Chemical Research in Toxicology

Article

pubs.acs.org/crt

Nonsynonymous Polymorphisms in the Human AS3MT Arsenic Methylation Gene: Implications for Arsenic Toxicity

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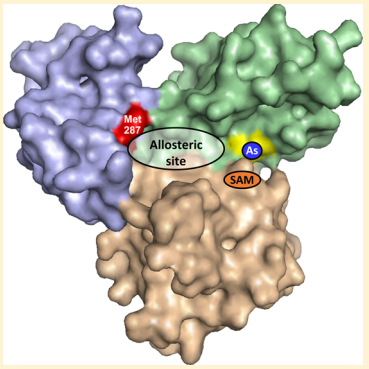
Supporting Information

ABSTRACT: Arsenic methylation, the primary biotransformation in the human body, is catalyzed by the enzyme As(III) S-adenosylmethionine (SAM) methyltransferases (hAS3MT). This process is thought to be protective from acute high-level arsenic exposure. However, with long-term low-level exposure, hAS3MT produces intracellular methylarsenite (MAs(III)) and dimethylarsenite (DMAs(III)), which are considerably more toxic than inorganic As(III) and may contribute to arsenic-related diseases. Several single nucleotide polymorphisms (SNPs) in putative regulatory elements of the hAS3MT gene have been shown to be protective. In contrast, three previously identified exonic SNPs (R173W, M287T, and T306I) may be deleterious. The goal of this study was to examine the effect of single amino acid substitutions in hAS3MT on the activity of the enzyme that might explain their contributions to adverse health effects of environmental arsenic. We identified five additional intragenic variants in hAS3MT (H51R, C61W, I136T, W203C, and R251H). We purified the eight polymorphic hAS3MT proteins and characterized their enzymatic properties. Each enzyme had low methylation activity through decreased affinity for substrate, lower overall rates of catalysis, or lower stability. We propose that amino acid substitutions in hAS3MT with decreased catalytic activity lead to detrimental responses to environmental arsenic and may increase the risk of arsenic-related diseases.



INTRODUCTION

Arsenic is a toxic metalloid that is the 20th most abundant element in the Earth's crust.[1] Arsenic in bedrock is released into groundwater that is used to grow food and as drinking water, causing a worldwide health hazard that affects tens of millions of people.[2] Inorganic arsenic (iAs) is transformed primarily to methylarsenite (MAs(III)) and dimenthylarsenite (DMAs(III)) and, to a lesser extent, to trimethylarsine (TMAs(III)) by the enzyme As(III) S-adenosylmethionine (SAM) methyltransferase (AS3MT in mammals and ArsM in microbes).[3,4]

Human AS3MT (hAS3MT) produces MAs(III) and DMAs(III) primarily in liver.[5] These trivalent products have been shown to be more toxic[6−8] and potentially more carcinogenic[9] than inorganic arsenic. MAs(III) and DMAs(III) are eventually excreted in urine, where they are abiotically oxidized in air to MAs(V) and DMAs(V).[10,11] In this study, the sums of trivalent and pentavalent inorganic and methylated urinary arsenic are termed iAs, MAs, and DMAs, respectively. High urinary levels of MAs relative to DMAs are a susceptibility factor correlated with arsenic-related diseases.[5,12−14] In contrast, a higher ratio of DMAs to MAs in urine is considered protective, perhaps because it reflects more rapid clearance of arsenic from the body.[15] Other factors that might influence urinary levels are redox pathways such as glutathione synthesis and reduction,[16] channels, permeases and pumps such as aquaglyceroporins, glucose permeases, phosphate permeases, and ABC ATPases that transport arsenicals in and out of tissues.[17]

The most frequent distribution of arsenic metabolites in human urine is 10−30% inorganic As, 10−20% MAs, and 60−70% DMAs, but there are large individual variations.[5,18] Some variations are associated with single nucleotide polymorphisms in the hAS3MT gene.[15] Most SNPs are neutral and have little effect on health or development.[19] However, a M287T SNP in hAS3MT has been associated with higher risk of arsenic-related diseases such as diabetes,[20] skin lesions,[21,22] and cancer.[13,23] Polymorphisms in the hAS3MT gene have been associated with altered arsenic metabolite patterns among different populations including those in Bangladesh, Argentina, Mexico, Taiwan, and Central Europe.[24−28] For example, one protective hAS3MT haplotype that produces low urinary excretion of MAs (~7.5%) and a higher percentage of DMAs (~78%) is found in indigenous populations in the Argentinean Andes exposed for generations to elevated arsenic in their water supply (0.8 mg/L).[15,28,29] The increased ratio of DMAs/MAs in urine in this population may reflect natural selection for SNPs in the noncoding region of the human hAS3MT gene that increases expression of the gene, leading to increased arsenic tolerance. Inhabitants of Camarones in the Arica y Parinacota Region in

Received: April 29, 2017
Published: May 24, 2017

DOI: 10.1021/acs.chemrestox.7b00113
Chem. Res. Toxicol. 2017, 30, 1481−1491

Chile exposed to >1 mg/L of arsenic in their drinking water have four protective genetic variants of the hAS3MT gene (G12390C, C14215T, T14458C, and G35991A).[30] These variants are associated with more efficient arsenic metabolism and suggest human adaptation to persistent high levels of arsenic.[28−30]

Until this study, only three SNPs that encode single amino acid changes in the hAS3MT protein, R173W, M287T, and T306I, have been identified.[31] These three exonic SNPs are found in the AS3MT coding region of African-Americans and Caucasian-Americans. They reported that the M287T SNP has a frequency of about 10% in both populations, the R173W SNP has a minor allele frequency of 0.8% in the African-American population, and the T306I SNP has a minor allele frequency of 0.8% in just one sample of Caucasian-Americans. The most common polymorphism, M287T, has been associated with lower overall methylation capacity, with lower primary (MAs/iAs) and secondary (DMAs/MAs) urinary methylation ratios (PMI and SMI, respectively) in different populations.[12,24−26,32]

In this study, we searched repositories of human genomic data and identified five new nonsynonymous missense variants. Including the three previously known,[31] these eight were further analyzed. The location of these single amino acid substitutions in the sequence of hAS3MT is shown in Supplemental Figure S1. The goal of the project was to determine the consequences of the single amino acid substitutions on the ability of these enzymes to methylate arsenic and whether structural information can be used to predict the effect of the substitutions. Knowledge of the enzymatic mechanism of AS3MT is crucial for understanding its paradoxical role in protection from arsenic exposure and its transformation of arsenic into more toxic methylated species, information that can only be acquired from studies with purified enzyme. We previously synthesized a gene for hAS3MT optimized for bacterial expression, which allowed purification of highly active AS3MT.[33] We introduced each mutation into the synthetic hAS3MT gene and purified the resulting enzymes. We compared their enzymatic properties and stability with the most common form of hAS3MT (termed wild type in this study) using either As(III) or MAs(III) as substrate. The location of each substitution in the structure of hAS3MT was identified using a homology structural model of hAS3MT,[33] allowing correlation of structure with enzymatic properties. From our results, individuals with any of the eight variants would be predicted to have a longer total arsenic retention time in the body, leading to elimination of more iAs and MAs and less DMAs with a lower urinary DMAs/MAs ratio. These individuals could be at greater risk for arsenic-related diseases.

MATERIALS AND METHODS

Reagents. SAM was purchased from Cayman Chemical Co., Ann Arbor, MI. A stock solution of tris(2-carboxyethyl)phosphine (TCEP) was prepared at 0.5 M and adjusted to pH 7.0. MAs(V) was reduced to trivalent MAs(III) using $Na_2S_2O_3$, $Na_2S_2O_5$, and H_2SO_4 and adjusted to pH 6.5 with NaOH, as described.[34] The identities of the reduction products were confirmed by high performance liquid chromatography (HPLC) coupled to inductively coupled mass spectroscopy (ICP-MS), as described.[35] The substrates of for methylation were the glutathione (GSH) conjugates $As(GS)_3$ and $MAs(GS)_2$, which were prepared by incubation of 1 mM As(III) or MAs(III) with a four-fold molar excess of GSH for 5 h at 23 °C in degassed buffers under argon, as described.[36] All other reagents were purchased from commercial sources and were of analytical grade or better.

Strains, Media, and Growth Conditions. *Escherichia coli* Stellar (Clontech Laboratories, Mountain View, CA) was used for plasmid DNA construction and replication. For most experiments, cultures of *E. coli* bearing the indicated plasmids were grown aerobically in Luria−Bertani (LB)[37] at 37 °C supplemented with 100 μg/mL ampicillin or 50 μg/mL kanamycin, as required, with shaking.[33] Bacterial growth was monitored by measuring the optical density at 600 nm ($A_{600\ nm}$).

Construction of hAS3MT Variants. The synthetic hAS3MT gene[33] was cloned as an *Eco*RI/*Sal*I digest from pUC57-Kan-hAS3MT into expression vector pMAL-c2x that produces a fusion with the maltose binding protein gene at the 5′ end and eight histidine residues at the 3′ end. The eight mutations in the synthetic hAS3MT gene[33] were introduced by site-directed mutagenesis using a QuickChange mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotides used for mutagenesis are listed in Supplemental Table S1. Each hAS3MT mutation was confirmed by commercial DNA sequencing (Sequetech, Mountain View, CA).

Protein Expression and Purification. Wild-type AS3MT (87 837 Da) and variant enzymes with were purified by Ni-NTA chromatography, as described.[33] Protein concentrations were estimated from $A_{280\ nm}$ using ε = 39 080 M^{-1} cm^{-1}.[38] hAS3MT-containing fractions were rapidly frozen and stored at −80 °C until use. Thioredoxin (Trx) and thioredoxin reductase (TR) were prepared as described.[33] All buffers were degassed by bubbling with argon for 30 min before use.

Assays of Arsenic Methylation. hAS3MT activity was assayed with two different procedures. The time-resolved Förster resonance energy transfer (TR-FRET) assay measures conversion of SAM to S-adenosylhomocysteine (SAH) at short times using an EPIgeneous Methyltransferase Assay kit (Cisbio Bioassays, Bedford, MA).[35] The assay was carried out using a low volume 384-well microtiter plate in a buffer consisting of 50 mM NaH_2PO_4, pH 8,.0, containing 0.3 M NaCl, 1 μM purified hAS3MT, 0.5 mM GSH, 1 μM Trx, 0.3 μM TR, and 0.03 mM NADPH and 10 μM of either $As(GS)_3$ or $MAs(GS)_2$, unless otherwise indicated. The plates were incubated at 37 °C for 2 min with shaking in an Eppendorf ThermoMixer C before addition of SAM at 10 μM, final concentration (unless otherwise indicated), to initiate the reaction. The reactions incubated for 1, 2, and 5 min and were terminated and developed by addition of the SAH-d2 and anti-SAH-Lumi4-Tb detection reagents. The plates were incubated for 1 h, and fluorescence was measured at both 665 and 620 nm with excitation at 337 nm in a Synergy H4 Hybrid Multi-Mode microplate reader. The homogeneous time-resolved fluorescence (HTRF) was calculated from the ratio of emission at 665 and 620 nm. The concentration of SAH was calculated with a calibration curve constructed with known concentrations of SAH.[35] The reaction was linear over 5 min, and initial rates were calculated from the slope.

For measurement at longer times and for speciation of the products, high pressure liquid chromatography (HPLC) was used, with arsenic concentrations determined by inductively coupled plasma mass spectrometry (ICP-MS).[33] The assay mixture contained 1 μM purified hAS3MT, 2.5 mM GSH, 10 μM Trx, 1.5 μM TR, 0.3 mM NADPH, and 10 μM of either $As(GS)_3$ or $MAs(GS)_2$ (unless otherwise indicated) in a buffer consisting of 50 mM NaH_2PO_4 and 0.3 M NaCl, pH 8. SAM was added at a final concentration 0.5 mM to initiate the reaction at 37 °C. For initial rate determinations of SAM kinetics, the reactions were carried out for 5, 10, and 20 min at the indicated SAM concentrations. The reaction was linear over this time period, and the slope was used to estimate the initial rate. To recover all of the arsenic, the reactions were terminated by addition of H_2O_2 at 10% (v/v), final concentration, which also oxidizes all arsenicals, so the products will be termed MAs and DMAs. The assay solution was immediately passed through a 3 kDa cutoff Amicon ultrafilter (Millipore, Billerica, MA), and speciation of arsenic in the filtrate was determined by HPLC (PerkinElmer Series 2000) with a C18 300A reverse-phase column (Chromservis s.r.o., Brno, Czech Republic), with the arsenic concentration measured by ICP-MS using an ELAN 9000 (PerkinElmer, Waltham, MA). As(III), MAs(III), DMAs(V), MAs(V), and As(V) were used at 1 μM as standards.

DOI: 10.1021/acs.chemrestox.7b00113
Chem. Res. Toxicol. 2017, 30, 1481−1491

Methylation in cells of *E. coli* BL21(DE3) expressing hAS3MT wild type and mutants was assayed by growing cells in 2 mL of LB medium in the presence of 0.3 mM IPTG, 100 μg/mL kanamycin, and 10 μM of either As(III) or 2 μM MAs(III) at 37 °C for 12 h. The cell were harvested, washed, and suspended in ST-1 medium[39] with 2 μM MAs(III) and then grown for 3 h at 37 °C. Arsenicals were speciated by HPLC using a C18 reverse phase column, and the amount of arsenic was estimated by ICP-MS.

Assays of Thermal Stability. Thermal stability was assayed by incubation of the enzyme at 42 °C for the indicated times in a buffer consisting of 50 mM NaH_2PO_4 and 0.3 M NaCl, pH 8. The reaction was terminated rapid cooling on ice, and methylation activity determined with the TR-FRET assay for 5 min at 37 °C. $t_{1/2}$ values were calculated using SigmaPlot (Systat Software, Inc., San Jose, CA).

Sources of Single Nucleotide Polymorphisms. hAS3MT SNPs were identified from the Ensembl genome browser, which contains 1000 genomes, NHLBI GO Exome Sequencing Project (ESP), Exome Aggregation Consortium (ExAC), NHLBI Exome Sequencing Project, CLINSEQ SNP: CSAgilent and the HapMap Project repositories of human genome data.

Homology Model of the hAS3MT Structure with Polymorphic Residues. A homology model of hAS3MT[33] was built on the structure of PhAs(III)-bound CmArsM (PDB ID: 4KW7) from residues 44−371 (residue numbers based on the CmArsM sequence) using a fully automated protein structure homology modeling server SWISS-MODEL (http://swissmodel.expasy.org/) (Figure 1A). The model quality was estimated based on the QMEAN scoring function. The final homology model incorporated 308 of those 328 residues. To place SAM in the model, in silico docking with SAM was carried out using the PATCHDOCK server. The docked hAS3MT model with SAM was superimposed with the As(III)-bound structure of CmArsM (PDB ID: 4FSD)[40] to acquire the arsenic atom in the As(III) binding site of hAS3MT. PyMOL v1.3 was used to visualize the structural models.[41] The model for each of the polymorphic variants was built similarly and superposed with each other to locate the position of the polymorphic residues (Figure 1B).

RESULTS

Missense Polymorphisms in hAS3MT and Population Frequencies. Because of the importance of arsenic metabolic reaction in humans, one objective was to determine whether the human hAS3MT gene, like many other human methyltransferase genes, includes additional functional genetic polymorphisms. From database searches, we identified 891 sequence variants in the AS3MT gene. Eight nonsynonymous missense variants that result in single amino acid changes in the AS3MT protein were chosen for further analysis using the following criteria: (1) previously known (R173W, M287T, and T306I), (2) location in the As(III) (C61W and W203C) or SAM binding (H51R and I136T) domains, and (3) high PolyPhen2 scores[42] (R251H, R173W, and W203C). The PolyPhen2 algorithm predicts the possible impact of an amino acid substitution on the structure and function of a human protein.[42] On the basis of the Exome Aggregation Consortium (ExAC) database, which has a large sample size, the order of the minor allele frequency (MAF) of these missense SNPs is as follows: WT > M287T (9.4%) > C61W (~0.5%) > R173W (~0.11%) > R251H (~0.1%) > T306I (~0.02%) > W203C (~0.004%) > H51R (~0.002%) (Table 1). Information on the frequency of I136T is not available. Additional details of each polymorphism in individual populations are given in Supplemental Tables S2−S9. The M287T SNP has highest allele frequency (8−11%) and genotype (13−24%) frequency in the global population. A homozygous recessive genotype has been found only for the M287T polymorphism in several populations, with a frequency of about 1% (Supplemental Table S8). Genotype frequencies of C61W, I136T, W203C, and T306I are unknown. Other missense SNPs (H51R, R173W, and R251H) were identified only in heterozygous individuals. For the 1000 Genomes Project, all donors were over 18 and declared themselves to be healthy at the time of collection (http://www.internationalgenome.org/faq/can-i-get-phenotype-gender-and-family-relationship-information-samples/).

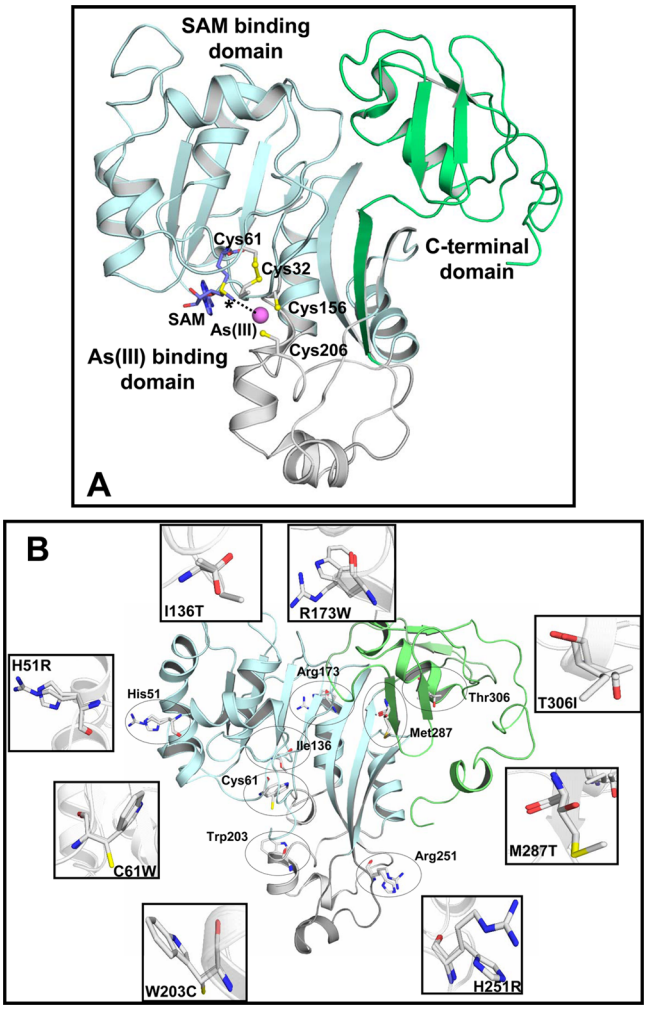


Figure 1. Homology model of hAS3MT and polymorphisms. (A) Structural model of the human AS3MT consists of three domains, the N-terminal SAM binding domain (light blue), the central As(III) binding domain (gray), and the C-terminal domain (green). Shown are the location of the four conserved cysteine residues, SAM and As(III). SAM (in ball-and-stick) occupies its binding site with its methyl group (*) poised to be donated to the arsenic atom (pink ball). (B) Location of the eight residues altered by the SNPs (circled) are shown in stick form, and the predicted structures of the amino acid substitutions are shown on the boarder superimposed on the original residues. Four (H51R, C61W, I136T, and R173W) are located in the large N-terminal domain that includes the SAM binding domain, two (W203C and R251H) are located in the As(III) binding domain, and two (M287T and T306I) are in the C-terminal domain.

Homology Model of hAS3MT and Polymorphisms. We modeled the structure of hAS3MT and its variants on the structure of the CmArsM As(III) SAM methyltransferases from the eukaryotic alga *Cyanidioschyzon merolae* sp. 5508.[40] We mapped the location of the eight residues that are represented with the intragenic polymorphisms on this hAS3MT homology

DOI: 10.1021/acs.chemrestox.7b00113
Chem. Res. Toxicol. 2017, 30, 1481−1491

Table 1. Frequencies of the Eight Missense hAS3MT Polymorphisms in the 1000 Genomes Project, ExAC, NHLBI Exome Sequencing Project, or PUSHMAN Population[a]

dbSNP rs no. (MAF from ExAC)	residue change	allele change	allele frequency (numbers of individuals): top, ancestral; bottom, variants	genotype frequency (numbers of individuals): top, homozygous dominant; middle, heterozygous; bottom, homozygous recessive	population
rs201702937 (0.002%)	H51R	a152g	A: 0.9998 (5007) G: 0.000199 (1)	A|A: 0.9996 (2503) A|G: 0.00039 (1) G|G: 0	1000 Genomes Project (African, American, East Asian, European, South Asian)
rs80317306 (0.50%)	C61W	t183g	T: 0.995 (120200) G: 0.005 (646)	NA	Exome Aggregation Consortium (ExAC) individuals (African/African American, Latino, East Asian, Finnish, Non-Finnish European, South Asian and others)
rs112056792 (NA)	I136T	t407c	T: 0.500 (1) C: 0.500 (1)	NA	BUSHMAN POP (Northern Kalahari of Africa)
rs35232887 (0.11%)	R173W	c517t	C: 0.999 (5003) T: 0.001 (5)	C|C: 0.998 (2499) C|T: 0.002 (5) T|T: 0	1000 Genomes Project (African, American, East Asian, European, South Asian)
rs370022454 (0.004%)	W203C	g609t	G: 0.999734 (3763) T: 0.000265675 (1)	NA	NHLBI Exome Sequencing Project (African-American)
rs139656545 (0.10%)	R251H	g752a	G: 0.998 (4997) A: 0.002 (11)	G|G: 0.996 (2493) A|G: 0.004 (11) A|A: 0	1000 Genomes Project (African, American, East Asian, European South Asian)
rs11191439 (9.4%)	M287T	t860c	T: 0.923 (4622) C: 0.077 (386)	T|T: 0.854 (2138) C|T: 0.138 (346) C|C: 0.008 (20)	1000 Genomes Project (African, American, East Asian, European, South Asian)
rs34556438 (0.02%)	T306I	c917t	C: 0.99951 (8164) T: 0.000489716 (4)	NA	NHLBI Exome Sequencing Project (European-American)

[a]Original data were mined from the Ensembl genome browser database (http://useast.ensembl.org/index.html) accessed April 5, 2017. For the 1000 Genomes Project, all donors were over 18 and declared themselves to be healthy at the time of collection. The health conditions for other data source populations are unknown.

mode (Figure 1A) and superimposed the side chains of the variants on those of the wild-type residues (Figure 1B). His51, Cys61, and Ile136 are in the SAM binding domain. Arg173, Trp203, and Arg251 are in the arsenic binding domain. Met287 and Thr306 are in the C-terminal domain.

Polymorphisms Affect hAS3MT Methylation Activity. The enzymatic activity of the polymorphic AS3MTs was determined. Each variant was expressed in *E. coli* cells in about the same amount as wild-type hAS3MT, as shown by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE)[43] (Supplemental Figure S2). Arsenic methylation in cells of *E. coli* provides an initial screen for the effects of the mutations. Methylation in cells expressing the eight variants was compared with wild-type AS3MT as a positive control and an inactive C206S mutant[33] as a negative control (Supplemental Figure S3). Cells expressing six of the eight SNPs (H51R, I136T, R173W, R251H, M287T, and T306I) methylated As(III) to varying degrees, but only M287T activity was comparable to the wild type. Cells expressing C61W and W203C derivatives were unable to methylate As(III) (Supplemental Figure 3AS). Like the C206S mutant, cells expressing W203C derivative were unable to methylate MAs(III), but the cells expressing the C61W derivative retained ability to methylate MAs(III) (Supplemental Figure 3BS).

Purified wild-type hAS3MT rapidly methylated As(III) to the methylated species and more slowly to the dimethylated species (Figure 2A), consistent with our previous observations.[33] At 30 min of reaction time, MAs accounted for approximately about 25% of total arsenic, and DMAs accounted for about 30%, with 45% remaining as iAs. The methylation activity of purified single amino acid polymorphic enzymes were compared with wild-type hAS3MTat 30 min using either As(III) (Figure 2B) or MAs(III) (Figure 2C) as substrate. With As(III) as substrate, the reaction measures a combination of both the first (As → MAs) and second methylation (MAs → DMAs) steps when assayed with HPLC−ICP−MS. MAs(III) is both the product of the first methylation step and is the substrate of the second methylation step, so with MAs(III) as substrate, the reaction measures only the second round of methylation (MAs → DMAs). Using As(III) as substrate, the eight polymorphic enzymes showed lower methylation activity compared to wild-type hAS3MT in the following order: WT > M287T > R251H > T306I > R173W > H51R > I136T. The C61W and W203C derivatives exhibited essentially no catalytic activity and were comparable to the inactive C206S mutant.

Using MAs(III) as substrate to assay the second round of methylation, the order of methylation activity was similar to the results with As(III) as substrate with one exception. The C61W enzyme, which was inactive with As(III) as substrate, had catalytic activity with MAs(III) (Figure 2C). Most characterized microbial ArsMs and animal AS3MTs have four conserved cysteine residues, which are Cys32, Cys61, Cys156, and Cys206 in hAS3MT (Supplemental Figure S1). Cys32 and Cys61 are required for methylation of both As(III) and MAs(III), while Cys156 and Cys206 are required only for methylation of MAs(III) but not As(III).[33] Most fungal AS3MT have only three conserved cysteines and lack the cysteine corresponding to Cys32 in hAS3MT. The natural three-cysteine enzyme of *Aspergillus fumigatus* methylates MAs(III) but not As(III), similar to the C61W hAS3MT polymorphic enzyme.[44]

Effect of Polymorphisms on the Ratio of DMAs to MAs. The efficiency of arsenic methylation is often expressed

DOI: 10.1021/acs.chemrestox.7b00113
Chem. Res. Toxicol. 2017, 30, 1481−1491

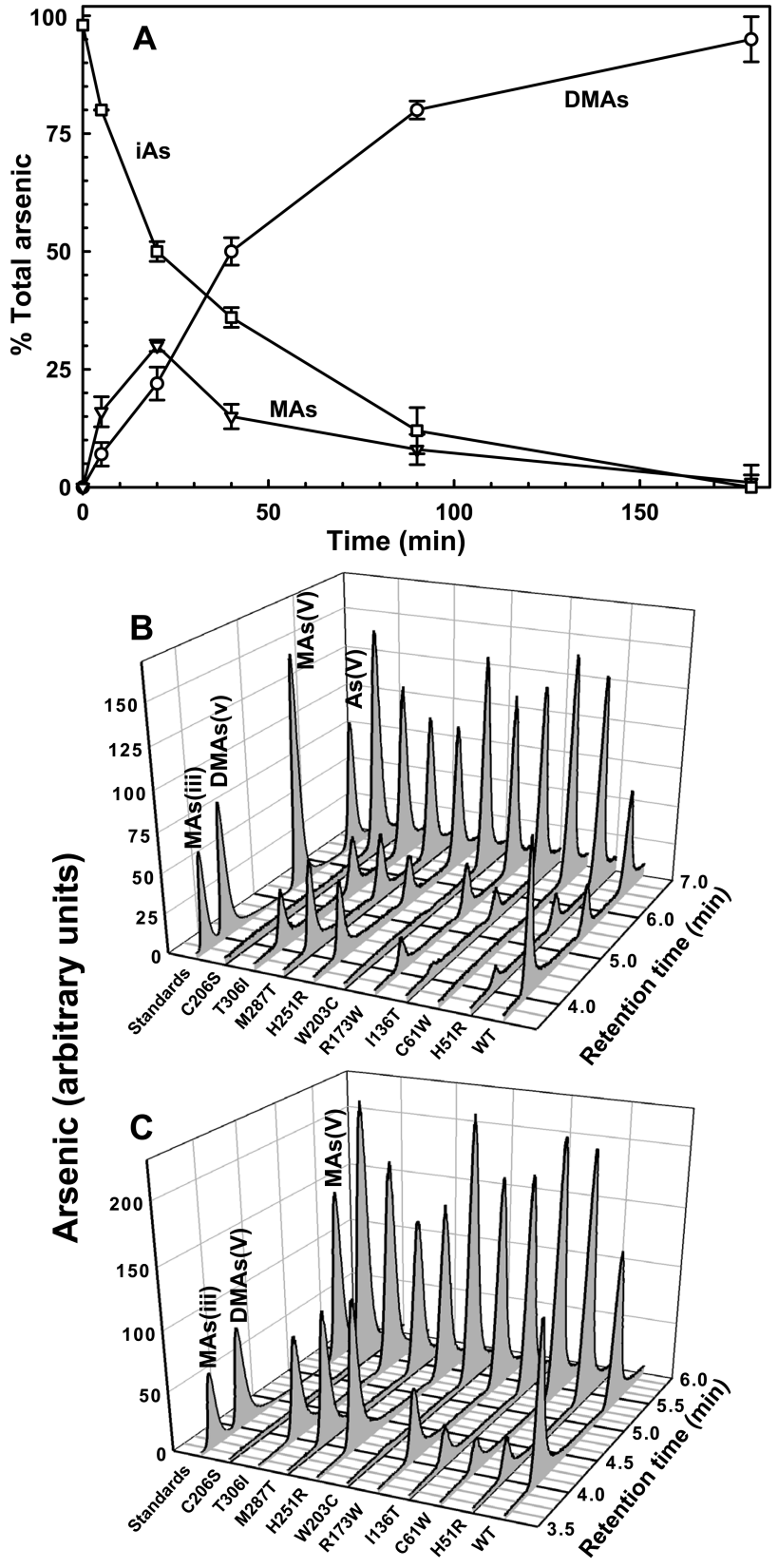


Figure 2. Arsenic methylation by wild-type hAS3MT and polymorphic variants. (A) Time course of As(III) methylation by wild-type hAS3MT. (B) As(III) methylation by wild-type hAS3MT and polymorphic variants. (C) MAs(III) methylation by wild-type hAS3MT and polymorphic variants. Methylation was assayed in mixture of 1 μM purified hAS3MT, 0.5 mM SAM, 2.5 mM GSH, 10 μM Trx, 1.5 μM TR, 0.3 mM NADPH, and 10 μM As(III) (A and B) or MAs(III) (C) in phosphate buffer, pH 8, at 37 °C. Samples were withdrawn at the indicated times (A) or 30 min (B and C), and the reaction terminated by addition of 10% (v/v) H_2O_2, final concentration, and arsenic species analyzed by HPLC−ICP−MS. The data are the means ± SE (n = 3).

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as the ratio between the product and substrate.[15,18,45] The primary methylation index (PMI) is defined as the ratio of MAs/iAs, and the secondary methylation index (SMI) is the ratio of DMAs/MAs.[18] The SMI in urine has frequently been used as a operational indicator of methylation capacity of individuals exposed to inorganic arsenic and in studies of interindividual variability in susceptibility to adverse health effects associated with chronic exposure.[46] Here we compared the in vitro methylation indexes of the eight polymorphic enzymes with wild-type hAS3MT calculated from the data in Figure 2B. The results show differences in both the PMI and SMI between the wild-type and polymorphic enzymes. In every case, both the SMI and PMI were lower than the wild-type values (Figure 3A). The wild-type enzyme had the highest

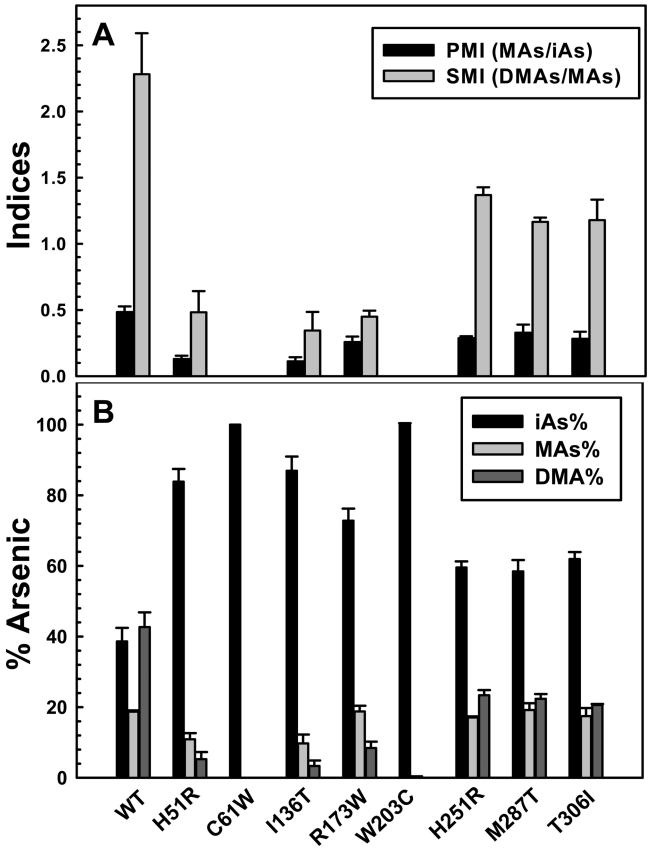

Figure 3. Efficiency of arsenic methylation. (A) Effect of polymorphisms on the PMI and SMI of arsenic methylation by wild-type hAS3MT and variants. (B) Conversion of iAs into MAs and DMAs by each hAS3MT expressed as a percentage of total arsenic. Methylation was assayed in mixture of 1 μM purified hAS3MT, 0.5 mM SAM, 2.5 mM GSH, 10 μM Trx, 1.5 μM TR, 0.3 mM NADPH, and 10 μM As(III) in phosphate buffer, pH 8, at 37 °C. Samples were withdrawn at 30 min, the reaction terminated by addition of 10% (v/v) H_2O_2, final concentration, and arsenic species were analyzed by HPLC–ICP–MS. The data are the means ± SE (n = 3).

DMAs/MAs ratio of 2.3 ± 0.3. The SMI values of M287T, R251H, and T306I (approximately 1.2) were lower than the wild-type enzyme but higher than the other SNPs. The SMI values of the H51R, I136T, R173W enzymes were approximately 0.45. By definition, the PMI and SMI of the inactive C61W and W203C enzymes are zero.

To obtain a more complete picture of the methylation cycle, the percentages of iAs, MAs, and DMAs were calculated (Figure 3B). The data demonstrate that SNPs with low methylation efficiency have higher percentages of iAs and MAs and lower percentages of DMAs compared to wild-type hAS3MT. After 30 min of reaction, wild-type AS3MT total arsenic was 38% As, 19% MAs, and 43% DMAs. The M287T, R251H, and T306I enzymes showed 58–61% inorganic As, 18–20% MAs, and 20–23% DMAs. H51R, I136T, R173W, C61W, and W203C had the highest amount of starting material iAs (83–100%) and lowest DMAs (0–8%). These results demonstrate that the eight nonsynonymous missense variants of hAS3MT have lower arsenic methylation capacity compared with wild-type hAS3MT, from which we predict may lead to individual variations in arsenic methylation and slower clearance of arsenic from the body, factors that could increase the risk of arsenic-related diseases.

Kinetic Analysis of hAS3MT Polymorphic Enzymes. Kinetics governs fluxes of metabolites through intracellular pathways. Enzymes catalyze the individual steps of cellular reactions, and their rates dictate which pathways predominate. The rate of production of individual methylated species, and hence the PMI and SMI, depends on the kinetics of each step, which may vary in different polymorphic variants. How do the kinetic properties of the polymorphic enzymes compare with the wild type? The kinetic parameters for each substrate, As(III), MAs(III), and SAM, were individually determined for wild-type and variant hAS3MTs. With the TR-FRET assay, the first (As → MAs) (Figure 4A) and second (MAs → DMAs) (Figure 4B) methylation steps can be assayed independently.[35] In both steps, wild-type hAS3MT exhibited the highest maximal rates (V_{max}) compared with the polymorphic enzymes. The R251H and T306I variants showed higher V_{max} values compared to the other polymorphic enzymes. The V_{max} values of the I136T, R173W, M287T, and H51R variants were significantly lower than the others in both reaction steps.

The affinity (K_m) of each enzyme for As(III) and MAs(III) was determined (Table 2). Wild-type and polymorphic hAS3MT enzymes each had K_m values in the range of 1–2 μM, indicating that the amino acid substitutions did not affect binding of As(III) (except for C61S and W203C, which do not methylate As(III)). The apparent K_m values for MAs(III) were lower than for As(III) for each enzyme, in the range of 0.4–0.8 μM, suggesting that MAs(III) is a better substrate than As(III). The exceptions were W203C, which does not methylate MAs(III), and C61S, which has a three-fold reduction in affinity for MAs(III).

The TR-FRET assay measures formation of SAH from SAM and thus cannot be used for determination of SAM kinetics,[35] so the kinetics for SAM as substrate was determined by formation of DMAs from MAs(III) as a function of SAM concentration using HPLC–ICP–MS. This measures only the second methylation step but was used because the C61S variant cannot methylate As(III). The V_{max} values for wild-type hAS3MT, R251H, and T306I were similar to each other and were higher than other polymorphic enzymes (Figure 4C). The affinity of each polymorphic enzymes for SAM was similar to that of the wild type, in the range of 14–30 μM with one exception. The K_m for I136T for SAM was 137 μM, eight-fold lower affinity than wild-type hAS3MT. From the homology model of hAS3MT, Ile136 is in the SAM binding domain, suggesting that a threonine substitution affects folding of the SAM binding domain.

The specificity constant or catalytic efficiency (k_{cat}/K_m) of an enzyme is a useful metric for comparing the relative rates of an

DOI: 10.1021/acs.chemrestox.7b00113
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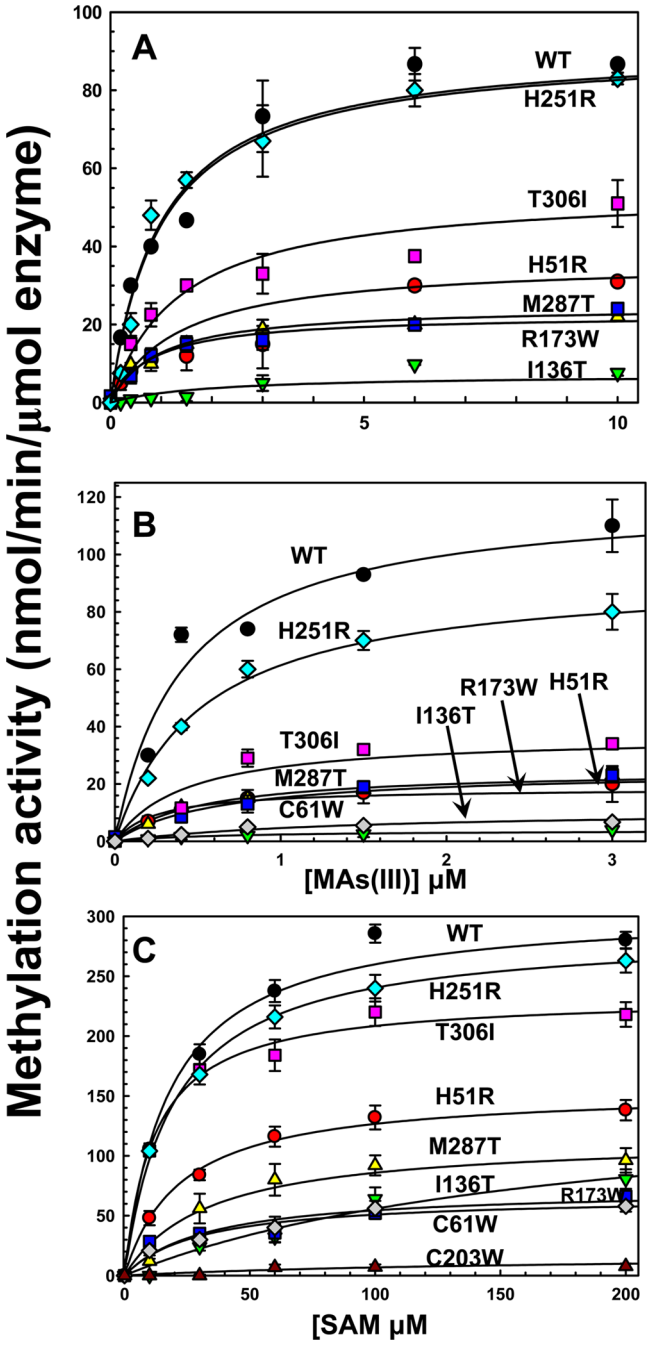

Figure 4. Kinetics of wild-type hAS3MT and polymorphic variants. (A) Methylation as a function of As(III) concentration. (B) Methylation as a function of MAs(III) concentration. Reactions were performed with. Assays were individually in the presence of 1 μM of purified protein, 10 μM SAM, 0.5 mM GSH, 1 μM Trx, 0.3 μM TR, 0.03 mM NADPH, and the indicated concentrations of As(III) or MAs(III) at 37 °C. Methylation was measured using the TR-FRET assay. (C) Methylation as a function of SAM concentration. Activity was measured in the presence of 1 μM of purified protein, 10 μM MAs(III), 2.5 mM GSH, 10 μM Trx, 1.5 μM TR, and 0.3 mM NADPH with the indicated concentrations of SAM. Samples were withdrawn at 30 min, the reaction terminated by addition of 10% (v/v) H_2O_2, final concentration, and arsenic species were analyzed by HPLC–ICP–MS. Data are the means ± SE (n = 3). Derived kinetic constants are summarized in Table 2.

enzyme for multiple substrates. The k_{cat}/K_m of the wild type and SNPs was calculated (Table 2). The wild type specificity constant for MAs(III) was three-fold higher than for As(III), clearly showing that the methylated species is a better substrate for hAS3MT than inorganic arsenic. In every case, the catalytic efficiency of the SNPs was lower than wild-type hAS3MT, unambiguously demonstrating that each single amino acid substitution resulted in a less active enzyme.

Thermal Stability of the Eight Polymorphic hAS3MTs. A possible consequence of a single amino substitution in a protein is improper folding that results in decreased stability.[42,47,48] The thermal stability of the seven active missense variants was compared with wild-type hAS3MT (Table 3). The enzymes were incubated at 42 °C for varying lengths of time followed by determination of methylation activity (Figure 5). Wild-type hAS3MT had a half-life of 20 min at 42 °C. The SNPs lost activity much faster, with half-lives ranging from 1–5 min. R251H has relatively higher stability compared to other polymorphic enzymes, which may in part account for its higher catalytic efficiency compared to the others (Table 2). However, these in vitro studies do not rule out the possibility of in vivo stabilizing or destabilizing factors.

DISCUSSION

A growing number of single nucleotide polymorphisms in the gene for human AS3MT have been identified. The goal of this study was to characterize the products of SNPs that produce single amino acid changes in the AS3MT enzyme. To date, only the three most frequent in human populations, R173W (rs35232887), M287T (rs11191439), and T306I (rs34556438), have been studied. M287T, the most frequent polymorphism, has been associated with a lower SMI, that is, higher urinary MAs and lower DMAs, in various populations.[20,22,25,32] This SNP has been associated with higher negative outcomes. For example, in individuals with the M287T polymorphism, they are at an increased risk of diabetes,[20] premalignant arsenic skin lesions,[22] basal cell carcinoma,[13] and perhaps bladder cancer.[23] On the other hand, there are no epidemiological studies of the R173W or T306I SNPs in human populations. Most individuals carrying these haplotypes are heterozygous, so each expresses a wild-type AS3MT gene, which affects the phenotype. A few homozygous M287T individuals have been identified,[27,49] and these have higher MAs and lower DMAs compared with wild-type hAS3MT.

There are only a few biochemical studies of these SNPs. In a ground-breaking study, Wood et al.[31] identified three non-synonymous SNPs in hAS3MT with single amino changes R173W, M287T, and T306I and examined the enzymatic activity of the three variants. In that study, the human cDNA was altered by site directed mutagenesis to introduce these three changes, which were then expressed in COS-1 cells, which exhibited little endogenous AS3MT expression. The advantage of this approach is that the enzyme is likely to have natural post-translational modifications. Cytosol containing the T306I variant had only 5% the amount of immunoreactive protein and no detectable methylation activity. The R173W variant had only 20% of the amount of immunoreactive protein and about 31% of the activity of the wild type, although it did not appear to be degraded more rapidly than the wild-type protein. The K_m of the R173W variant was approximately 3 μM for As(III) and 9 μM for SAM, compared with appropriately 5 μM for As(III) and 12 μM for SAM for the wild type. These values were not significantly different from each other. There was twice as much M287T immunoreactive protein in the cytosol, which did not appear to result from less rapid degradation. The

DOI: 10.1021/acs.chemrestox.7b00113
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Table 2. Kinetic Parameters for Arsenic Methylation Catalyzed by hAS3MT

hAS3MT	K_m (μM)			V_{max} (nmol/min/μmole hAS3MT)			K_{cat}/K_m (S^{-1}[d]M^{-1})		
	As(III)[a]	MAs(III)[b]	SAM[c]	As(III)[a]	MAs(III)[b]	SAM[c]	As(III)[a]	MAs(III)[b]	SAM[c]
WT	0.97 ± 0.1	0.40 ± 0.06	17 ± 1	91 ± 3	120 ± 20	304 ± 4	1564 ± 50	5000 ± 36	186 ± 3
H51R	1.5 ± 0.2	0.25 ± 0.13	22 ± 2	30 ± 6[d]	18 ± 8[d]	152 ± 4[d]	333 ± 10[d]	1200 ± 14[d]	71 ± 1[d]
C61W		1.40 ± 0.1[d]	30 ± 1[d]		10 ± 3[d]	64 ± 1[d]		119 ± 12[d]	22 ± 1[d]
I136T	1.9 ± 0.25[d]	0.64 ± 0.1	137 ± 5[d]	8 ± 3[d]	3.8 ± 0.1[d]	148 ± 8[d]	70 ± 8[d]	99 ± 5[d]	11 ± 1[d]
R173W	1.5 ± 0.25	0.47 ± 0.3	28 ± 2	25 ± 6[d]	22 ± 5[d]	68 ± 8[d]	277 ± 13[d]	582 ± 20[d]	25 ± 1[d]
W203C									
R251H	1.0 ± 0.15	0.52 ± 0.08	19 ± 1	90 ± 4	93 ± 10	288 ± 1 2	1500 ± 54	2980 ± 59[d]	158 ± 3[d]
M287T	1.1 ± 0.5	0.75 ± 0.1	33 ± 3[d]	20 ± 5[d]	30 ± 7[d]	116 ± 8[d]	303 ± 21[d]	1000 ± 14[d]	37 ± 2[d]
T306I	1.0 ± 0.3	0.65 ± 0.1	14 ± 1	54 ± 10	44 ± 9	232 ± 8	900 ± 40[d]	1833 ± 33[d]	173 ± 2

[a]Activity was measured at 0, 1, 2, and 5 min in the presence of 10 μM SAM, 0.5 mM GSH, 1 μM Trx, 0.3 μM TR, 0.03 mM NADPH, and As(III) at concentrations up to 10 μM using the TR-FRET assay at 37 °C. The data are the means ± SE (n = 3). [b]Activity was measured with the TR-FRET assay at 0, 1, 2, and 5 min in the presence of 10 μM SAM, 0.5 mM GSH, 1 μM Trx, 0.3 μM TR, 0.03 mM NADPH, and MAs(III) at concentrations up to 10 μM at 37 °C. [c]Activity was measured at 0, 5, 10, and 20 min in the presence of 10 μM MAs(III), 2.5 mM GSH, 10 μM Trx, 1.5 μM TR, 0.3 mM NADPH, and SAM at concentrations up to 600 μM by HPLC–ICP–MS at 37 °C. [d]Indicates statistically significant differences (p < 0.02) in V_{max} or K_m values between wild-type hAS3MT and variants-catalyzed methylation.

Table 3. Temperature-Dependent Half-Life ($t_{1/2}$) of hAS3MT and Polymorphic Variants[a]

polymorphisms	$t_{1/2}$ (min)
WT	20.0 ± 1.5
H51R	2.3 ± 0.8[c]
C61W	1.0 ± 0.5[b]
I136T	1.5 ± 0.6[b]
R173W	1.9 ± 0.5[b]
W203C	no activity
R251H	5.0 ± 1.0[c]
M287T	2.7 ± 0.6[c]
T306I	3.1 ± 0.5[c]

[a]Proteins were incubated at 42 °C for 0, 2, 6, 10, and 15 min. The control was kept on ice for 15 min. Methylation activity was assayed at 37 °C with the TR-FRET assay using 1μM hAS3MT and variants (or 10μM C61W), 10 μM SAM, 1μM Trx, 0.3 μM TR, 30 μM NADPH, 0.5 mM GSH, and 10μM of either As(III) or MAs(III). The data are the means ± SE (n = 3). [b]<0.001 when compared with wild-type hAS3MT. [c]<0.05 when compared with wild-type hAS3MT.

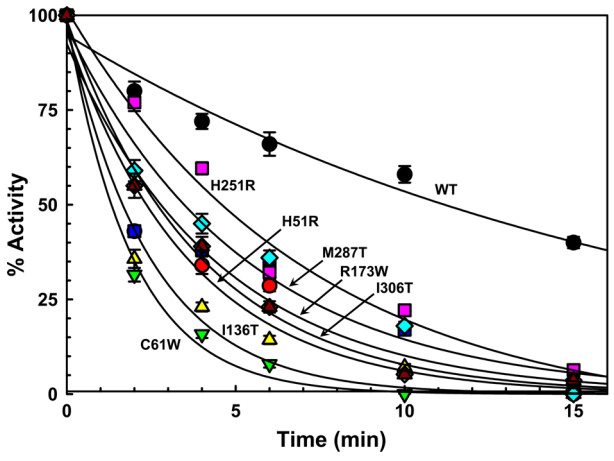

Figure 5. Temperature stability of wild-type hAS3MT and polymorphic variants. Wild-type hAS3MT and variants were heated at 42 °C for the indicated times, following which methylation activity was assayed with the TR-FRET assay at 37 °C using 1 μM enzyme, 10 μM SAM, 1 μM Trx, 0.3 μM TR, 30 μM NADPH, 0.5 mM GSH, and 10 μM As(III) (or 10 μM MAs(III) for C61W). The data are the mean ± SE (n = 3).

M287T enzyme had 3.5-fold more activity than the wild type after correction for the amount of immunoreactive protein. The K_m of this variant was approximately 11 μM for As(III), about half the affinity of the wild type and 5 μM for SAM, about twice the affinity of the wild type. These values are significantly different from each other, but not enough to explain any physiological differences.

There was one other notable biochemical study of the M287T variant.[50] In this study, wild-type hAS3MT cDNA and a site-directed M287T mutant were expressed in *E. coli*, purified, and assayed using an assay including Trx and TR with or without GSH. In the absence of GSH, the ratio of DMAs to MAs was low. In the presence of GSH, significantly more DMAs(III) was produced from As(III). GSH is the major intracellular thiol, so the activity in the presence of GSH is more likely to reflect the physiological activity. This is a significant finding, which led us to include Trx, TR, and GSH in our assays. In the presence of GSH, the K_m for both wild-type hAS3MT and M287T enzymes was approximately 1.6 μM for As(III) and 0.7–0.8 μM for MAs(III). The V_{max} values for both were relatively the same for both enzymes as well, approximately 6 pmol/μg protein/min with As(III) and 14–16 pmol/μg protein/min with MAs(III). From that study, the authors concluded that there were insufficient differences between the wild-type and M287T enzymes to account for differences in the SMI, and the reason for the increased susceptibility in individuals with the SNP could not be attributed to differences in catalytic activity between the variant and wild-type hAS3MT.

As valuable as these contributions were, they were not conclusive. Wood et al.[31] used crude cytosolic preparations. Enzyme kinetics are meaningful only when conducted with purified enzymes. The hAS3MT enzymes used by Ding et al.[50] had little activity, and methylation of iAs required equimolar or excess enzyme over substrate over long time periods. These are not catalytic conditions. At most a few turnovers would occur during the reaction, not sustained catalysis. Our approach was to use highly active purified enzymes to analyze the catalytic properties of the three most frequent exonic SNPS, as well as five additional less common ones, and correlate their activity and stability with structural information. We used a synthetic hAS3MT gene as the starting material for expression and mutant construction.[33] We showed previously that the product

DOI: 10.1021/acs.chemrestox.7b00113
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of the synthetic gene can be used in catalytic amounts with the Trx/TR/GSH assay. A large excess of substrate over enzyme ensures that there are multiple rounds of methylation during the assay time, which, with the TR-FRET assay, is linear up to 5 min.[35] In addition, the TR-FRET assay allows the two methylation reactions (iAs → MAs and MAs → DMAs) to be determined independently, an accomplishment not possible in previous studies. With this assay, it is clear that six of the variants had similar affinity for As(III) and MAs(III) as wild-type hAS3MT. The W203C enzyme lacked measurable activity. The C61W variant was not active with As(III), and Cys61 has been shown to be required for methylation of As(III).[33]

The kinetics of AS3MT sheds light on its role in metabolism, how its activity is controlled, and how a drug or an agonist might affect the metabolism of arsenic. Kinetic analyses have predictive physiological value. Kinetics governs the rates of metabolic pathways in vivo, which, in turn, allows our body to respond to arsenic exposure. The K_m for SAM of the active variants was also similar to that of the wild type except for I136T, consistent with the location of Ile136 near the SAM binding site. With each variant the V_{max} was reduced, and none of the variants approached the catalytic efficiency (k_{cat}/K_m) of the wild-type enzyme.

From the kinetics, we can conclude that each single amino acid substitution produces a less active enzyme. What is the connection between the substitution and the effect on activity? The homology structural model is informative.[33] The structure of a protein is the key to its function, allowing visualization of substrate and allosteric binding sites. The consequences of amino acid substitutions in polymorphic variants are most easily understood by the structural changes they produce. For that reason, we constructed the homology model of hAS3MT and mapped the location of the eight polymorphisms on its surface (Figure 1). His51, Cys61, and Ile136 are in the SAM binding domain. Arg173, Trp203, and Arg251 are in the arsenic binding domain. Met287 and Thr306 are in the C-terminal domain. The PolyPhen2 score of each polymorphic enzyme was calculated. A high PolyPhen2 scores indicates possible deleterious effects on the structure and activity of an enzyme. The M287T enzyme has a low PolyPhen2 score, which suggests little effect on hAS3MT structure. T306I has an intermediate score. H51R, C61W, R173W, I136T, and W203C have higher PolyPhen2 scores, predicting possibly harmful effects on the enzyme structure and reduced catalytic activity (Supplemental Table S10). Arg173 is in the As(III) binding site, so we predict that the R173W substitution may affect affinity for As(III).[40] A C61W eliminates one of the four conserved cysteine residues (Cys32, Cys61, Cys156, and Cys206) involved in substrate binding and specificity. The C61W substitution prevents formation of a disulfide bond between Cys44 and Cys61 that is required for As(III) methylation. As predicted, the C61W enzyme methylated MAs(III) but was unable to methylate As(III). His51 is located near the start of the N-terminal domain but not near the SAM binding site, and the H51R substitution does not affect the K_m for SAM. In contrast, Ile is near the SAM binding site, and the I136T substitution reduces the affinity for SAM eight-fold.

All of the substituted residues are on the surface of the protein except for Thr306, which is buried inside the enzyme, so a T306I substitution is likely to disrupt the structure. The M287T SNP has been proposed to lower the SMI by specifically reducing the rate of the second methylation step.[24] However, our results indicate that the first and second methylation steps are reduced by about the same amounts (78% and 75%, respectively), so there must be some other explanation. Met287 is located on the surface at the entrance to a cleft in AS3MT to which small molecule inhibitors bind (Figure 6).[51] We proposed that this cleft is an allosteric site that

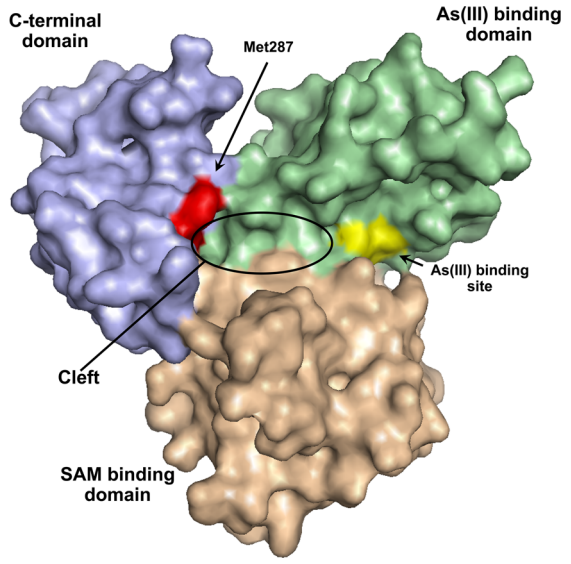

Figure 6. Molecular surface model of hAS3MT. The surface model of hAS3MT was built on the structure of PhAs(III)-bound CmArsM (PDB ID: 4KW7) from residues 44−371 (residue numbers based on the CmArsM sequence) using a fully automated protein structure homology modeling server SWISS-MODEL (http://swissmodel.expasy.org/). PyMOL v1.3 was used to visualize the structural model.[22] The putative allosteric site (circled) is located at the interface of the N-terminal (SAM binding) domain (wheat) and As(III) binding domain (light green). The C-terminal domain is in light blue, Met287 in red, and the As(III) binding site in yellow.

binds physiological molecules that regulate methylation activity by modulating a conformational change at the cleft. We speculate that the M287T substitution hinders binding of putative modulators or retards the allosteric conformational change, reducing the rate of methylation of this variant. This property is consistent with observed epidemiological studies of individuals with the M287T polymorphism.

Another factor that contributes to the reduced activity of most the variants is that they are less stable than the wild type. From measurements of temperature stability, the variants denature between 4- and 20-fold faster than wild-type hAS3MT. Each of the eight identified SNPS is thus deleterious to one degree or another because it destabilizes the structure of the enzyme. The few examples of protective AS3MT polymorphisms are located outside of the coding sequence in putative regulatory elements.

In conclusion, using purified hAS3MT variants, we demonstrate that the eight identified amino acid substitutions in hAS3MT led to decreased catalytic activity through decreased affinity for As(III) or SAM and lower stability. The loss of methylation capacity could lead to increased harmful responses to environmental arsenic. Since arsenic levels in food and water in the United States and other developed countries are generally below the EPA and WHO recommended levels, there is little selective pressure against detrimental genotypes. In contrast, in regions of the world with high arsenic exposure,

DOI: 10.1021/acs.chemrestox.7b00113
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increased expression of AS3MT is protective because it leads to faster clearance of arsenic from the body.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.7b00113.

Oligonucleotide primers for mutagenesis, population frequency of hAS3MT polymorphisms, PolyPhen2 scores for missense variants, AS3MT sequence alignments, expression of hAS3MT variants, arsenic methylation in cells of *E. coli* (PDF)

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Funding

This work was supported by National Institutes of Health Grant No. R01 ES023779.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Drs. Donald W. Bowden, Director, Center for Diabetes Research, Wake Forest School of Medicine, and Helen Tempest, Department of Human and Molecular Genetics, Florida International University Herbert Wertheim College of Medicine, for advice and suggestions on the genetics of arsenic metabolism.

■ ABBREVIATIONS

AS3MT, As(III) SAM methyltransferase; As(III), arsenite; DMAs(III), dimethylarsenite; DMAs(V), dimethylarsenate; ESP, Exome Sequencing Project; EXAC, Exome Aggregation Consortium; GSH, reduced glutathione; LB medium, Luria–Bertani medium; MAs(III), methylarsenite; MAs(V), methylarsenate; MBP, maltose-binding protein; MAF, minor allele frequency; PMI, primary methylation index; Rs, reference SNP ID; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SNPs, single nucleotide polymorphisms; SMI, secondary methylation index; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TR-FRET, time-resolved Förster resonance energy transfer; TCEP, tris(2-carboxyethyl)-phosphine; TMAs(III), trimethylarsine; Trx, thioredoxin; TR, thioredoxin reductase

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DOI: 10.1021/acs.chemrestox.7b00113
Chem. Res. Toxicol. 2017, 30, 1481−1491