Probing the *S*-adenosylmethionine-binding site of rat guanidinoacetate methyltransferase

Effect of site-directed mutagenesis of residues that are conserved across mammalian non-nucleic acid methyltransferases

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Most mammalian non-nucleic acid methyltransferases share three sequence motifs. To gain insight into the *S*-adenosylmethionine (AdoMet)-binding site of guanidinoacetate methyltransferase, we mutated several conserved residues that are found in or near motifs I and II. Conversion of either of two glycine residues of motif I (Gly⁶⁷ and Gly⁶⁹) to an alanine resulted in an inactive enzyme. These enzymes, although having UV absorption, fluorescence and far-UV CD spectra virtually identical with those of the wild-type enzyme, seem to be conformationally different from the wild-type enzyme as judged by near-UV CD spectra and the extent of urea denaturation, and

are apparently not capable of binding AdoMet. Mutation of Tyr¹³⁶ of motif II to a valine resulted in a decrease in k_{cat}/K_m values for substrates. Changing this residue to a phenylalanine caused only a minor change in k_{cat}/K_m for AdoMet. This suggests that the aromatic side chain stabilizes the binding of AdoMet. Mutagenic changes of Glu⁸⁹, which is the residue corresponding to the conserved acidic residue on the C-terminal side of motif I, indicated its contribution to AdoMet binding. These results are consistent with the idea that both motifs I and II are crucial in forming the AdoMet binding site of guanidinoacetate methyltransferase.

INTRODUCTION

Most mammalian non-nucleic acid methyltransferases are monomeric proteins of rather small size and share three regions of sequence similarity (motifs I, II and III from the N-terminal side) [1]. The sequence of motif I is glycine-rich and is reminiscent of the nucleotide binding motifs found in dehydrogenases and kinases; motif II is characterized by the presence of a conserved aspartate residue that is surrounded by hydrophobic residues. Motif III has rather well-defined fingerprint residues [2]. The three motifs are always found in the same order on the polypeptide chain and separated by similar intervals.

To gain insight into the structural and functional roles of these motifs, we have chosen guanidinoacetate methyltransferase (EC 2.1.1.2) on which to perform site-directed mutagenesis studies. Guanidinoacetate methyltransferase catalyses the *S*-adenosylmethionine (AdoMet)-dependent methylation of guanidinoacetate to form creatine, and is believed to be a major enzyme involved in the metabolic conversion of AdoMet to *S*-adenosylhomocysteine (AdoHcy) in mammalian livers [3]. Rat guanidinoacetate methyltransferase protein consists of a single polypeptide chain containing 235 amino acid residues [4], and has the three sequence motifs at positions 63–71, 133–136 and 159–165 respectively. A previous mutagenesis study has shown that conversion of the conserved aspartate residue of motif II (Asp¹³⁴) to either a glutamate or asparagine resulted in an enzyme with a greatly reduced affinity for AdoMet [5]. The decrease in binding energy due to mutation was greater than could be accounted for by the loss of a single hydrogen bond or electrostatic interaction. This suggests that, although being not directly involved in the binding, the aspartate residue might be a major structural determinant of the AdoMet site. The occurrence of Asp¹³⁴ in the AdoMet-binding pocket is corroborated by the finding that AdoMet becomes covalently linked to Tyr¹³⁶ on irradiation by UV [6]. In contrast, motif III of guanidinoacetate methyl-

transferase has no apparent role in substrate binding and catalysis; the enzymes in which each of the fingerprint residues is replaced by a similar amino acid have k_{cat} and K_m values essentially identical with those of the wild-type enzyme [2]. The present paper describes the results of a further study aimed at defining the role of the regions or residues that are conserved across mammalian non-nucleic acid methyltransferases, and shows that glycine at positions 67 and 69 (motif I) is crucial to AdoMet binding in guanidinoacetate methyltransferase, and the aromatic side chain of Tyr¹³⁶ (motif II) stabilizes its binding.

MATERIALS AND METHODS

Materials

AdoMet (chloride salt), AdoHcy and adenosine deaminase (type VI) were obtained from Sigma. AdoMet was purified by passage through a C₁₈ cartridge (Sep-Pak; Waters Associates) as described previously [7]. *S*-Adenosyl-L-[methyl-³H]methionine ([methyl-³H]AdoMet) (82.5 Ci/mol) was purchased from Amersham. AdoHcy hydrolase used in the assay of guanidinoacetate methyltransferase activity was a recombinant rat enzyme produced in *Escherichia coli* [8]. The polyclonal antibody against rat guanidinoacetate methyltransferase was raised in rabbits and purified by chromatography on a guanidinoacetate methyltransferase-coupled Sepharose 4B column before use as described by Shutz et al. [9]. Other chemicals were of the highest purity available from commercial sources and were used without further purification.

Wild-type recombinant guanidinoacetate methyltransferase

Wild-type recombinant rat guanidinoacetate methyltransferase was produced in *E. coli* JM109 [10] transformed with pUCGAT9-1 containing the coding region of rat guanidinoacetate methyl-

Table 1 Oligonucleotides used for mutagenesis

The codons changed are underlined, and the nucleotides changed are shown in bold.

Mutant	Mutagenic oligonucleotide (3' → 5')	Codon change
G67A	CTTCA CCG GAAACCGT	GGC → GCG
G69A	CCCGAA ACG CTACCGGT	GGG → GCG
E89Q	TAATA AG TTACGTTG	GAA → CAA
E89A	CTAATA CG TACGTTGC	GAA → GCA
E89K	CTAATA T TTACGTTGC	GAA → AAA
D92N	TACGTT GT TACCCAG	GAT → AAT
Y133V	TAAGAT CA GCTGTGTATAGG	TAC → GTC
Y136V	CTGTGT CA AGGTGACAGA	TAT → GTT
W143L	TTCTCTGG AC GTGTGAGTG	TGG → CTG
W143F	TTCTCTGG AG GTGTGAGTG	TGG → TTC

transferase cDNA downstream of the *lac* promoter [4]. The enzyme was purified to electrophoretic homogeneity by a three-step procedure including ammonium sulphate fractionation, Sephacryl S-200 gel filtration and DEAE-cellulose chromatography [4]. The molar concentration of the enzyme was determined either spectrophotometrically by using $\epsilon = 5.98 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm or from protein concentration by taking the molecular mass as 26 kDa [4]. Protein concentrations were determined by the method of Lowry et al. [11] with recombinant guanidinoacetate methyltransferase as the standard.

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis was used to prepare cDNAs encoding mutant forms of guanidinoacetate methyltransferase. Mutagenic oligonucleotides (listed in Table 1) were synthesized on a Cyclone Plus DNA synthesizer (MilliGen/Biosearch). Mutants were designated by the one-letter symbol of the amino acid residue being mutated followed by its sequence number and the symbol of the substituted residue. Mutagenesis was performed by the method of Kunkel et al. [12], with a Mutan-K site-directed mutagenesis kit (Takara Shuzo, Kyoto, Japan). Clones containing the desired mutation were identified by nucleotide sequence analysis across the mutation site by the dideoxy chain termination method [13].

Purification of mutant enzymes

The mutant forms of guanidinoacetate methyltransferase were produced in *E. coli* JM109 transformed with pUC118 plasmid containing the respective mutant sequences. The cells were grown in 2 × YT medium (1 × YT = 10 g of bacto-yeast extract, 16 g of bacto-tryptone and 5 g of NaCl per litre) containing 50 mg/l ampicillin at 37 °C. When D_{600} reached 0.3, isopropyl-1-thio- β -D-galactopyranoside was added to a final concentration of 1 mM and culturing was continued for an additional 16 h to induce the enzymes. All mutant enzymes were purified from the soluble fractions of *E. coli* extracts, and were purified to electrophoretic homogeneity by a procedure similar to that used for the purification of the wild-type enzyme [4].

Enzyme assay

To avoid product inhibition by AdoHcy, the guanidinoacetate methyltransferase activity was determined in the presence of AdoHcy hydrolase and adenosine deaminase. The wild-type and mutant forms of guanidinoacetate methyltransferase were incubated with various concentrations of AdoMet and guanidino-

acetate, together with excess recombinant rat AdoHcy hydrolase and calf intestinal mucosa adenosine deaminase in 0.05 M potassium phosphate at pH 8.0 and 30 °C. The apparent kinetic constants were obtained by varying the concentration of one substrate at a constant high concentration (usually more than 5 times the K_m) of the other. The product inosine was quantitated by HPLC. Details of the assay procedure are described elsewhere [5].

Urea denaturation

Stock urea solution was freshly prepared before each experiment from ultrapure urea purchased from Wako Chemicals (Osaka, Japan). The wild-type and mutant forms of guanidinoacetate methyltransferase (0.1 mg/ml) were incubated in 0.1 M Tris/HCl, pH 7.5, containing 5 mM dithiothreitol and various concentrations of urea, for 30 min at 25 °C. Denaturation was followed by monitoring the change in intrinsic protein fluorescence. The wild-type and mutant guanidinoacetate methyltransferases showed virtually identical fluorescence spectra with an emission maximum at 337 nm when excited at 280 nm. On denaturation with 8 M urea, the emission maximum was shifted to 351 nm with a slight reduction in fluorescence intensity. A wavelength of 326 nm, which shows the greatest fluorescence difference between the native and denatured states, was used to follow denaturation. The reversibility of denaturation was ascertained by the recovery of original spectra on removal of urea by dialysis. Assuming that no intermediate exists in the transition from the native to denatured state, the free energy change of unfolding at a given urea concentration (ΔG_D) was calculated from the equation $\Delta G_D = -RT \ln K_D$, where K_D was the equilibrium constant between the folded and unfolded states. The plot of ΔG_D against urea concentration was always linear, and the free energy change of unfolding at zero urea concentration, $\Delta G_D(\text{H}_2\text{O})$, was obtained by the equation, $\Delta G_D = \Delta G_D(\text{H}_2\text{O}) - m[\text{urea}]$, where m was the slope of the plot.

Photoaffinity labelling with AdoMet

Photoaffinity labelling of the wild-type and mutant enzymes was performed as described previously [6]. Briefly, a mixture of the enzyme and [*methyl*- ^3H]AdoMet (1.2×10^4 c.p.m./nmol) in 0.1 M Tris/HCl, pH 8.0/1 mM EDTA/1 mM dithiothreitol was placed in the wells of multititre plate in 50 μl aliquots and irradiated at a distance of 7 cm with a 15 W germicidal lamp at 0 °C. The protein was separated from excess reagent by gel column centrifugation as described by Penefsky [14], and assayed for protein concentration and bound radioactivity.

Other analytical methods

SDS/PAGE [15] and immunoblot analysis [8] were performed as described. Spectrophotometric and absorbance measurements were made with a Hitachi 320 recording spectrophotometer. Fluorescence spectra were recorded with a Hitachi F-3010 fluorescence spectrophotometer and CD spectra with a Jasco J-500C spectropolarimeter.

RESULTS AND DISCUSSION

Figure 1 shows the alignments of regions around motifs I and II of mammalian non-nucleic acid methyltransferases. To investigate the role of the key residues in or near the motifs we prepared in the present study mutant forms of guanidinoacetate methyltransferase with single substitutions at Gly⁶⁷, Gly⁶⁹, Glu⁸⁹, Asp⁹², Tyr¹³³, Tyr¹³⁶ and Trp¹⁴³.

Enzyme	Motif	
	I	II
rGAMT	63 VLEVGFGMA...17...E...43...YDTY	
rGNMT	60 VLDVACGTG...16...D...44...FDAV	
rDHPBMT	91 ILDVGC GGG...16...D...50...FDAV	
rPIMT	82 ALDVGSGSG...19...D...42...YDAI	
rPNMT	76 LIDIGSGPT...17...D...75...ADAL	
hNNMT	59 LIDIGSGPT...17...D...71...ADCV	
mTEMt	62 LIDIGSGPT...17...D...69...ADCV	
bHIOMT	183 ICDLGGGSG...18...D...28...KDAL	
rCOMT	62 VLELGAYCG...19...E...41...VDTL	
rHMT	56 ILSIGGGAG...21...E...94...WDFI	
Consensus	oL ^D oGsGsG.....D.....oDso	
	E	E

Figure 1 Alignments of sequences around motifs I and II of mammalian non-nucleic acid methyltransferases

The sequences are from GenBank/EMBL/DBJ databases. The enzymes are abbreviated and their accession numbers are given in parentheses: rGAMT, rat guanidinoacetate methyltransferase (J03588); rGNMT, rat glycine N-methyltransferase (X06150); rDHPBMT, rat dihydroxypolyprenylbenzoate methyltransferase (L20427); rPIMT, rat protein isopartyl methyltransferase (D11475); rPNMT, rat phenylethanolamine N-methyltransferase (X14211); hNNMT, human nicotinamide N-methyltransferase (U08021); mTEMt, mouse thioether methyltransferase (M88694); bHIOMT, bovine hydroxyindole O-methyltransferase (J02671); rCOMT, rat catechol O-methyltransferase (M60754); rHMT, rat histamine methyltransferase (D10693). The number at the left of each sequence indicates the position of the first residue and the numbers within the sequence represent the numbers of intervening amino acids. The letters o and s denote hydrophobic and small neutral amino acids respectively.

Purification of mutant enzymes

The cell-free extracts of *E. coli* transfected with respective mutant plasmids all contained a 26 kDa protein, which was immunoreactive with anti-(rat guanidinoacetate methyltransferase) IgG (results not shown). The amounts of the proteins produced were variable: G67A was produced much less efficiently than the wild-type recombinant rat guanidinoacetate methyltransferase and other proteins. The proteins could be purified to electrophoretic homogeneity by a simple procedure similar to that used for the

purification of the wild-type enzyme. The purified mutants were obtained in a yield ranging from 10 to 20 mg from 1 litre of the bacterial culture except for G67A, which was obtained in an amount as low as 0.2 mg/litre.

Properties of motif I mutant enzymes

The consensus sequence of motif I is reminiscent of the glycine-rich sequence of the nucleotide-binding motif of dehydrogenases and kinases [16]. The nucleotide-binding motif has a sequence GXGXXG (X denotes any amino acid) and this portion forms a turn joining the first β -strand and the α -helix of the nucleotide-binding $\beta\alpha\beta$ fold (Rossmann fold). Mutation of glycines of this sequence has been shown to exert profound effects on nucleotide binding and catalysis in a number of enzymes [8,17–19]. Thus, to examine the functional significance of motif I of guanidinoacetate methyltransferase, we mutated each of Gly⁶⁷ and Gly⁶⁹ to alanine. The mutants G67A and G69A showed no activity even at AdoMet and guanidinoacetate concentrations as high as 8 and 10 mM respectively (Table 2).

The UV absorption, fluorescence and far-UV CD spectra of both G67A and G69A were almost identical with those of the wild-type enzyme (results not shown), but a distinctive difference was noted in the near-UV CD spectrum between the wild-type and mutant enzymes (Figure 2). Whereas the shapes of the spectra were nearly identical, the values of mean residue ellipticity at approx. 282 nm was reduced to two-thirds in the mutant enzymes, indicating some conformational differences. The mutant enzymes were also conformationally less stable than the wild-type enzyme, as shown by the urea denaturation experiment. The free energy change of unfolding at each urea concentration (ΔG_D) was calculated from the equilibrium constant between the folded and unfolded states, as described in the Materials and methods section. As shown in Figure 3, plots of ΔG_D against urea concentration were linear in all cases, and linear extrapolation to zero urea concentration [20] gave $\Delta G_D(\text{H}_2\text{O})$ values ($\pm$ S.D.) of 7.50 ± 0.21 , 3.93 ± 0.11 and 5.06 ± 0.19 kcal/mol (31.4 ± 0.88 , 16.44 ± 0.46 and 21.17 ± 0.79 kJ/mol) for wild-type, G67A and G69A respectively. Thus changing the glycine residue at these

Table 2 Kinetic constants of motif I and motif II mutant enzymes

Initial velocity measurements were made at pH 8.0 and 30 °C as described in the Materials and Methods section. Values of kinetic constants were obtained from fits to the Michaelis–Menten equation. Values are given $\pm$ S.D. with two or three determinations being made for each point. Abbreviation: n.d., not detected.

Enzyme	k_{cat} (min ⁻¹)	AdoMet		Guanidinoacetate	
		K_m (μ M)	k_{cat}/K_m (min ⁻¹ · μ M ⁻¹)	K_m (μ M)	k_{cat}/K_m (min ⁻¹ · μ M ⁻¹)
WT	4.73 $\pm$ 0.15	4.91 $\pm$ 0.35	0.96	10.71 $\pm$ 1.41	0.44
G67A	n.d.				
G69A	n.d.				
E89Q	3.15 $\pm$ 0.16	12.03 $\pm$ 4.10	0.26	13.11 $\pm$ 2.52	0.24
D92N	3.43 $\pm$ 0.16	4.82 $\pm$ 0.75	0.71	10.01 $\pm$ 1.78	0.34
Y133F	3.92 $\pm$ 0.08*	4.59 $\pm$ 0.31*	0.79	12.58 $\pm$ 1.96*	0.31
Y133V	2.06 $\pm$ 0.02	9.68 $\pm$ 0.39	0.21	270 $\pm$ 12	7.63 $\times 10^{-3}$
Y136F	5.73 $\pm$ 0.06*	6.47 $\pm$ 0.17*	0.89	61.23 $\pm$ 0.39	0.09
Y136V	1.36 $\pm$ 0.15	69.65 $\pm$ 15.81	0.02	12060 $\pm$ 3360	1.13 $\times 10^{-4}$
W143F	3.02 $\pm$ 0.08	6.42 $\pm$ 0.52	0.47	35.51 $\pm$ 3.92	0.09
W143L	2.36 $\pm$ 0.08	6.32 $\pm$ 0.96	0.37	27.20 $\pm$ 2.48	0.09

* From [5].

Figure 2 Near-UV CD spectra of the wild-type and mutant forms of guanidinoacetate methyltransferase

The spectra were taken at 25 °C in 20 mM potassium phosphate, pH 7.2, containing 1 mM EDTA, in cells with a path length of 10 mm. Protein concentrations were 0.5 mg/ml. $[\theta]$, mean residue ellipticity.

Figure 3 Plots of free energy change of unfolding (ΔG_D) as a function of urea concentration

Wild-type and mutant forms of guanidinoacetate methyltransferase (0.1 mg/ml) were incubated in 0.1 M Tris/HCl, pH 7.5, containing 5 mM dithiothreitol and various concentrations of urea, and ΔG_D values were calculated as described in the Materials and methods section.

positions does not affect the backbone structure but induces a conformational difference that makes the enzyme less stable.

It has been shown that AdoMet bound to guanidinoacetate methyltransferase becomes covalently linked to Tyr¹³⁶ on UV irradiation [6]. We exploited this property to test whether the glycine mutants are capable of binding AdoMet. The wild-type and mutant enzymes were exposed to UV in the presence of [*methyl*-³H]AdoMet as described in the Materials and methods section. Whereas the wild-type enzyme showed the incorporation of radioactivity as expected from a dissociation constant of approx. 0.1 mM [6], no significant incorporation of radioactivity was observed with the G67A and G69A enzymes at concentrations of AdoMet as high as 0.5 and 1.0 mM. Although the possibility cannot be excluded that AdoMet does bind to the mutants but different geometries of the active sites make it difficult or impossible for the photochemical reaction to take place, rather pronounced conformational differences as elucidated by CD spectra and the urea denaturation experiment

suggest that the mutant enzymes are not capable of binding AdoMet. Although glycine at positions 67 and 69 is essential to the catalytic activity of guanidinoacetate methyltransferase, it should be noted that not all mammalian non-nucleic acid methyltransferases contain two glycines at the corresponding positions: glycine N-methyltransferase has an alanine in the place of the first glycine and catechol O-methyltransferase has a tyrosine at the place of the second glycine (Figure 1).

The GXGXXG sequence of the nucleotide-binding motif is followed by an acidic residue on the C-terminal side separated by approx. 20 residues. This residue is situated at the C-terminus of the second β strand and has been shown to be hydrogen-bonded to the 2'-hydroxy group of adenosine ribose [16]. Mammalian non-nucleic acid methyltransferases also have an acidic residue 17–21 residues away from motif I (Figure 1). In guanidinoacetate methyltransferase Glu⁸⁹ or possibly Asp⁹² corresponds to the conserved acidic residue. In view of the importance of the glycines of motif I in AdoMet binding, it might be expected that the acidic residue plays a similar role to that in nucleotide-binding proteins. Conversion of Glu⁸⁹ to glutamine, which has a similar bulk to glutamate and is still capable of forming hydrogen bonds, caused a decrease in $k_{\text{cat}}/K_m(\text{AdoMet})$ to one-third (Table 2), whereas mutation to an alanine or lysine resulted in an almost inactive enzyme (results not shown). The mutant in which Asp⁹² was replaced by asparagine showed a $k_{\text{cat}}/K_m(\text{AdoMet})$ value not very different from that of the wild-type enzyme (Table 2). Thus it is likely that Glu⁸⁹ interacts with AdoMet possibly through hydrogen bonding, and corresponds to the conserved acidic residue in methyltransferases.

Properties of motif II mutants

A previous investigation has shown that the conserved aspartate of motif II (Asp¹³⁴) plays a pivotal role in maintaining the active site structure of guanidinoacetate methyltransferase [5]. The sequence around motif II is rich in hydrophobic amino acids, and guanidinoacetate methyltransferase has tyrosines (Tyr¹³³ and Tyr¹³⁶) at positions -1 and +3 with respect to the conserved aspartate. It was shown previously that replacement of either residue with phenylalanine was virtually without effect on the k_{cat}/K_m value for AdoMet [5]. Because these residues are apparently at the AdoMet-binding site, it is possible that the aromatic ring of one of these residues helps to stabilize the binding of AdoMet through cation- π interaction with the positively charged sulphonium of AdoMet [21,22]. To test this possibility we mutated each of Tyr¹³³ and Tyr¹³⁶ to valine in the present study. Conversion of Tyr¹³³ to valine resulted in a catalytically less active enzyme with a 2-fold increase in K_m for AdoMet. Mutation of Tyr¹³⁶ to valine, on the other hand, exerted a decrease in k_{cat}/K_m for AdoMet to one-fiftieth and a decrease in k_{cat}/K_m for guanidinoacetate to 1/3900 (Table 2).

Guanidinoacetate methyltransferase follows an ordered mechanism in which AdoMet is the first reactant to bind [5]. The minimal kinetic mechanism of guanidinoacetate methyltransferase is represented as:

where GAA is guanidinoacetate and Cr is creatine. In this mechanism, the steady-state kinetic constants are described by individual rate constants as shown in Eqns. (2)–(4):

$$k_{\text{cat}} = \frac{k_3 k_4}{k_3 + k_4} \quad (2)$$

$$k_{\text{m}}(\text{AdoMe}) = \frac{k_3 k_4}{k_1(k_3 + k_4)} \quad (3)$$

$$k_{\text{m}}(\text{GAA}) = \frac{k_4(k_{-2} + k_3)}{k_2(k_3 + k_4)} \quad (4)$$

$k_{\text{cat}}/K_{\text{m}}$ for AdoMet is the association rate constant between the enzyme and AdoMet (k_1). From the magnitude of the difference in the values of wild-type and Y136V, the binding of AdoMet to the latter enzyme is calculated to be destabilized by 2.3 kcal/mol (9.6 kJ/mol). Thus it is possible that Tyr¹³⁶ contributes to the binding of AdoMet through cation- π interaction. k_{cat} represents the rate constant of the chemical step or of product release that is rate-limiting, and $k_{\text{cat}}/K_{\text{m}}(\text{GAA})$ includes the rate constant for the chemical transformation. If the release of the product from the enzyme is rate-limiting, as is usually the case with methyltransferases [23], and if the mutation affects the methyl transfer reaction, there would be little effect on k_{cat} , and $k_{\text{cat}}/K_{\text{m}}(\text{GAA})$ could be very low. If Tyr¹³⁶ indeed stabilizes AdoMet binding by interacting with the sulphonium sulphur, polarization of the latter would be enhanced and the methyl transfer reaction would be facilitated [22]. A large decrease in $k_{\text{cat}}/K_{\text{m}}(\text{GAA})$ by changing the residue from an aromatic to an aliphatic residue might be explained by such an interaction (Table 2). However, a low $k_{\text{cat}}/K_{\text{m}}(\text{GAA})$ would also be obtained if the binding of guanidinoacetate were hindered. There is no feasible test of this hypothesis because of the lack of known competitive inhibitors with respect to guanidinoacetate.

Recently the crystal structures of catechol O-methyltransferase (a small-molecule methyltransferase) [24], *HhaI* methyltransferase (a C5-cytosine DNA methyltransferase) [25] and *TaqI* methyltransferase (an N6-adenine DNA methyltransferase) [26] have been elucidated. DNA methyltransferases lack motifs II and III of non-nucleic acid methyltransferase but possess a glycine-rich sequence that shows a weak resemblance to motif I [1,2,27–29]. Despite having rather low degrees of sequence similarity, these enzymes show strikingly similar structures in the AdoMet-binding domains. These domains assume α/β structures, and the conserved glycine-rich motifs (GsGsG in non-nucleic acid methyltransferases, where s represents small neutral amino acids; FXGXG in DNA methyltransferases) are present in the tight loop joining the first β -strand and α -helix of the $\beta\alpha\beta$ fold [30]. Catechol O-methyltransferase has a molecular mass similar to that of guanidinoacetate methyltransferase and contains motifs I–III at similar positions. Catechol O-methyltransferase consists of eight α -helices and seven β -strands, and Gly⁶⁶ of motif I (see Figure 1) is situated at the end of the β 1-strand and corresponds to the conserved glycine in all dehydrogenases. The last residue of the β 2-strand is Glu⁹⁰, which is hydrogen-bonded to the hydroxyl group of AdoMet. Motif II (residues 132–135) exists between the α 6-helix and the β 4-strand [24], and forms part of the AdoMet-binding pocket. The present and previous [5] site-directed studies establish the importance of Gly⁶⁷, Gly⁶⁹, Glu⁸⁹, Asp¹³⁴ and Tyr¹³⁶ for AdoMet binding in guanidinoacetate methyltransferase. Thus it is highly likely that motifs I and II of all non-nucleic acid methyltransferases occur in the loop regions, and the conserved residues therein are crucial

in the formation of the AdoMet site. It has also been reported that changing Gly¹⁷⁷ of *EcoKI* methyltransferase (a conserved residue corresponding to Gly⁶⁷ of guanidinoacetate methyltransferase) to aspartate abolishes AdoMet binding [31].

In catechol O-methyltransferase, Trp¹⁴³, which is located 10 residues on the C-terminal side of the conserved aspartate, is shown to make an edge-to-face contact with the adenine ring of AdoMet [24]. Although the tryptophan is not a conserved residue of the methyltransferases, it is interesting that guanidinoacetate methyltransferase has a tryptophan (Trp¹⁴³) at a similar position. To investigate the possible contribution of the residue to AdoMet binding in guanidinoacetate methyltransferase, we mutated Trp¹⁴³ to phenylalanine or leucine. As shown in Table 2, only minor changes in the catalytic property were observed, suggesting the absence of such interactions.

This work was supported in part by a grant for scientific research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- Kagan, R. M. and Clarke, S. (1994) Arch. Biochem. Biophys. **310**, 417–427
- Gomi, T., Tanihara, K., Date, T. and Fujioka, M. (1992) Int. J. Biochem. **24**, 1639–1649
- Mudd, S. H. and Poole, J. R. (1975) Metab. Clin. Exp. **24**, 721–735
- Ogawa, H., Date, T., Gomi, T., Konishi, K., Pitot, H. C., Cantoni, G. L. and Fujioka, M. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 694–698
- Takata, Y., Konishi, K., Gomi, T. and Fujioka, M. (1994) J. Biol. Chem. **269**, 5537–5542
- Takata, Y. and Fujioka, M. (1992) Biochemistry **31**, 4369–4374
- Fujioka, M. and Ishiguro, Y. (1986) J. Biol. Chem. **261**, 6346–6351
- Gomi, T., Date, T., Ogawa, H., Fujioka, M., Aksamit, R. R., Backlund, Jr., P. S. and Cantoni, G. L. (1989) J. Biol. Chem. **264**, 16138–16142
- Shutz, G., Kieval, S., Groner, B., Sippel, A. E., Kurtz, D. T. and Feigelson, P. (1977) Nucleic Acids Res. **4**, 71–84
- Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene **33**, 103–119
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
- Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987) Methods Enzymol. **154**, 367–382
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
- Penefsky, H. S. (1979) Methods Enzymol. **56**, 527–530
- Laemmli, U. K. (1970) Nature (London) **227**, 680–685
- Wierenga, R. K. and Hol, W. G. J. (1983) Nature (London) **302**, 842–844
- Parsonage, D., Wilke-Mounts, S. and Senior, A. E. (1987) J. Biol. Chem. **262**, 8022–8026
- Reinstein, J., Brune, M. and Wittinghofer, A. (1988) Biochemistry **27**, 4712–4720
- McGrath, J. P., Capon, D. J., Goeddel, D. V. and Levinson, A. D. (1984) Nature (London) **310**, 644–649
- Pace, C. N. (1986) Methods Enzymol. **131**, 266–280
- Dougherty, D. A. and Stauffer, D. A. (1990) Science **250**, 1558–1560
- McCurdy, A., Jimenez, L., Stauffer, D. A. and Dougherty, D. A. (1992) J. Am. Chem. Soc. **114**, 10314–10321
- Coward, J. K., Slisz, E. P. and Wu, F. Y.-H. (1973) Biochemistry **12**, 2291–2297
- Vidgren, J., Svensson, L. A. and Liljas, A. (1994) Nature (London) **368**, 354–358
- Cheng, X., Kumar, S., Postai, J., Pflugrath, J. W. and Roberts, R. J. (1993) Cell **74**, 299–307
- Labahn, J., Granzin, J., Schluckebier, G., Robinson, D. P., Jack, W. E., Schildkraut, I. and Saenger, W. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 10957–10961
- Postai, J., Bhagwat, A. S., Postai, G. and Roberts, R. J. (1989) Nucleic Acids Res. **17**, 2421–2435
- Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Postai, J., Roberts, R. J. and Wilson, G. G. (1994) Nucleic Acids Res. **22**, 1–10
- Klimasauskas, S., Timinskas, A., Menkevicius, S., Butkiene, D., Butkus, V. and Janulaitis, A. (1989) Nucleic Acids Res. **17**, 9823–9832
- Schluckebier, G., O'Gara, M., Saenger, W. and Cheng, X. (1995) J. Mol. Biol. **247**, 16–20
- Willcock, D. F., Dryden, D. T. F. and Murray, N. E. (1994) EMBO J. **13**, 3902–3908