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Research paper

Mutational analysis of residues in human arsenic (III) methyltransferase (hAS3MT) belonging to 5 Å around S-adenosylmethionine (SAM)

Xiangli Li [a], Zhirong Geng [a, *], Jiayin Chang [a], Xiaoli Song [b], Zhilin Wang [a, *]

[a] State Key Laboratory of Coordination Chemistry, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, PR China
[b] School of Chemistry and Chemical Engineering, Yangzhou University, Yangzhou 225002, PR China

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ABSTRACT

The functions of residues 57-RY-58, G60, L77, 80-GSGR-83, I101, T104, 134-GY-135, N155, V157 and 160-LV-161 in human arsenic (III) methyltransferase (hAS3MT) 5 Å around S-adenosylmethionine (SAM) have not been studied. Herein, sixteen mutants were designed by substituting these residues with Ala. Mutants G60A, G80A, I101A, N155A and L160A were completely inactive. Only MMA was detected when mutants R57A, Y58A, G82A and T104A were used as the enzymes, which suggested that their catalytic activities were seriously impaired compared with that of wild type (WT). The catalytic capacities of other mutants were also lower than that of WT-hAS3MT. The $K_{M(SAM)}$ values of mutants were 1.9–8.7 times that of WT, suggesting their affinities to SAM were weakened. As evidenced by the experimental data herein, earlier literature and the model of hAS3MT-SAM, 57-RYYG-60, G78, G80, G82 and 155-NCV-157 interacted with the methionine of SAM, and 101-IDMT-104 and 135-YIE-137 were associated with the nucleotide adenosine of SAM. Since C156 and L160 were the common residues between 5 Å around SAM and 5 Å around As, and C156S and L160A were inactive, we proposed that C156 and L160 functioned in the methyl transfer process. G78, G80 and G82 belonging to the consensus GxGxG were located in a loop connecting the first β-strand and α-helix in the Rossmann fold core. Y59, N155, C156 and L160 oriented S^{+}-CH_3 during its approach to the arsenic lone pair, and further activated methyl transfer. G78, D102, M103, T104, I136 and N155 formed hydrogen bonds with SAM.

1. Introduction

Arsenic has dual roles, one being potent toxin and carcinogens threatening human health, and the other being drugs against cancers such as acute promyelocytic leukemia [1–5]. Both of them are closely related with the metabolism of arsenic. Studying the mechanism of arsenic metabolism is crucial to relieve the toxicity of arsenic and to better exert its drug function. Arsenic (III) methyltransferase (AS3MT) is a main enzyme catalyzing the arsenic methylation which is a primary pathway of its metabolism, although N-6 adenine-specific DNA methyltransferase 1 also catalyzes some of MMA^{III} methylation [6–8]. AS3MT with reductant and methyl donor S-adenosylmethionine (SAM) catalyzes arsenic methylation [7,9,10], the mechanism of which has been studied for many years [11,12]. SAM binding to AS3MT is prerequisite for the methylation of substrate inorganic arsenic (iAs). It has been proposed that during methylation, SAM first binds AS3MT and then iAs binds it as the second substrate [13,14].

Active sites and As-binding sites of AS3MT have been studied. Residues C157 and C207 in recombinant mouse AS3MT, C156 in rat AS3MT, C61, C156 and C206 in human AS3MT (hAS3MT) and C72, C174 and C224 in *Cyanidioschyzon merolae* arsenite S-adenosylmethyltransferase (CmArsM) have been proved as their As-binding sites and active sites [15–19]. hAS3MT belongs to the Rossmann fold SAM-dependent methyltransferase, with highly conserved glycine-rich sequence of GxGxG as the hallmark

Abbreviations: iAs, inorganic arsenic; MMA, monomethylated arsenicals; DMA, dimethylated arsenicals; AS3MT, arsenic (III) methyltransferase; SAM, S-adenosylmethionine; IPTG, isopropyl β-D-thiogalactopyranoside; ATR-FTIR, attenuated total reflection Fourier transform infrared spectrometry; WT, wild-type; HPLC-ICP-MS, high performance liquid chromatography-inductively coupled plasma-mass spectrometry; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CmArsM, cyanidioschyzon merolae arsenite S-adenosylmethyltransferase.

* Corresponding authors. Tel.: +86 25 83686082; fax: +86 25 83317761.
E-mail addresses: gengzr@nju.edu.cn (Z. Geng), wangzl@nju.edu.cn (Z. Wang).

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[7,20,21]. The predicted SAM-binding motifs of hAS3MT obtained via sequence alignment of various SAM-dependent methyltransferases are motif I 74-ILDLGSGSG-82, motif II 101-IDMT-104, motif III 147-ESHDIVVSN-155 and motif IV174-VLKHGGELYF-183 [6,7,20]. Parts of the functions of residues have been investigated [17,20,22]. The model of wild type (WT) hAS3MT with SAM shows that the residues belonging to 5 Å around SAM are 57-RYYG-60, 76-DLGSGSGRD-84, 101-IDMT-104, Q107, 134-GYIE-137, 155-NCV-157, 160-LV-161 and C206 [20,22]. Among them, the functions of residues Y59, D76, G78, S79, D84, D102, M103, Q107, I136, E137, C156 and C206 have been studied [17,20,22]. Mutants Y59A, D76N, G78A, D84N, D102N, C156S and C206S are completely inactive and the catalytic activities of mutants M103A, Q107A, I136A and E137A are lower than that of WT. The functions of the rest remain unknown hitherto.

To analyze the functions of overall residues located 5 Å around SAM and to determine the residues closely associated with SAM-binding, the functions of the remaining residues were evaluated. Sixteen mutants R57A, Y58A, G60A, L77A, G80A, S81A, G82A, R83A, I101A, T104A, G134A, Y135A, N155A, V157A, L160A and V161A were obtained by site-directed mutagenesis. Then, their catalytic activities and secondary conformation were gauged, and models of mutants with SAM were also built and analyzed. Mutants G60A, G80A, I101A, N155A and L160A were completely deprived of catalytic activities. Only MMA was produced when mutants R57A, Y58A, G82A and T104A were used as the enzymes, suggesting their catalytic activities were seriously weakened. The catalytic capacities of L77A, S81A, R83A, G134A, Y135A, V157A and V161A were also decreased. As indicated by the experimental data in this study, earlier literature and hAS3MT-SAM model, residues 57-RYYG-60, 76-DLGSGSGRD-84, 101-IDMT-104, 134-GYIE-137, 155-NCV-157, 160-LV-161 and C206 in hAS3MT synergistically formed the SAM-binding domain, especially the hydrophobic residues G60, L77, G78, G80, G82, I101, M103, G134, I136, V157, L160 and V161, the aromatic residues Y58 and Y59, and the polar with charge residues R57, D76, R83, D84, D102, E137, N155, C156 and C206. 57-RYYG-60, 78-GSGSG-82 and 155-NC-157 interacted with methionine of SAM. Meanwhile, 101-IDMT-103 and 135-YIE-137 closely contacted with nucleotide adenosine of SAM. C156 and L160, as common residues between 5 Å around SAM and 5 Å around As, functioned in the methyl transfer process. G78, G80 and G82 belonging to the consensus GxGxG were located in a loop connecting the first β-strand and α-helix in the Rossmann fold core. Y59, N155, C156 and L160 interacting with S^{+}-CH_3 of SAM assisted to orient S^{+}-CH_3 during its approach to the arsenic lone pair and further activated methyl transfer. G78, D102, M103, T104, I136 and N155 formed hydrogen bonds with SAM.

2. Materials and methods

Caution: Arsenic has been known as a carcinogen and should be handled carefully [23].

2.1. Materials

SAM, GSH, isopropyl β-D-thiogalactopyranoside (IPTG) and bovine serum albumin (BSA) were purchased from Sigma. Arsenicals were bought from J&K Chemical Ltd. Phosphate-buffered saline (PBS, pH 7.0) buffer was prepared by mixing appropriate volumes of Na_2HPO_4 and NaH_2PO_4 into a 25 mM stock solution. $NaAsO_2$ (As^{3+}), $Na_2HAsO_4{\cdot}7H_2O$ (As^{5+}), disodium methylarsonate (MMA^{5+}) and dimethylarsinic acid (DMA^{5+}) were obtained from J&K Chemical Ltd.

2.2. Protein expression and purification

Sixteen mutants (R57A, Y58A, G60A, L77A, G80A, S81A, G82A, R83A, I101A, T104A, G134A, Y135A, N155A, V157A, L160A and V161A) and WT-hAS3MT were prepared as described previously [24]. The primers for site-directed mutagenesis are summarized in Table 1. Protein expression and purification were performed according to the protocols detailed in previous literature [17,24]. The method of Bradford based on a BSA standard curve was used to determine protein concentrations [25].

2.3. Determining the catalytic activity of the hAS3MT mutants

Solutions (100 μl) containing 11 μg enzyme, 7 mM GSH, 1 μM iAs^{3+} and 1 mM SAM in PBS (25 mM, pH 7.0) were incubated at 37 °C for 2 h, and then the reaction was terminated by adding H_2O_2 to a final concentration of 3%. Finally, the arsenic species were separated on an anion-exchange column by HPLC (PRP X-100 250 mm × 4.6 mm i.d., 5 μm, Hamilton) and analyzed by ICP-MS (Elan 9000, PerkinElmer) [26,27]. The arsenic species were eluted with 12 mM $(NH_4)_2HPO_4$ as the mobile phase, the pH of which was adjusted to 6.0 with H_3PO_4. To determine the iAs kinetic parameters, various iAs concentrations (0.5–500 μM) were used without changing other conditions. In the SAM kinetic experiments, 0.05–1 mM SAM were used. Working curve prepared using 5, 10, 25, 50 and 100 μg/L standard arsenic species was used to calculate the amounts of arsenic species obtained from the reaction. The methylation rates were calculated as mole equivalents of methyl groups that were transferred from SAM to iAs^{3+} (i.e., 1 pmol CH_3 per 1 pmol MMA or 2 pmol CH_3 per 1 pmol DMA) [28]. Noncompetitive substrate inhibition Eq. (1): $V = [S] * V_{max}/(K_M + [S] + [S]^2/$

Table 1
Primers used for site-directed mutagenesis.

	Primer	Sequence
R57A	+	5′-CGAAGAAGTAGCCCTA**GCG**TATTATG-3′
	−	5′−GCCATAATA**CGC**TAGGGCTACTTCTTCG-3′
Y58A	+	5′-CGAAGAAGTAGCCCTAAGA**GCG**TATGG-3′
	−	5′-AGACCACAGCCATA**CGC**TCTTAGGG-3′
G60A	+	5′-AGCCCTAAGATATTAT**GCG**TGTGG-3′
	−	5′-CAGACCACA**CGC**ATAATATCTTAGGGC-3′
L77A	+	5′-GCTGGATTTTGGAT**GCG**GGTAG-3′
	−	5′−TCCACTACC**CGC**ATCCAAAATCCAG-3′
G80A	+	5′-CTGGGTAGT**GCG**AGTGGTAGAGATTG-3′
	−	5′-GCAATCTCTACCACT**CGC**ACTACCC-3′
S81A	+	5′-CTGGGTAGTGGA**GCG**GGTAGAGATT-3′
	−	5′−GCAATCTCTACC**CGC**TCCACTACC-3′
G82A	+	5′-GT**GCG**AGAGATTGCTATGTACTTAGCC-3′
	−	5′-GGCTAAGTACATAGCAATCTCT**CGC**AC-3′
R83A	+	5′-GAAGTGGT**GCG**GATTGCTATGTA-3′
	−	5′-GGCTAAGTACATAGCAATC**CGC**ACC-3′
I101A	+	5′-AAAAGGACACGTGACTGGA**GCG**GAC-3′
	−	5′-TGGCCTTTGGTCATGTC**CGC**TCC-3′
T104A	+	5′-GACTGGAATAGACATG**GCG**AAAGGC-3′
	−	5′-TCCACCTGGCCTTT**CGC**CATGTCTA-3′
G134A	+	5′-GCATCTAATGTGACTTTTATTCAT**GCG**TAC-3′
	−	5′-TCTCCCAACTTCTCAATGTA**CGC**ATG-3′
Y135A	+	5′-GACTTTTATTCATGGC**GCG**ATTGAG-3′
	−	5′-ACTTCTCAAT**CGC**GCCATGAATAAA-3′
N155A	+	5′-GCCATGATATTGTTGTATCA**GCG**TGTG-3′
	−	5′-GGCACAAGGTTAATAACACA**CGC**TGATAC-3′
V157A	+	5′-TATTGTTGTATCAAACTGT**GCG**ATTAACC-3′
	−	5′-GGCACAAGGTTAAT**CGC**ACAGTTTGATAC-3′
L160A	+	5′-ACTGTGTTATTAAC**GCG**GTGCCTGA-3′
	−	5′-GTTTATCAGGCAC**CGC**GTTAATAACAC-3′
V161A	+	5′-CAAACTGTGTTATTAACCTT**GCG**CCTG-3′
	−	5′-GCACTTGTTGTTTATCAGG**CGC**AAG-3′
Whole	+	5′-CGGGATATCATGGCTGCACTTCGTGAC-3′
	−	5′-CGGGTCGACTTAGTGATGGTGATG-3′