Identification of a Tyrosine Residue in Rat Guanidinoacetate Methyltransferase That Is Photolabeled with *S*-Adenosyl-L-methionine†

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ABSTRACT: Exposure of rat guanidinoacetate methyltransferase to ultraviolet light in the presence of *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([*methyl*-^{3}H]AdoMet) results in covalent linking of radioactivity to the enzyme protein. The incorporation of radioactivity shows no lag and is linear with respect to time up to 1 h. The photolabeling is saturable with [*methyl*-^{3}H]AdoMet, and the binding constant of the enzyme for AdoMet determined in this experiment is similar to that obtained by equilibrium dialysis. Low concentrations of competitive inhibitors *S*-adenosyl-L-homocysteine and sinefungin effectively prevent the photoinduced labeling by AdoMet. Although guanidinoacetate methyltransferase is irreversibly inactivated upon ultraviolet irradiation in the absence of AdoMet, the enzyme inactivated by 1-h exposure to ultraviolet irradiation has been shown to bind AdoMet with an affinity identical to that of the native enzyme. These results indicate that photolabeling occurs at the active site. Following proteolysis of the [*methyl*-^{3}H]-AdoMet-labeled enzyme with chymotrypsin, a radioactive peptide is isolated having a sequence Asp-Thr-X-Pro-Leu-Ser-Glu-Glu-Thr-Trp. The peptide corresponds to residues 134–143, with X being modified Tyr-136. The same peptide is photolabeled when [*carboxy*-^{14}C]AdoMet is used. High-performance liquid chromatography of this peptide after acid hydrolysis and phenyl isothiocyanate derivatization suggests that the entire molecule of AdoMet is attached to Tyr-136.

S-Adenosyl-L-methionine- (AdoMet-)[1] dependent methyltransferases catalyze the transfer of methyl groups from AdoMet to a wide variety of compounds including nucleic acids, proteins, and small molecules. Whereas the amino acid sequence is determined for more than 70 methyltransferases, the X-ray crystallographic structure is not available for any enzyme, and little is known about structure–function relationships for this class of enzymes. Rat liver GAMT (EC 2.1.1.2), which catalyzes the formation of creatine from guanidinoacetate and AdoMet, is a simple protein consisting of a single polypeptide chain of 235 amino acid residues and is available in large amounts by the recombinant DNA procedure (Ogawa et al., 1988). Thus the enzyme is considered to provide a useful system for the study of structure–function relationships and has been the subject of investigation in this laboratory for the past several years. Previous chemical modification and site-directed mutagenesis studies (Fujioka et al., 1988; Takata et al., 1991; Konishi & Fujioka, 1991) and a limited proteolysis study (Fujioka et al., 1991) have indicated that Cys-15 is juxtaposed to Cys-90 and Cys-219 in the three-dimensional structure, and the flexibility of the N-terminal segment around residues 15–20 is important for the catalytic functioning of the enzyme. The portions of the enzyme containing these residues are apparently distant from the AdoMet-binding site. In an effort to localize the AdoMet-binding site in the primary sequence of GAMT, we attempted in this study to use AdoMet as a photoaffinity label and to determine the modified residue. The unmodified AdoMet has been shown to photolabel a number of AdoMet-dependent methylases including phenylethanolamine *N*-methyltransferase (Yu, 1983; Hurst et al., 1984), catechol *O*-methyltransferase (Yu, 1984), protein carboxyl methyltransferase (Hurst et al., 1984), *Eco*RII methyltransferase (Som & Friedman, 1990, 1991), and *dam* methylase (Wenzel et al., 1991). We report here that a covalent adduct is produced when GAMT is irradiated with UV light in the presence of AdoMet and the identification of a tyrosine residue modified with AdoMet. To our knowledge, this is the first report of a mammalian methyltransferase in which a residue within the binding region of AdoMet is identified.

Experimental Procedures

Materials. AdoMet (chloride salt), AdoHcy, sinefungin, and adenosine deaminase (type VI) were obtained from Sigma. The amino acid calibration mixture and PITC were purchased from Wako (Osaka, Japan), standard PTH-amino acids were from Pierce, and α-chymotrypsin was from Worthington. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine ([*methyl*-^{3}H]AdoMet) (82.5 Ci/mol) and *S*-adenosyl-L-[*carboxy*-^{14}C]methionine ([*carboxy*-^{14}C]AdoMet) (47.8 mCi/mmol) were obtained from Amersham and Du Pont-New England Nuclear, respectively. Solvents for HPLC were supplied by Cica-Merck (Tokyo, Japan). AdoMet was purified by passage through a C_{18} cartridge (Sep-Pak; Waters Associates) as described previously (Fujioka & Ishiguro, 1986). Iodoacetic acid (Merck) was recrystallized from hot chloroform. Other chemicals were of the highest purity available from commercial sources and were used without further purification. GAMT used in the present study was a recombinant enzyme (rGAMT) produced in

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[1] Abbreviations: GAMT, guanidinoacetate methyltransferase; rGAMT, recombinant rat guanidinoacetate methyltransferase, AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; PITC, phenyl isothiocyanate; PTC, phenylthiocarbamoyl; PTH, phenylthiohydantoin; DTT, dithiothreitol; HPLC, high-performance liquid chromatography.

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Escherichia coli JM109 (Yanisch-Perron et al., 1985) transformed with plasmid pUCGAT9-1 that contained the coding sequence of rat GAMT cDNA. The procedures for expression and purification of the enzyme were described previously (Ogawa et al., 1988). rGAMT lacks the N-terminal acyl group present in the liver enzyme but otherwise exhibits the same kinetic and physical properties as the liver enzyme. Molar concentrations of the enzyme were determined either by spectrophotometry using $\epsilon = 5.98 \times 10^4$ M^{-1} cm^{-1} at 280 nm or from protein concentrations using $M_r = 26000$ (Ogawa et al., 1988). Protein concentrations were determined by the method of Lowry et al. (1951) with purified rGAMT as the standard. Recombinant rat AdoHcy hydrolase used for measurements of GAMT activity was obtained as described (Gomi et al., 1989).

Enzyme Assay. The GAMT activity was determined spectrophotometrically by a coupled assay with AdoHcy hydrolase and adenosine deaminase (Fujioka et al., 1988). GAMT was incubated with 20 μM AdoMet and 0.5 mM guanidinoacetate in the presence of sufficient amounts of AdoHcy hydrolase and adenosine deaminase in 2.0 mL of 50 mM potassium phosphate (pH 8.0), and the decrease in absorbance at 265 nm that accompanied the overall conversion of AdoMet to inosine was monitored.

Photolabeling of rGAMT with Radioactive AdoMet. rGAMT was photolabeled with either [*methyl*-^{3}H]AdoMet (1.2×10^4 cpm/nmol) or [*carboxy*-^{14}C]AdoMet (5.9×10^4 cpm/nmol) in 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM DTT. Fifty-microliter aliquots of the reaction mixture were placed in the wells of a multititer plate on ice and irradiated at a distance of 7 cm from a 15-W germicidal lamp (Hitachi Ltd., Tokyo). No preincubation was found to be necessary prior to irradiation. The radioactivity incorporated into the enzyme was determined by scintillation counting after isolation of the protein by gel column centrifugation as described by Penefsky (1979) or by the filter paper disk method of Bollum (1968). Both methods gave the same results. The filter paper disk assay was carried out as follows. An aliquot (10–20 μL) withdrawn from the irradiation mixture was placed on a Toyo Roshi 51 filter paper disk (2.5 cm in diameter), and the disk was immediately immersed in 10% trichloroacetic acid. It was then washed five 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 toluene scintillation fluid.

Isolation of Chymotryptic Peptides Photolabeled by Radioactive AdoMet. A 2.5-mL mixture of rGAMT (40–50 μM) and 0.2 mM radioactive AdoMet was exposed to UV light in 50-μL aliquots for 1 h under the conditions described above. The rGAMT protein was separated from small molecules by gel filtration through a column of Sephadex G-25 equilibrated and eluted with 20 mM potassium phosphate (pH 7.2), containing 1 mM EDTA, followed by dialysis against the same buffer and then against water. The sample was S-carboxymethylated with iodoacetate after treatment with DTT (Darbre, 1986), and the resulting protein was digested with α-chymotrypsin (substrate-to-enzyme ratio, 100:1 w/w) in 0.1 M NH_4HCO_3 for 16 h at 37 °C. The chymotryptic digest was fractionated on a TSK ODS 120T column (0.46 × 25 cm) (Tosoh; Tokyo, Japan) using a gradient of acetonitrile in 0.05% trifluoroacetic acid. Rechromatography of the radioactive fraction was carried out on the same column with an acetonitrile gradient in 5 mM ammonium acetate (pH 6.8). Chromatographic conditions are described in the figure legends.

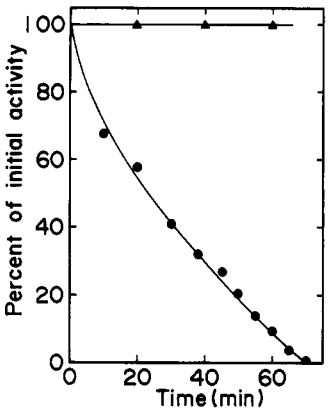

FIGURE 1: Inactivation of rGAMT by UV irradiation. rGAMT (17 μM) was UV irradiated in 0.1 M Tris-HCl (pH 8.0), containing 1 mM EDTA and 1 mM DTT. At the time indicated, aliquots (10–20 μL) were withdrawn from the incubation mixture and assayed for residual activity as described under Experimental Procedures. The enzyme not irradiated (▲) showed no loss of activity.

Peptide Sequencing. The amino acid sequences of isolated radioactive peptides were determined on an Applied Biosystems 470A gas-phase sequenator. PTH-amino acids were identified and quantified by HPLC with an Applied Biosystems 120A analyzer. In addition, aliquots of each step were determined for radioactivity.

Other Analytical Procedures. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out according to Laemmli (1970). Spectrophotometric and absorbance measurements were made with a Hitachi 320 spectrophotometer and fluorescence measurements with a Hitachi F-3010 fluorescence spectrophotometer. The binding of AdoMet to the native and photoinactivated rGAMT was determined by the equilibrium dialysis method. The enzymes (45–50 μM) were allowed to equilibrate with 0.02–0.3 mM [*methyl*-^{3}H]-AdoMet in 0.1 M Tris-HCl (pH 8.0), containing 1 mM EDTA and 1 mM DTT, for 16 h at 4 °C.

RESULTS

Effect of UV Irradiation on Guanidinoacetate Methyltransferase Activity. When rGAMT was UV irradiated in Tris-HCl buffer (pH 8.0), containing DTT and EDTA, there was a time-dependent loss of enzyme activity. The inactivation did not follow a simple decay kinetics. The rate of activity loss beyond 80% inactivation was greater than that expected from the initial phase of inactivation, and no activity was detectable after 70-min irradiation (Figure 1). The presence of 0.2 mM AdoMet in the incubation mixture did not change the inactivation pattern appreciably. No loss of activity was observed over the test period when the mixtures were not exposed to UV light. The inactivation is not due to UV-induced protein cross-linking of fragmentation; sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed no appreciable change in the intensity of the M_r 26000 band of rGAMT over a period of 1 h, at which time the enzyme activity decreased to <10%. Prolonged irradiation, however, caused a gradual disappearance of the M_r 26000 protein and the appearance of small amounts of high and low molecular weight materials (data not shown). rGAMT that had been UV irradiated for 1 h, despite being virtually inactive, retained the ability to bind AdoMet with a dissociation constant essentially identical to that of the untreated enzyme. An equilibrium dialysis study showed that both enzymes bound AdoMet with a rather high K_d of 0.085 mM in 0.1 M Tris-HCl (pH 8.0), containing 1 mM EDTA and 1 mM DTT, at 4 °C. The UV-treated enzyme had a UV absorbance at 280 nm not

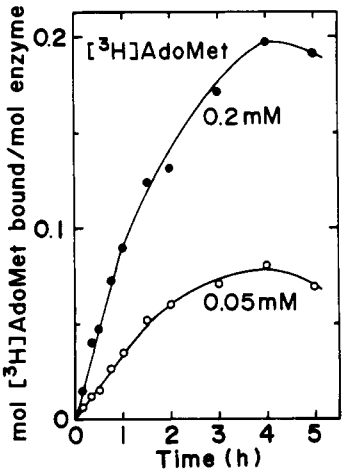

FIGURE 2: Time course of UV-induced radioactivity incorporation from [*methyl*-^{3}H]AdoMet. rGAMT (17 μM) was UV irradiated in the presence of 0.2 (●) and 0.05 mM (○) [*methyl*-^{3}H]AdoMet (1.2 × 10^4 cpm/nmol), and radioactivity incorporation was determined as described under Experimental Procedures.

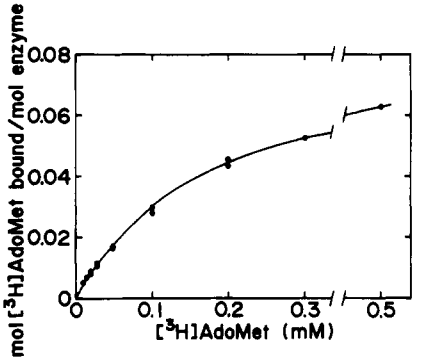

FIGURE 3: Concentration dependence of photolabeling of rGAMT with [*methyl*-^{3}H]AdoMet. rGAMT (35 μM) was exposed to UV light in the presence of various concentrations of [*methyl*-^{3}H]AdoMet for 30 min. The curve is drawn with K_d = 0.1 mM.

appreciably different from that of the native enzyme, whereas its fluorescence intensity at 337 nm (excitation wavelength, 280 nm) was decreased by 26% compared with the nonirradiated enzyme.

Photoincorporation of [methyl-^{3}H]AdoMet into rGAMT. Exposure of rGAMT to UV irradiation in the presence of [*methyl*-^{3}H]AdoMet resulted in incorporation of radioactivity into the protein. No radioactivity was fixed in the absence of UV light. The binding was covalent; the bound radioactivity was associated with the enzyme protein after precipitation with 10% trichloroacetic acid or ammonium sulfate and also withstood guanidine hydrochloride treatment and proteolysis. The uptake of radioactivity by the enzyme was linear up to about 1 h and then declined, and a maximum incorporation was reached at about 4 h (Figure 2). The extent of labeling was poor, and a maximum of 17–20% of the enzyme was radiolabeled with 0.2 mM AdoMet. That the radioactivity incorporation proceeds linearly for at least 1 h is consistent with the finding that the enzyme retains the ability to bind AdoMet normally over this period of irradiation. The decrease in radioactivity observed after 4 h is obviously due to enzyme destruction as seen by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Photolabeling of rGAMT as a function of AdoMet concentration was studied by exposing the enzyme (35 μM) to UV light for 30 min in the presence of 0.01–0.5 mM [*methyl*-^{3}H]AdoMet (Figure 3). The binding data conformed to a simple reversible bimolecular reaction between the enzyme and AdoMet, and a dissociation constant of 0.10 ± 0.01 mM was obtained by a least-squares method. This value is close to the one obtained in the equilibrium binding study performed under similar conditions (see above). When rGAMT that had been UV irradiated for 1 h in the absence of AdoMet was photolabeled with [*methyl*-^{3}H]AdoMet, the data points fell on the same curve (not shown), again confirming that the exposure did not affect AdoMet binding.

Table I: Effect of AdoMet Analogues on Photolabeling[a]

compound	concn (μM)	inhibition of photolabeling (%)
AdoHcy	5	25
	50	79
	200	96
	200	98[b]
sinefungin	160	62
	480	84
	960	99
ATP	500	33
	1000	44

[a] rGAMT (17 μM) was UV irradiated for 1 h with 0.1 mM [*methyl*-^{3}H]AdoMet in the absence and presence of the compounds indicated. The percent inhibition was obtained by comparing the radioactivities found in the presence and absence of the test compound. [b] rGAMT that had been UV irradiated for 1 h in the absence of AdoMet prior to photolabeling was used.

It has been shown previously that GAMT binds substrates in an obligatory order: AdoMet is bound first followed by guanidinoacetate (Fujioka et al., 1991). The product of the reaction AdoHcy and an AdoMet analogue sinefungin compete with AdoMet in binding (Konishi & Fujioka, 1991). Although these compounds had little or no effect on photoinduced inactivation of rGAMT, at low concentrations they protected the UV-dependent incorporation of [*methyl*-^{3}H]AdoMet (Table I). Complete protection was achieved with AdoHcy and sinefungin when these compounds were present in 2- and 10-fold molar excess over AdoMet, respectively. That AdoHcy is a more effective inhibitor compared with sinefungin is explained on the basis of their affinities for the enzyme. A K_i value of 0.35 μM has been obtained kinetically for the former and one of 5.7 μM for the latter at 30 °C and pH 8.0.[2] High concentrations of ATP caused only a moderate reduction in the amount of radioactivity incorporated. Since ATP does not bind to GAMT, this is likely due to optical shielding by the compound. Under the conditions of Table I, no appreciable radioactivity was incorporated into AdoHcy hydrolase and bovine serum albumin, which do not bind AdoMet. Thus the results described above indicate that AdoMet becomes covalently bound at the AdoMet-binding site of rGAMT.

Isolation of Peptide Photolabeled with [methyl-^{3}H]AdoMet. In order to localize the sites photolabeled with AdoMet in the primary structure, chymotryptic peptides were prepared from rGAMT that had been UV irradiated with [*methyl*-^{3}H]AdoMet for 1 h, and radioactive peptides were isolated by HPLC. As only a small fraction of the enzymes was radiolabeled under the conditions (see above) and it is possible that multiple residues at the binding site are labeled, we used in this experiment AdoMet of higher specific radioactivity (1.2 × 10^5 cpm/nmol) than that used for the incorporation experiments. To isolate radioactive peptides, we first determined the elution profile of radioactivity by subjecting a small fraction of the digest to HPLC. Fractions were collected manually at 1-min intervals, and aliquots from each fraction were counted.

[2] T. Gomi, K. Tanihara, T. Date, and M. Fujioka, manuscript submitted for publication.

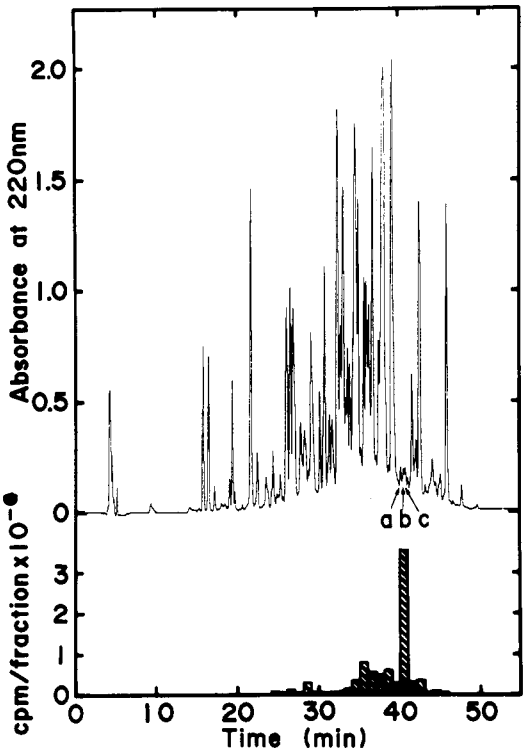

FIGURE 4: HPLC profile of the chymotryptic digest derived from rGAMT photolabeled with [*methyl*-^{3}H]AdoMet. rGAMT (42 μM) was exposed to UV light in the presence of 0.2 mM [*methyl*-^{3}H]-AdoMet (1.2×10^5 cpm/nmol) for 1 h. The resulting enzyme was S-carboxymethylated with iodoacetate and digested with chymotrypsin as described under Experimental Procedures. A fraction of the digest (~5 nmol) was fractionated on a TSK ODS 120T column with a gradient of acetonitrile in 0.05% trifluoroacetic acid. The concentration of acetonitrile was varied from 0 to 48% over a period of 50 min starting at 10 min. The flow rate was 0.8 mL/min. The effluent was monitored by absorbance at 220 nm and collected manually in 1-min fractions. Aliquots from each fraction were assayed for radioactivity. The shaded bars indicate the total radioactivity found in each fraction.

Table II: Amino Acid Sequence Data

	radioactive peptide from enzyme labeled with					
	[*methyl*-^{3}H]AdoMet			[*carboxy*-^{14}C]AdoMet		
cycle	residue	pmol	cpm	residue	pmol	cpm
1	Asp	351	9294	Asp	91	68
2	Thr	167	3063	Thr	20	73
3	X		1266	X		270
4	Pro	23	152	Pro	4	34
5	Leu	9	142	Leu	2	27
6	Ser	11	53	Ser	2	20
7	Glu	6	46			
8	Glu	6	22			
9	Thr	3	20			
10	Trp	2	15			

Whereas radioactivity was distributed in several fractions, about 41% of the recovered radioactivity was found in a single fraction collected between 40 and 41 min, which contained 3 small peaks of absorbance (a, b, and c) (Figure 4). Thus, in the chromatography of the rest of the digest, each of these peaks was collected separately, and peak b was found to be the only peptide that contained radioactivity. Rechromatography in a different solvent system revealed that peak b consisted of a number of peptides, and the major peptide eluting at 21 min contained greater than 75% of the radioactivity loaded (Figure 5).

Automated Edman degradation of the radioactive peptide isolated above gave a sequence Asp-Thr-X-Pro-Leu-Ser-Glu-Glu-Thr-Trp (Table II). This sequence uniquely fits to

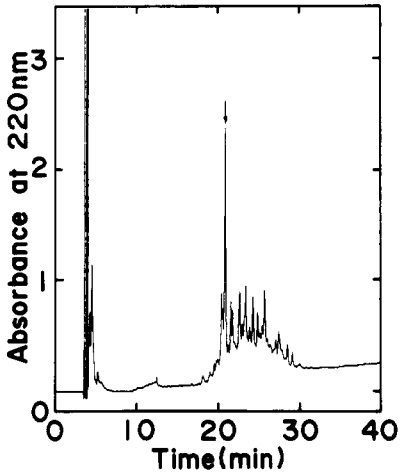

FIGURE 5: Rechromatography of peak b of Figure 4. The effluent corresponding to peak b of Figure 4 was rechromatographed on a TSK ODS 120T column with a gradient of acetonitrile in 5 mM ammonium acetate (pH 6.8). Acetonitrile gradient: 0–16% between 10 and 15 min; 16–80% between 15 and 60 min. The radioactive peptide is indicated by an arrow.

residues 134–143, with X corresponding to Tyr. Whereas an unidentified PTH derivative appeared in cycle 3, most of the radioactivity was found in the first cycle of sequencing, suggesting that the radioactive methyl group of the photoadduct or a portion containing it was unstable to sequencing procedures. To confirm that Tyr-136 is the site of modification, we performed a similar experiment using [*carboxy*-^{14}C]-AdoMet. The extent of labeling by this compound was about the same as that obtained with [*methyl*-^{3}H]AdoMet, and a peptide of major radioactivity could be isolated as above. This peptide had an identical sequence, but radioactivity was recovered mainly in cycle 3 (Table II).

Nature of the Photoproduct. Although the fact that radioactivity is introduced from both methyl-labeled and carboxy-labeled AdoMet indicates the presence of the methionine moiety in the photoadduct, it is not known whether it contains the adenosine portion of the AdoMet molecule. In order to gain insight into the nature of the product, the decapeptide obtained above was subjected to amino acid analysis by the PITC precolumn derivatization method (Bidlingmeyer et al., 1984). In addition to the peaks of PTC derivatives of aspartate, glutamate, serine, threonine, proline, and leucine, two peaks were observed at 11.7 and 19.2 min in HPLC (Figure 6A), which coincided with the peaks observed when AdoMet, carried through acid hydrolysis and PITC treatment, was analyzed (Figure 6B). The 19.2-min peak was at the position of PTC-Met, whereas the 11.7-min peak originated from the adenine moiety (Figure 6C). The peak corresponding to PTC-tyrosine, which should appear at around 17 min, was not observed, indicating that tyrosine was not recovered from the adduct by acid treatment. Also, no unidentified peak was found under the chromatographic conditions used. Although the nature of the modified tyrosine is not known, the results obtained above indicate that the entire molecule of AdoMet is covalently attached to the peptide.

Discussion

Exposure of rGAMT to UV light results in a rather rapid, irreversible loss of enzyme activity. Almost all activity is lost within 70 min under the conditions of Figure 1. Although extensive enzyme destruction is observed upon prolonged irradiation, this inactivation occurring early in the incubation is not due to protein aggregation or peptide bond cleavage. The kinetics of activity loss suggests that an initial photo-

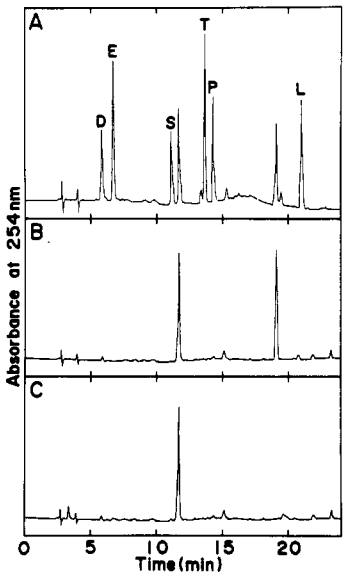

FIGURE 6: Amino acid analysis of the radioactive peptide from rGAMT photolabeled with [*methyl*-^{3}H]AdoMet. (A) The radioactive peptide from the [^{3}H]AdoMet-modified enzyme was hydrolyzed in vacuo in the vapor phase of 5.7 M HCl containing 0.1% phenol and 10 mM DTT for 24 h at 108 °C. The hydrolysate was treated with PITC as described by Bidlingmeyer et al. (1984) and then subjected to reverse-phase HPLC on Develosil ODS-7 (0.46 × 25 cm) (Nomura Chemicals, Nagoya, Japan) with a linear gradient from 0.1 M ammonium acetate to 60% acetonitrile (v/v) at a flow rate of 1.0 mL/min. The effluent was monitored by absorbance at 254 nm. (B) AdoMet and (C) adenine were treated similarly with HCl and PITC and chromatographed under the same conditions.

chemical reaction at certain residue(s) triggers a conformational change that renders the residue(s) critical for activity susceptible to photoreaction. A more remote possibility would be that reaction occurs at multiple residues independently and, although modification at either one of these residues does not diminish activity, complete loss of activity results when all are modified.

In the presence of radioactive AdoMet, UV irradiation causes covalent linking of radioactivity to the protein. Despite the loss of catalytic activity, the radioactivity incorporation proceeds linearly for at least 1 h (Figures 1 and 2). This requires that the inactive enzyme can still bind AdoMet and AdoMet is not degraded during the test period. An equilibrium dialysis study showed that rGAMT that had been inactivated by 1-h exposure to UV light in the absence of AdoMet bound AdoMet with a K_d value identical to that of the nonirradiated enzyme. Also, the UV-treated enzyme can be photolabeled with AdoMet exhibiting the same concentration dependence. Although the nature of this rGAMT which is catalytically inactive and retains the ability to bind AdoMet normally has not been explored extensively, the decrease in intensity of intrinsic protein fluorescence suggests some structural changes. (The UV absorption spectrum is not appreciably changed.) On the basis of the results of chemical modification studies (Fujioka et al., 1988; Konishi & Fujioka, 1991) and limited proteolysis (Fujioka et al., 1991), it has been proposed that the N-terminal region of GAMT up to the residue around 30 is on the protein surface and is flexible. Cross-linking of Cys-15 with Cys-90 or Cys-219 by a disulfide bond (Takata et al., 1991) or mixed disulfide formation between Cys-15 and glutathione (Konishi & Fujioka, 1991) results in an enzyme with little or no activity but with the ability to bind AdoMet almost normally. Thus it is not inconceivable that photoinduced modification of some residues (probably the ones occurring on the protein surface) and/or the resulting conformational change cause(s) inactivation without affecting the affinity for AdoMet significantly. AdoMet was found to be stable to UV light; AdoMet exposed to irradiation for 3 h exhibited the same behavior in HPLC as the untreated compound and served as the substrate of GAMT. The UV stability of AdoMet is also reported by Som and Friedman (1990).

In affinity-labeling experiments with substrate analogues, linear portions are often observed in the high concentration range of binding curves, and this is usually considered as representing nonspecific binding. In the AdoMet concentration dependence of rGAMT photolableing (Figure 3), the portion of the curve above 0.25 mM AdoMet may appear to be linear. However, in the present case it is not likely that nonspecific binding occurs to a significant extent. (a) Unmodified AdoMet, which binds stoichiometrically at the active site, is used. (b) The photoincorporation data of Figure 3 can be described in terms of a simple reversible bimolecular reaction between rGAMT and AdoMet with a K_d of 0.1 mM. This value of K_d is close to the one found in the equilibrium binding study. The curve is not a hyperbola because AdoMet concentration is varied between 0.29[E] and 14.3[E]. The values obtained by subtracting the ones that appear due to nonspecific binding (by use of a straight line going through the origin and parallel to the apparently linear portion) cannot be fitted to a bimolecular kinetics. (c) The UV-induced incorporation of AdoMet is prevented completely by competitive inhibitors such as AdoHcy and sinefungin (Table I). AdoHcy hydrolase, which binds AdoHcy but not AdoMet, is not photolabeled by AdoMet under the same conditions. (d) The labeling is linear with respect to time, and no lag is observed. This excludes the possibility that UV-induced denaturation of the enzyme is prerequisite to photoadduct formation. Thus, the available data are consistent with the idea that the photoinduced covalent linking of AdoMet takes place solely at the active site, though it is possible that more than one residue within the binding pocket becomes photolabeled.

Using AdoMet radiolabeled at different positions (methyl group and carboxy group), we could obtain the same major radioactive chymotryptic peptide. Automated sequencing on an Applied Biosystems gas-phase sequenator shows that it is the peptide containing residues 134–143, with a modified tyrosine at position 136. In sequencing the peptide from the [*methyl*-^{3}H]AdoMet-modified enzyme, however, the majority of radioactivity was found in the first cycle and much lesser amounts in the following two cycles. In contrast, with the peptide derived from the [*carboxy*-^{14}C]AdoMet-modified enzyme most of the radioactivity was released in cycle 3, together with an unidentified PTH-amino acid (Table II). These results indicate that although Tyr-163 is modified with AdoMet, the methyl group of the photoadduct is labile to sequencing methods. Although the chemistry leading to loss of the methyl group is not known, a similar instability of the AdoMet methyl group in sequencing is noted by Reich and Everett (1990) and by Som and Friedman (1991). The presence of the methyl and carboxy groups of AdoMet in the photoadduct and the result of amino acid analysis of the decapeptide containing the adduct (Figure 6) indicate that the entire AdoMet molecule is covalently linked to Tyr-136.

The primary structures of a number of AdoMet-dependent methyltransferases are now known. Most of the enzymes of known sequence are DNA or RNA methylases of prokaryotes, and the sequences of only seven enzymes are reported among mammalian methyltransferases. Mammalian methyltransferases are characterized by the presence of two conserved

sequences, Leu-(Asp/Glu)-(Val/Leu/Ile)-Gly-Xaa-Gly-Xaa-Gly and Leu-(Lys/Arg)-Pro-Gly-Gly-Xaa-Leu. The sequences occur in GAMT at positions 64–71 and 159–165, respectively. Tyr-136 is not included in either of these segments. Whereas relevance of the conserved regions in AdoMet binding is still not clear, replacement of Lys-160, Pro-161, Gly-162, Gly-163, or Leu-165 with other amino acids results in structural alterations at around residue 20.[2] The same area is perturbed upon formation of the E·AdoMet/sinefungin·guanidinoacetate ternary complex (Fujioka et al., 1991; Konishi & Fujioka, 1991). Thus, it may be suggested that the region of GAMT comprising residues 160–165 is located at or near the active site. It is possible that the AdoMet-binding pocket is constructed from noncotiguous segments of the molecule. The AdoMet-binding sites of *Eco*RII methylase (a cytosine DNA methylase) (Som & Friedman, 1991) and *Eco*RI methylase (an adenine DNA methylase) (Reich & Everett, 1990) have recently been probed by photoaffinity labeling with unmodified AdoMet and 8-azido-AdoMet, respectively. In *Eco*RII methylase the major product of photoreaction, *S*-methylcysteine, is contained in a conserved area of cytosine methylases, whereas the photolabeled region of *Eco*RI methylase is not included in the conserved sequences of adenine methylases. No sequence similarity is found among the modified regions of GAMT, *Eco*RI methylase, and *Eco*RII methylase.

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Registry No. GAMT, 9029-75-8; Tyr, 60-18-4; AdoMet, 29908-03-0.

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