat Liver Guanidinoacetate Methyltransferase: Reactivity and Function of Sulfhydryl Groups

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ABSTRACT: Rat liver guanidinoacetate methyltransferase, produced in *Escherichia coli* by recombinant DNA technique, possesses five cysteine residues per molecule. No disulfide bond is present. Analysis of the chymotryptic peptides derived from the iodo[^{14}C]acetate-modified enzyme shows that Cys-90, Cys-15, Cys-219, and Cys-207 are alkylated by the reagent in order of decreasing reactivity. Incubation of the enzyme with excess 5,5′-dithiobis(2-nitrobenzoate) (DTNB) in the absence and presence of cystamine [2,2′-dithiobis(ethylamine)] causes the appearance of 4 and 5 mol of 2-nitro-5-mercaptobenzoate/mol of enzyme, respectively. Reaction of the methyltransferase with an equimolar amount of DTNB results in an almost quantitative disulfide cross-linking of Cys-15 and Cys-90 with loss of a large portion of the activity. The methyltransferase is completely inactivated by iodoacetate following nonlinear kinetics. Comparison of the extent of inactivation with that of modification of cysteine residues and the experiment with the enzyme whose Cys-15 and Cys-90 are cross-linked suggest that alkylation of Cys-15 and Cys-90 results in a partially active enzyme and that carboxymethylation of Cys-219 completely eliminates enzyme activity. The inactivation of guanidinoacetate methyltransferase by iodoacetate or DTNB is not protected by substrates. Furthermore, disulfide cross-linking of Cys-15 and Cys-90 or carboxymethylation of Cys-219 does not impair the enzyme's capacity to bind *S*-adenosylmethionine. Thus, these cysteine residues appear to occur outside the active-site region, but their integrity is crucial for the expression of enzyme activity.

Guanidinoacetate methyltransferase (*S*-adenosyl-L-methionine:guanidinoacetate *N*-methyltransferase, EC 2.1.1.2), which catalyzes the transfer of the methyl group of *S*-adenosylmethionine (AdoMet)[1] to guanidinoacetate to form creatine, was purified to homogeneity from the livers of pig (Im et al., 1979) and rat (Ogawa et al., 1983). The enzymes from both sources are monomeric proteins with a relatively small molecular size. A cDNA clone encoding rat liver guanidinoacetate methyltransferase has recently been obtained, and the complete amino acid sequence of the enzyme is deduced from its nucleotide sequence. Also, by introducing the cDNA into plasmid pUC118, a recombinant plasmid that expresses guanidinoacetate methyltransferase in *Escherichia coli* has been constructed. The recombinant enzyme, which occupies about 5% of bacterial soluble proteins, exhibits kinetic properties indistinguishable from those of the rat liver enzyme and appears to be structurally identical with the liver enzyme except for the absence of the N-terminal blocking group (Ogawa et al., 1988).

It was reported earlier that guanidinoacetate methyltransferase from pig liver was progressively inactivated during purification and that this inactivation was reversed by a thiol (Cantoni & Vignos, 1954). A more recent investigation in this laboratory has shown that the rat liver enzyme also undergoes this type of inactivation and possesses multiple SH groups, the integrity of which appears crucial for activity (Ogawa et al., 1983). However, detailed studies of their reactivity and functional role have been hampered by limited supply of the enzyme because of its relatively low abundance in the liver and the difficulty of purifying it in good yield. As sufficient quantities of guanidinoacetate methyltransferase amenable to mechanistic study are now available by the recombinant DNA procedure, we extended the study on the function and reactivity of SH groups in guanidinoacetate methyltransferase. The present paper reports the results obtained by using several disulfides and iodoacetate as sulfhydryl modification reagents.

Experimental Procedures

Materials. Guanidinoacetate methyltransferase used in the present study was produced in *E. coli* MV1304 transformed with plasmid pUCGAT9-1, which contained the cDNA for rat liver guanidinoacetate methyltransferase linked to the *lac* promoter (Ogawa et al., 1988). The enzyme was purified by ammonium sulfate fractionation, Sephadex G-100 gel filtration, and DEAE-cellulose chromatography as described previously (Ogawa et al., 1988). The purified enzyme gave a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Prior to experiments, the enzyme was exhaustively dialyzed against 20 mM potassium phosphate, pH 7.2/1 mM EDTA to remove the dithiothreitol added during purification. Molar concentrations of the enzyme were calculated on the basis of M_r = 26 200 (Ogawa et al., 1983, 1988). Protein concentration was determined by the method of Lowry

[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 2-PDS, 2,2′-dithiodipyridine; 4-PDS, 4,4′-dithiodipyridine; TNB, 2-nitro-5-mercaptobenzoic acid; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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et al. (1951) with guanidinoacetate methyltransferase as the standard. *S*-Adenosylhomocysteinase was purified from rat liver by the procedure of Fujioka and Takata (1981).

AdoMet (grade II) obtained from Sigma Chemical Co. was purified before use by reverse-phase chromatography as described previously (Fujioka & Ishiguro, 1986), and its concentration was determined spectrophotometrically by using a value of $\epsilon_{260} = 15.4 \times 10^3$ M^{-1} cm^{-1}. Iodoacetic acid (Merck) was recrystallized from hot chloroform. Iodo[2-^{14}C]acetic acid (55 mCi/mmol) and *S*-adenosyl-L-[*methyl*-^{14}C]methionine (54 mCi/mmol) were purchased from Amersham. Adenosine deaminase, DTNB, cystamine [2,2′-dithiobis(ethylamine)], and α-chymotrypsin were obtained from Sigma and 2-PDS and 4-PDS from Aldrich Chemical Co. The amino acid calibration mixture, standard phenylthiohydantoin amino acids, and phenyl isothiocyanate were from Wako Pure Chemical Industries. Other chemicals were of the highest purity available from commercial sources.

Assay of Guanidinoacetate Methyltransferase Activity. The guanidinoacetate methyltransferase activity was determined spectrophotometrically by a coupled assay with *S*-adenosylhomocysteinase and adenosine deaminase. The assay was carried out at 30 °C in a reaction mixture containing 17.5 μM AdoMet, 0.5 mM guanidinoacetate, and sufficient amounts of *S*-adenosylhomocysteinase and adenosine deaminase in 2.0 mL of 50 mM potassium phosphate, pH 8.0. The decrease in absorbance at 265 nm due to the conversion of adenosine to inosine was continuously followed in a spectrophotometer.

Reaction of Guanidinoacetate Methyltransferase with 5,5′-Dithiobis(2-nitrobenzoate), 2,2′-Dithiodipyridine, and 4,4′-Dithiodipyridine. Solutions of DTNB, 2-PDS, and 4-PDS were prepared fresh before each experiment, and their concentrations were determined from molar extinction coefficients: DTNB, $\epsilon_{324} = 1.778 \times 10^4$ M^{-1} cm^{-1} (Riddles et al., 1983); 2-PDS, $\epsilon_{233} = 1.39 \times 10^4$ M^{-1} cm^{-1} (Grassetti & Murray, 1967); 4-PDS, $\epsilon_{247} = 1.63 \times 10^4$ M^{-1} cm^{-1} (Grassetti & Murray, 1967). Guanidinoacetate methyltransferase was incubated with the disulfide in 0.1 M Tris-HCl, pH 8.0/0.2 M ammonium sulfate/1 mM EDTA, and the reaction was followed spectrophotometrically. The amount of SH groups reacting with the reagents was calculated by using the molar extinction coefficients of the products: TNB, $\epsilon_{412} = 1.415 \times 10^4$ M^{-1} cm^{-1} (Riddles et al., 1983); 2-thiopyridone, $\epsilon_{343} = 7.06 \times 10^3$ M^{-1} cm^{-1} (Grassetti & Murray, 1967); 4-thiopyridone, $\epsilon_{324} = 1.98 \times 10^4$ M^{-1} cm^{-1} (Grassetti & Murray, 1967). The extent of inactivation was monitored by measuring the residual enzyme activity. An aliquot (≤20 μL) removed from the reaction mixture was directly added to the assay mixture (2.0 mL), and the decrease in absorbance at 265 nm was followed. The amounts of reagents present in the assay mixture had no effect on the activity measurement.

Labeling of Cysteine Residues Cross-Linked by Treatment with 5,5′-Dithiobis(2-nitrobenzoate). Guanidinoacetate methyltransferase (54 nmol) was incubated with an equimolar concentration of DTNB in a volume of 2.0 mL under the conditions described above. When the reaction had gone to completion as determined by the absorbance change at 412 nm, the reaction mixture was dialyzed against 20 mM Tris-HCl, pH 8.0/1 mM EDTA and then against water. To avoid oxidation of unreacted SH groups, all dialysis solutions were prepared with oxygen-free water that had been boiled for 20 min and then bubbled with nitrogen while cooling, and dialysis was carried out under positive nitrogen tension. The modified enzyme, after lyophilization, was treated under nitrogen with 8 mM unlabeled iodoacetate in 0.5 mL of 0.5 M Tris-HCl, pH 8.0/4 M guanidine hydrochloride/1 mM EDTA for 1 h at 37 °C. Following dialysis against 20 mM Tris-HCl, pH 8.0/3 M guanidine hydrochloride and against water, the carboxymethylated enzyme was incubated with 13.7 mM 2-mercaptoethanol in 0.5 mL of 4 M buffered guanidinium chloride, pH 8.0, at 50 °C. After 4 h, iodo[^{14}C]acetate (490 cpm/nmol) was added to a final concentration of 13.7 mM, and the mixture was further incubated for 1.5 h at 37 °C. The reaction mixture was then dialyzed as above, and the ^{14}C-labeled enzyme was lyophilized.

Inactivation of Guanidinoacetate Methyltransferase by Iodoacetate. Guanidinoacetate methyltransferase was incubated with iodoacetate in the dark in 0.1 M Tris-HCl, pH 8.5, at 30 °C. The extent of inactivation was determined by measuring the residual enzyme activity on an aliquot removed from the reaction mixture as described above. The concentration of iodoacetate present in the assay mixture did not interfere with the assay.

Determination of Total Sulfhydryl Groups. The SH contents of the native and modified guanidinoacetate methyltransferases were determined by the reaction with DTNB in the presence of 6 M guanidine hydrochloride. After exhaustive dialysis of the enzyme sample against oxygen-free 0.1 M potassium phosphate, pH 7.3/1 mM EDTA, its SH content was determined as described by Riddles et al. (1983).

Proteolytic Digestion and Isolation of Peptides. Digestion of guanidinoacetate methyltransferase with chymotrypsin was carried out overnight at 37 °C, using a protease to protein ratio of 1:100 (w/w) in 0.1 M NH_4HCO_3, pH 7.9.

Chymotryptic peptides were separated by HPLC on a Tosoh CCP 8000 liquid chromatograph with a TSK gel ODS-120T reverse-phase column (0.46 × 25 cm) (Tosoh).

Amino Acid Analysis. Samples were hydrolyzed in vacuo in 5.7 M HCl/0.1% phenol/0.1% 2-mercaptoethanol for 24 h at 108 °C. Amino acid composition was determined based on reverse-phase separation (Gomi et al., 1986) of phenylthiocarbamoyl derivatives (Heinrikson & Meredith, 1984).

Sequence Analysis. Amino acid sequences of isolated peptides were determined by automated Edman degradation on an Applied Biosystems 470A gas-phase sequencer equipped with a 120A high-performance liquid chromatograph system.

Equilibrium Dialysis. Guanidinoacetate methyltransferase with a disulfide cross-link between Cys-15 and Cys-90 was prepared by incubating the enzyme with an equimolar amount of DTNB, followed by dialysis against 20 mM potassium phosphate, pH 7.2/1 mM EDTA. To prepare the enzyme having Cys-219 carboxymethylated, the cross-linked enzyme was treated with 3 mM iodoacetate until the residual activity measured after treatment with dithiothreitol fell <5% of the initial value. Dithiothreitol (20 mM) was then added to cleave the disulfide. After 30 min at 30 °C, the carboxymethylated enzyme was freed from excess reagents by dialysis against 20 mM potassium phosphate, pH 7.2/1 mM EDTA. The binding of AdoMet to the native and modified enzymes was determined by equilibrium dialysis in 20 mM potassium phosphate, pH 7.2, at 4 °C with [*methyl*-^{14}C]AdoMet as the ligand. The enzymes (7–11 μM) were allowed to equilibrate with 1.25–100 μM [^{14}C]AdoMet.

Other Analytical Procedures. Spectrophotometric and 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-500C. Radioactivity was determined in an Aloka liquid scintillation spectrometer, LSC 903.

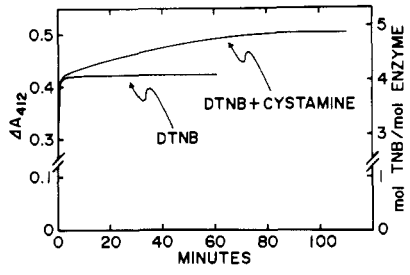

FIGURE 1: Reaction of guanidinoacetate methyltransferase with DTNB in the presence and absence of cystamine. Guanidinoacetate methyltransferase (7.3 μM) was incubated with 70 μM DTNB in the presence and absence of 350 μM cystamine in 0.1 M Tris-HCl, pH 8.0/0.2 M ammonium sulfate/1 mM EDTA at 20 °C.

RESULTS

Number of Sulfhydryl Groups in Guanidinoacetate Methyltransferase. In the presence of 6 M guanidine hydrochloride at pH 7.3, a total of 4.9–5.2 mol of SH groups/mol of guanidinoacetate methyltransferase was titrated with DTNB. Since the primary structure of the enzyme predicted by the nucleotide sequence of the cDNA shows the presence of five half-cystine residues (Ogawa et al., 1988), it is concluded that guanidinoacetate methyltransferase is devoid of disulfide links.

Reaction of Guanidinoacetate Methyltransferase with Disulfides. Addition of excess DTNB (10-fold molar excess) to guanidinoacetate methyltransferase at pH 8.0 and 20 °C in the absence of denaturant resulted in a rapid release of TNB from the reagent. A release of TNB corresponding to 3.29 mol/mol of enzyme occurred within the time of mixing, followed by a slower reaction. A total of 4.07 mol of TNB/mol of enzyme was released (Figure 1). After exhaustive dialysis of the reaction mixture against 20 mM potassium phosphate, pH 7.5/1 mM EDTA, the absorption spectrum of the modified enzyme showed a peak at 328 nm in addition to an absorption maximum at 280 nm, indicating the formation of protein–TNB mixed disulfide. By use of a value of $\epsilon_{324} = 8.89 \times 10^3$ M^{-1} cm^{-1} for the molar absorptivity of half DTNB, the modified enzyme was calculated to contain 2.23 mol of bound TNB/mol of enzyme.[2] The discrepancy between the amount of TNB molecules released and that of TNB molecules bound to the enzyme suggests that three SH groups react with DTNB under these conditions, and one of the mixed disulfides formed undergoes reaction with a neighboring SH to form a disulfide.

Wilson et al. (1980) reported that protein SH groups that occur in a hydrophobic environment and react slowly with DTNB can be studied by using mixtures of DTNB and colorless disulfides such as cystamine. If the nonchromophoric disulfide reacts with the SH group, the thiol produced from the disulfide will quickly react with DTNB. The time course of appearance of TNB after the addition of a mixture of DTNB and cystamine is shown in Figure 1. Following the formation of 4 mol of TNB/mol of enzyme, a very slow reaction released an additional 1 equiv of TNB. The result suggests that the SH group which is inaccessible to DTNB can react with cystamine, and the reaction of the other SH groups with DTNB is not appreciably influenced by the presence of cystamine.

When the reaction of guanidinoacetate methyltransferase with limited amounts of DTNB was allowed to go to completion, the results shown in Table I were obtained. The amount of TNB appearing after the addition of DTNB equivalent to the enzyme was roughly twice that of the reagent. Upon further addition of DTNB, the increment of TNB corresponded to the amount of reagent added. The spectrum of the enzyme treated with an equimolar amount of DTNB showed a very weak absorption in the region of 320–400 nm. The modified enzyme was estimated to contain ~0.05 mol of bound TNB/mol of enzyme. Thus, the result implies that guanidinoacetate methyltransferase has one SH group very reactive toward DTNB, and the resulting TNB mixed disulfide is susceptible to disulfide interchange with an SH group present in close proximity. Similar results were obtained when 2-PDS and 4-PDS in amounts equivalent to the enzyme were used: 1.86 mol of 2-thiopyridone and 1.52 mol of 4-thiopyridone were formed per mole of enzyme. Appearance of turbidity after addition of increased concentrations of the dithiodipyridines precluded determination of total number of SH groups reactive with these reagents.

Table I: Reaction of Guanidinoacetate Methyltransferase with Limited Amounts of DTNB[a]

DTNB added (mol/mol of enzyme)	TNB released (mol/mol of enzyme)	activity remaining (%)
1.0	1.90	16
1.5	2.62	9
2.0	3.05	0
3.0	3.92	0

[a] Guanidinoacetate methyltransferase (12 nmol/mL) was treated successively with 1.0, 0.5, 0.5, and 1.0 equiv of DTNB. After each addition, the reaction was allowed to go to completion (monitored by the change in absorbance at 412 nm), and the residual activity was determined on an aliquot removed from the reaction mixture. Values are corrected for small volume changes.

To demonstrate that the disulfide is formed between two specific cysteine residues, the following experiment was carried out. Guanidinoacetate methyltransferase was modified with an equimolar amount of DTNB, and the modified enzyme was first treated with unlabeled iodoacetate under denaturing conditions and then with iodo[^{14}C]acetate after reduction with 2-mercaptoethanol (see Experimental Procedures). Since the native guanidinoacetate methyltransferase contains no disulfide bond, this treatment should label the cysteine residues that were enganged in disulfide cross-linking. Figure 2 shows the HPLC analysis in a trifluoroacetic acid solvent system of the chymotryptic peptides derived from the enzyme thus radiolabeled. The effluent was monitored by absorbance at 220 nm and collected in 1.6-mL fractions. When aliquots from each fraction were determined for radioactivity, >75% of the radioactivity applied to the column was found in only one fraction. In the control sample treated similarly but in the absence of DTNB, no appreciable radioactivity was found in this and other fractions, indicating that nonspecific oxidation of SH groups did not occur during the manipulation. Rechromatography of the radioactive fraction using an acetonitrile gradient in ammonium acetate showed that it contained several peptides, of which two were radioactive (Figure 3, peptides I and II). Amino acid analysis (Table II) and Edman degradation (Table III) established the following sequences for the two peptides: peptide I, Ile-Ile-Glu-Cys(Cm)-Asn-Asp-Gly-Val-Phe; peptide II, Phe-Ala-Pro-Gly-Glu-Asp-Cys(Cm)-Gly-Pro-Ala-Trp. As expected, the majority of radioactivity was released with the phenylthiohydantoin derivative of (carboxymethyl)cysteine. Peptide II had an uncleaved Phe–Ala bond. Peptides I and II uniquely fit residues 87–95 and residues 9–19, respectively, of the guanidinoacetate

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

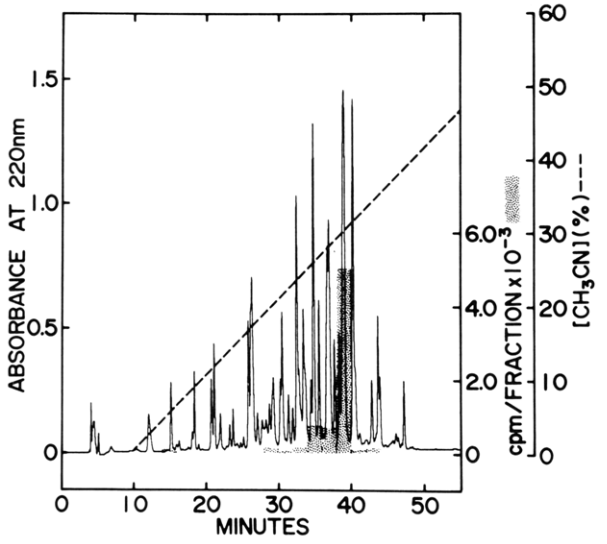

FIGURE 2: HPLC profile of a chymotryptic digest of guanidinoacetate methyltransferase modified with an equimolar amount of DTNB. The DTNB-treated enzyme was carboxymethylated with unlabeled and ^{14}C-labeled iodoacetate as described under Experimental Procedures prior to chymotryptic digestion. About 30 nmol of chymotryptic peptides were fractionated 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. Shadowed bars represent the total radioactivity associated with each fraction collected at 2-min intervals.

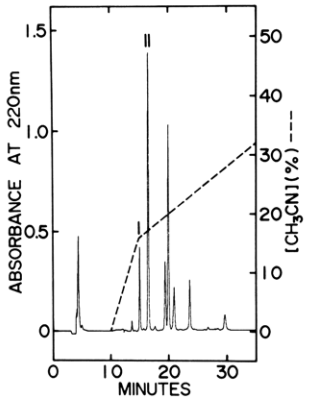

FIGURE 3: Rechromatography of the radioactive fraction of Figure 2. The major radioactive fraction of Figure 2 was rechromatographed on a TSK ODS-120T column using an acetonitrile gradient in 5 mM ammonium acetate, pH 6.8.

methyltransferase sequence (Ogawa et al., 1988). The specific radioactivities of peptides I and II were about the same and very close to that of iodo[^{14}C]acetate used to label the enzyme. (The molar concentrations of peptides were determined by amino acid analysis.) Thus, the results described above indicate that a disulfide bond is formed between the SH groups of Cys-15 and Cys-90. That the cross-linking is intramolecular was shown by identical mobilities of the native and modified enzymes on nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown).

While the reaction of guanidinoacetate methyltransferase with 2 equiv of DTNB totally abolished activity, the enzyme treated with 1 or 1.5 equiv of DTNB retained a small fraction of activity (Table I). Since the result of peptide analysis as well as the stoichiometry between the amount of DTNB added and that of TNB released indicates that Cys-15 and Cys-90 are quantitatively cross-linked by a disulfide bond when the enzyme was treated with a slight molar excess of the reagent, it appears that the disulfide formation alone is not sufficient to eliminate enzyme activity, and modification of other cysteine

Table II: Amino Acid Compositions of Isolated Peptides

amino acid	residues/peptide I	II	III	IV
Asx	1.92	0.92	1.09	0.93
Glx	1.06	0.98	0.14	2.87
Cys(Cm)	1.05	0.94	1.01	0.94
Ser	0.00	0.02	0.10	0.07
Gly	1.11	2.02	0.09	0.00
His	0.00	0.00	0.02	0.02
Arg	0.01	0.03	1.15	1.00
Thr	0.00	0.00	0.00	0.98
Ala	0.04	2.01	2.29	0.04
Pro	0.00	2.16	2.20	0.12
Tyr	0.03	0.06	2.00	0.05
Val	0.99	0.02	1.13	0.96
Met	0.04	0.00	0.08	1.00
Ile	1.84	0.01	0.08	0.93
Leu	0.00	0.03	1.12	0.00
Phe	1.00	1.00	0.16	0.05
Lys	0.02	0.01	0.04	0.02
corresponding residue no. from sequence	87–95	9–18	212–222	202–211

Table III: Amino Acid Sequence of Peptides I and II

cycle	peptide I residue	nmol	cpm	peptide II residue	nmol	cpm
1	Ile	1.47	3	Phe	2.81	4
2	Ile	1.77	0	Ala	2.50	4
3	Glu	0.96	0	Pro	2.86	3
4	Cys(Cm)	1.00	459	Gly	2.01	47
5	Asn	1.18	151	Glu	2.26	10
6	Asp	0.80	30	Asp	2.03	14
7	Gly	0.77	10	Cys(Cm)	1.98	923
8	Val	0.75	2	Gly	1.71	131
9	Phe	0.63	0	Pro	2.07	35
10				Ala	2.46	11
11				Trp	0.92	11

residue(s) is required for complete loss of activity. The catalytic activity associated with the enzyme modified with a 1.5-fold molar excess of DTNB was about the same whether the assay was conducted under the standard assay conditions or in the presence of increased concentrations of AdoMet (6-fold) and guanidinoacetate (5-fold).

The DTNB inactivation of guanidinoacetate methyltransferase was fully reversed within 30 min by treatment with 20 mM dithiothreitol at pH 8.0.

Inactivation by Iodoacetate. Guanidinoacetate methyltransferase was inactivated by iodoacetate in a time- and concentration-dependent manner at pH 8.5 and 30 °C. No detectable activity remained after prolonged incubation. Amino acid analysis of the iodo[^{14}C]acetate-inactivated enzyme showed that radioactivity was associated only with *S*-(carboxymethyl)cysteine. Greater than 90% of the radioactivity applied to the column was eluted at the position of this amino acid. Furthermore, no peak other than those corresponding to standard amino acids and (carboxymethyl)cysteine was present in the chromatogram (data not shown). Thus, iodoacetate reacts only with cysteine residues under these conditions, even though the reagent potentially reacts with several types of amino acid side chains in proteins (Grundlach et al., 1959a,b).

In the inactivation of guanidinoacetate methyltransferase with iodoacetate, a semilogarithmic plot of the residual activity against time was not linear; a rapid decrease in activity was followed by a slower reaction that eventually led to total loss of activity (Figure 4). The slow phase of the inactivation was apparently linear and had a pseudo-first-order rate constant of 0.017 min^{-1}. Extrapolation of this phase to the zero-time

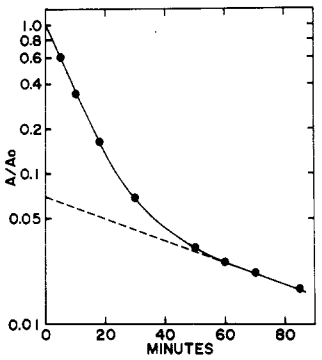

FIGURE 4: Inactivation of guanidinoacetate methyltransferase by iodoacetate. Guanidinoacetate methyltransferase (25 μM) was incubated with 3 mM iodoacetate at pH 8.5 and 30 °C. At times indicated, aliquots were removed for measurements of the residual activity.

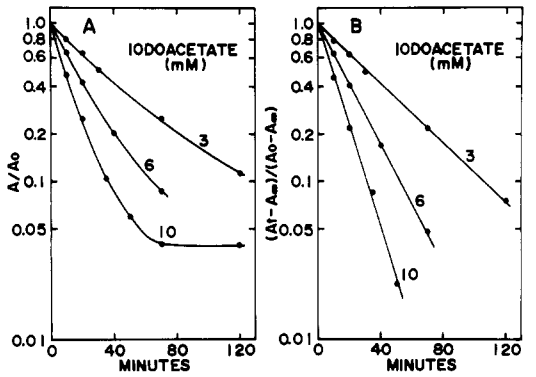

FIGURE 5: Inactivation by iodoacetate of guanidinoacetate methyltransferase with Cys-15 and Cys-90 cross-linked. The cross-linked enzyme prepared as described under Experimental Procedures was incubated at pH 8.5 and 30 °C with iodoacetate at the concentrations indicated. At time intervals, dithiothreitol (50 mM) was added to aliquots (30 μL) removed from the incubation mixture, and the residual activity was determined after 30 min. Panel B shows semilogarithmic plots obtained after the remaining activity was subtracted. A_0 and A_t represent enzyme activities at time 0 and t, and A_∞ is the remaining activity.

axis yielded a value of about 7% of the initial activity.

The kinetics of inactivation shown in Figure 4 suggests that guanidinoacetate methyltransferase has more than two modifiable cysteine residues, and alkylation of each of these differentially affects enzyme activity. Under the circumstances, assessment of reactivities and estimation of the numbers of essential and nonessential residues by the statistical method of Tsou (1962) would be extremely difficult. To obtain information on these points and also to identify the residues modified, we carried out the following experiments. Guanidinoacetate methyltransferase was incubated with 3 mM iodo[^{14}C]acetate under the conditions of Figure 4 for 18 min to achieve 85% inactivation. After removal of the radioactive reagent, the modified enzyme was carboxymethylated with unlabeled iodoacetate under denaturing conditions. Chymotryptic peptides derived from the enzyme sample were separated by HPLC, and radioactive peptides were purified under the same conditions as described in Figures 2 and 3. Three major radioactive peptides were obtained (not shown). Two of these peptides were identical with peptides I and II of Figure 3 as seen from their retention times in HPLC and amino acid compositions. The third peptide (peptide III) had an amino acid composition compatible with the cysteine-containing peptide comprising residues 212–222 (Cys-219) (Table II). Since all cysteine residues are fully carboxymethylated prior to proteolytic digestion, it is possible to determine the extent of modification by the radioactive reagent by dividing the specific radioactivity of peptide by that of the iodo[^{14}C]acetate used to inactivate the enzyme. Values of 0.52, 1.02, and 0.21 were found for the peptides containing Cys-15, Cys-90, and Cys-219, respectively. The sum of these values (1.75 mol of iodo[^{14}C]acetate incorporated/mol of enzyme) agrees well with the number of SH groups lost by iodoacetate treatment: with a 90% inactivated enzyme the number of SH groups titratable with DTNB under denaturing conditions was fewer by 1.79 compared with the native enzyme.

In addition to Cys-15 and Cys-90, which form a disulfide by the DTNB-mediated reaction, Cys-219 is carboxymethylated during the iodoacetate inactivation. To test whether modification of Cys-219 causes inactivation, guanidinoacetate methyltransferase having a disulfide link between Cys-15 and Cys-90 (prepared by the reaction with an equimolar concentration of DTNB) was incubated with iodoacetate, and the enzyme activity was determined after reduction by dithiothreitol. While greater than 95% of the activity of the native enzyme was recovered in the absence of iodoacetate as described above, there was a time- and concentration-dependent loss of recovery in its presence. The plot of logarithm of residual activity after treatment with dithiothreitol against time was not linear, as shown in Figure 5A, and prolonged incubation showed that ~4% of the initial activity did not disappear. When the remaining activity was subtracted from the observed values, the resulting plots were apparently linear at all concentrations of iodoacetate tested (Figure 5B). The apparent rate constant for inactivation was proportional to the iodoacetate concentration, and a second-order rate constant of 7.36 M^{-1} min^{-1} was calculated. As described above, when guanidinoacetate methyltransferase was treated with 1 equiv of DTNB, about 5% of the reagent is fixed to the third residue in the form of TNB mixed disulfide, and this modification also results in a loss of activity (cf. Table I). Since the activity found after prolonged incubated with iodoacetate is comparable in magnitude to the amount of TNB mixed disulfide present in the enzyme, it appears that the activity results from the reduction of the mixed disulfide rather than being an inherent property of the modified enzyme. This idea is supported by the observation that the same residual activity was obtained when *N*-ethylmaleimide was used instead of iodoacetate. The occurrence of the reaction to form TNB mixed disulfide would leave ~5% of Cys-15 and Cys-90 free and unprotected from the reaction with iodoacetate, and this should theoretically cause a deviation of the kinetic plot from linearity. However, the small percentage of unprotected enzyme would produce no experimentally discernible effect on the kinetics.

That the irreversible inactivation by iodoacetate under these conditions is due to the alkylation of Cys-219 was confirmed by peptide analysis of the iodo[^{14}C]acetate-inactivated enzyme. In the enzyme that had been inactivated to 10% residual activity (after treatment with dithiothreitol), the majority of radioactivity was associated with peptide III, which contains Cys-219. An incorporation of 0.81 mol of iodo[^{14}C]acetate/mol of peptide III was obtained. Prolonged incubation with iodo[^{14}C]acetate resulted in the appearance of another radioactive peptide, the amino acid composition of which was in agreement with the peptide containing Cys-207 (residues 202–211, peptide IV, Table II).

Guanidinoacetate methyltransferase that had been treated with excess DTNB was not irreversibly inactivated by iodoacetate or *N*-ethylmaleimide.

Lack of Effect of Substrates on Inactivation. The rate and extent of the reaction of guanidinoacetate methyltransferase

with DTNB as monitored by the absorbance change at 412 nm were not affected by the presence of AdoMet or guanidinoacetate even at concentrations greater than 20 times their respective K_m values. Also, the substrates did not protect the enzyme from inactivation by iodoacetate throughout the entire course of reaction. While AdoMet binds to the free enzyme [see below and Ogawa et al. (1983)], it is not known whether guanidinoacetate can bind to the free enzyme or if it binds only to the enzyme–AdoMet complex. If the latter is the case and the loss of activity is due to modification of a residue located at or near the guanidinoacetate-binding site, protection might be observed in the presence of a competitive inhibitor of guanidinoacetate and AdoMet. In an attempt to test this possibility, several compounds structurally related to guanidinoacetate were examined for their ability to act as a dead-end inhibitor. However, guanidinopropinonate, guanidinosuccinate, glycine, β-alanine, and γ-aminobutyrate all failed to inhibit the guanidinoacetate methyltransferase reaction even at 5 mM concentrations. Creatine (5 mM), the product of the reaction, also did not inhibit the reaction. Therefore, the experiment to test possible protection by guanidinoacetate analogues was not feasible.

Properties of Modified Guanidinoacetate Methyltransferases. The equilibrium dialysis study with [^{14}C]-AdoMet showed that guanidinoacetate methyltransferase having a cross-link between Cys-15 and Cys-90 and the enzyme with Cys-219 carboxymethylated both bound AdoMet as the native enzyme did. A K_d value of approximately 10 μM and a binding stoichiometry of 1 mol of AdoMet/mol of enzyme were obtained in each case (data not shown).

When excited at 280 nm, guanidinoacetate methyltransferase exhibited a fluorescence with an emission maximum at 330 nm, characteristic of a tryptophan-containing protein. The fluorescence intensity did not change upon addition of AdoMet or guanidinoacetate. Cross-linking of Cys-15 and Cys-90 and carboxymethylation of Cys-219 resulted in a decrease of the fluorescence intensity by 11 and 14%, respectively, without affecting the shape of the spectrum.

The circular dichroism spectra between 190 and 280 nm of the modified enzymes were essentially identical with that of the native enzyme (data not shown).

Discussion

Four of the five cysteine residues of guanidinoacetate methyltransferase are reactive toward iodoacetate in the native state. Analysis of chymotryptic peptides from the iodoacetate-modified enzyme shows that Cys-90, Cys-15, Cys-219, and Cys-207 are alkylated in order of decreasing reactivity. Cys-168 is not appreciably modified. The hydrophilicity profile of the enzyme predicted by the method of Hopp and Woods (1981) indicates that the regions containing residues 11–15 and 89–91 are highly exposed to the aqueous environment, while the region comprising residues 160–174 is buried in the hydrophobic interior of the protein (data not shown). In the reaction of the enzyme with DTNB, a disulfide bond is readily formed between Cys-15 and Cys-90. The DTNB-mediated disulfide cross-linking is caused by a nucleophilic attack of neighboring thiolate on the protein–TNB mixed disulfide formed. This type of reaction is reported with several enzymes (Holbrook & Jeckel, 1967; Kleppe & Damjanovich, 1969; Wassarman & Major, 1969; Connellan & Folk, 1969; Flashner et al., 1972; Talegdi & Straub, 1973). The facile formation of disulfide bond between Cys-15 and Cys-90 appears to indicate that they are in close proximity in the three-dimensional structure. However, the possibility exists that the initial modification of one of these residues distorts the protein conformation, thereby facilitating the cross-linking reaction with the other residue. Since both DTNB and iodoacetate require a thiolate anion for reaction, a very high reactivity of Cys-90 toward iodoacetate suggests that this residue is the site of attack of DTNB in the formation of disulfide, although it is possible that DTNB reacts with both Cys-90 and Cys-15 in a mutually exclusive manner. Besides Cys-15 and Cys-90, guanidinoacetate methyltransferase possesses two other cysteine residues that are less reactive toward DTNB and modified in the form of TNB mixed disulfide. Because of the occurrence of disulfide interchange between the TNB mixed disulfides and free SH groups under denaturing conditions, it was not possible to identify these residues by the method used to determine the cysteines involved in the disulfide cross-linking. However, the failure of the enzyme that has been treated with iodoacetate for a prolonged time to react with DTNB suggests that the same cysteine residues are modified by the two reagents. Analysis of reactivities of SH groups by the DTNB/cystamine system of Wilson et al. (1980) shows that one SH group that is unreactive toward DTNB can react slowly with cystamine (Figure 1). From the foregoing considerations, this residue seems to be Cys-168.

The inactivation of guanidinoacetate methyltransferase by iodoacetate exhibits complex kinetics. A semilogarithmic plot of the residual activity against time gives a curved line that becomes apparently linear as the reaction progresses (Figure 4). This type of plot would be obtained for the enzyme containing multiple modifiable residues if (1) modification of one of these leads to complete loss of activity and modification of the other residue(s) results in a partially active enzyme species or (2) modification at nonessential residue(s) decreases the rate of modification of the essential residue (Ray & Koshland, 1961). Under the conditions of Figure 4, Cys-15, Cys-90, and Cys-219 are modified. The slow phase of the inactivation has an apparent first-order rate constant of 0.017 min^{-1}. If it is assumed that the rate of modification of the residue responsible for this phase is not affected by modification of other residues, a fraction of 0.26 of this residue is calculated to be modified at 18 min when the enzyme is 85% inactivated. This value is comparable to the extent of radioactivity incorporation in Cys-219 (0.21) in the enzyme that has been 85% inactivated with iodo[^{14}C]acetate. The experiments with guanidinoacetate methyltransferase whose Cys-15 and Cys-90 are protected by cross-linking indicate that carboxymethylation of Cys-219 causes an all-or-none loss of activity with a rate constant of 0.022 min^{-1} (Figure 5B), comparable to that of the slow phase of Figure 4. From these observations and the fact that disulfide cross-linking of Cys-15 and Cys-90 largely but not completely destroys enzyme activity (Table I), the observed kinetics (Figure 4) appears to be consistent with the first possibility described above: carboxymethylation of Cys-219 eliminates all activity and alkylation of Cys-15 and/or Cys-90 results in 93% loss of activity, and these inactivation reactions proceed independently. If this postulate is correct, the rates at which Cys-15 and Cys-90 are modified further suggest that modification of both residues is required for the partial inactivation. When an enzyme has two residues that are involved in the expression of activity and modification of one residue completely destroys enzyme activity (with a rate constant of k_2) while modification of the other causes partial loss of activity (with a rate constant of k_1), the time course of activity loss will be given by eq 1 (Ray & Koshland, 1961), where F is the

$$A/A_0 = (1 - F)e^{-(k_1+k_2)t} + Fe^{-k_2t} \quad (1)$$

fractional activity compared to the original activity. Since F

is very small in the present case (0.07), the initial portion of the plot of Figure 4 would give an approximate value of (k_1 + k_2). With a value of 0.108 min^{-1} found for this phase and k_2 = 0.017 min^{-1} for the slow phase, the value of k_1 would be around 0.09 min^{-1}. At 18 min of incubation with iodoacetate, Cys-90 is fully carboxymethylated and a fraction of 0.52 of Cys-15 is carboxymethylated. When estimated from these values, the rate constant for modification of Cys-90 is too large and that of Cys-15 is too small compared to the value of 0.09 min^{-1}. Thus, it appears that modification of Cys-90 and Cys-15 each partially destroys activity, and a 93% loss of activity occurs when both are modified. If Cys-15 and Cys-90 are in close proximity, as suggested by their ability to form a disulfide, it would be difficult to imagine that modification of one residue exerts an influence on enzyme activity but that modification of the other does not.

The disulfide bond formation between Cys-15 and Cys-90 deprives the enzyme of a large portion of activity. The decrease of activity is apparently not due to lowering the affinities for substrates since the residual activity is not increased when assayed in the presence of increased concentrations of substrates. Also, the modified enzyme is shown to normally bind AdoMet. Although possible occurrence of Cys-219 at the guanidinoacetate-binding site cannot be rigorously ruled out by the data presently available, the fact that the introduction of a carboxymethyl group to Cys-219 does not affect the binding of AdoMet strongly suggests that it is not located in the active site. These results and the lack of effect of substrates on the rate of inactivation by iodoacetate and DTNB are indicative of Cys-15, Cys-90, and Cys-219 being located outside the active-site region and thus having no direct roles in binding and catalysis. The integrity of these residues appears to be crucial for the maintenance of the conformation required for catalysis. The occurrence of a gross conformation change upon modification of these residues is unlikely, however, because the modified enzymes show the fluorescence and circular dichroism spectra almost identical with those of the native enzyme.

Registry No. EC 2.1.1.2, 9029-75-8; DTNB, 69-78-3; L-Cys, 52-90-4.

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