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Identification of a S-adenosylmethionine (SAM) dependent arsenic methyltransferase in *Danio rerio*

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Abstract

Arsenic methylation is an important cellular metabolic process that modulates arsenic toxicity and carcinogenicity. Biomethylation of arsenic produces a series of mono-, di- and tri-methylated arsenic metabolites that can be detected in tissues and excretions. Here we report that zebrafish exposed to arsenite (As^{III}) produces organic arsenicals, including MMA^{III}, MMA^V and DMA^V with characteristic tissue ratios, demonstrating that an arsenic methylation pathway exists in zebrafish. In mammals, cellular inorganic arsenic is methylated by a SAM-dependent arsenic methyltransferase, AS3MT. A zebrafish arsenic methyltransferase homologue, As3mt, was identified by sequence alignment. Western blotting analysis showed that As3mt was universally expressed in zebrafish tissues. Prominent expression in liver and intestine correlated with methylated arsenic metabolites detected in those tissues. As3mt was expressed in and purified from *E. coli* for *in vitro* functional studies. Our results demonstrated that As3mt methylated As^{III} to DMA^V as an end product and produced MMA^{III} and MMA^V as intermediates. The activity of As3mt was inhibited by elevated concentrations of the substrate As^{III} as well as the metalloid selenite, which is a well-known antagonistic micronutrient of arsenic toxicity. The activity As3mt was abolished by substitution of either Cys160 or Cys210, which correspond to conserved cysteine residues in AS3MT homologues, suggesting that they are involved in catalysis. Expression in zebrafish of an enzyme that has a similar function to human and rodent orthologues in catalyzing intracellular arsenic biomethylation validates the applicability of zebrafish as a valuable vertebrate model for understanding arsenic-associated diseases in humans.

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Keywords

zebrafish; arsenic; arsenite; selenite; methylation; SAM; GSH; HPLC-ICP-MS; MMA^{III}; MMA^V; DMA^V

Introduction

Arsenic is an environmental toxicant, carcinogen as well as therapeutic drug (Tseng, 2008; de The and Chen, 2010; Kitchin and Conolly, 2010). Mechanisms of arsenic metabolism have been extensively studied. It has been found that trivalent arsenite (As^{III}) and pentavalent arsenate (As^V) uptake are facilitated by aquaglyceroporins (Liu *et al.*, 2002; Hamdi *et al.*, 2009) and phosphate transporters (Bun-ya *et al.*, 1996; Beene *et al.*, 2011) in prokaryotes and eukaryotes. The adventitious uptake and accumulation of arsenic via nutrient transporters drives the evolution of a variety of arsenic detoxification pathways. One of the major pathways is methylation, which has been studied in microbes, plants and mammals (Qin *et al.*, 2006; Drobna *et al.*, 2009; Ye *et al.*, 2012). Methylation of inorganic arsenicals produces different organic forms, which vary in ease of excretion, reactivity and carcinogenicity. In human, arsenic methylation produces dimethylarsinic acid (DMA^V) as a major product along with other species, including monomethylarsonous acid (MMA^{III}), monomethylarsonic acid (MMA^V), dimethylarsonous acid (DMA^{III}) and trimethylarsine oxide (TMAO) (Styblo *et al.*, 2002). All inorganic and methylated arsenicals can be detected in urine with different ratios depending on individual methylation efficiencies (Styblo *et al.*, 2002; Katsoyiannis *et al.*, 2007). Methylation has been viewed as a detoxification process for a long time since pentavalent arsenic species are less toxic than inorganic arsenic (Styblo *et al.*, 2002). However, the trivalent intermediates, MMA^{III} and DMA^{III}, which can also be produced and excreted as intermediates, are more toxic than inorganic As^V or As^{III}, which means under certain circumstances methylation activity can exacerbate arsenic toxicity (Petrick *et al.*, 2000; Hirano *et al.*, 2004; Drobna *et al.*, 2005). Therefore, arsenic methylation serves as one of decisive factor to determine the overall arsenic induced malignancy (Rehman, *et al.*, 2012). However, it is not well understood what causes interindividual variation in methylation efficiencies and in what situations arsenic methylation is beneficial for human health (Chung *et al.*, 2009; Kwong *et al.*, 2010; Wnek *et al.*, 2010).

The bona fide arsenic methyltransferase, AS3MT, was isolated from rat liver, the major site for arsenic methylation (Thomas *et al.*, 2004; Tseng, 2007). Currently, several AS3MT homologues have been identified from microbes to plants, each with similar function in arsenic methylation (Qin *et al.*, 2006; Meng *et al.*, 2011). Based on sequence alignment, two AS3MT homologues were identified in zebrafish. In this study, a closer homologue, *as3mt* was cloned and the enzyme function in arsenic methylation was studied using purified recombinant protein.

The As^{III} methylation by AS3MT is proposed to have two rounds of reaction; each round includes oxidative methylation followed by reduction. The first round reaction produces MMA^V which is then reduced to MMA^{III}, followed by a second round of methylation to DMA^V (Marapakala *et al.*, 2012). Some microbial homologues undergo a third round of methylation to TMA (trimethylarsinic acid), a gaseous form of arsenic (Qin *et al.*, 2009), or the oxidized form of TMAO (trimethylarsine oxide). It has been shown that intracellular glutathione (GSH) level regulates the formation of TMA and possibly serves as a broad determinant of the pattern and extent of formation of arsenic metabolites (Waters *et al.*, 2004). However, it is not clear which reaction step is rate-limiting. The detailed mechanisms are still lacking and questions such as whether toxic trivalent intermediates formed by

AS3MT and which conditions favor their accumulation remain unanswered. Currently reported biochemical experiments were done in conditions fostering a complete reaction using high enzyme concentrations and prolonged reaction times, thus the trivalent intermediate, MMA^{III}, has not been detected. In this study, under a well controlled condition dependent on SAM and a reductant (β -mercaptoethanol, β ME), we demonstrated that As3mt undergoes two rounds of step-wise methylation, with initial product of mono-methylated MMA^{III} and MMA^V, followed by the second and final round of step with product of DMA^V.

The lack of a three dimensional protein structure of AS3MT homologues prevented the complete elucidation of enzymatic mechanisms such as initial substrate binding and connection between different catalytic cycles. Currently, conserved cysteines have been identified in the sequences of a wide range of AS3MT homologues, and mutation of these cysteines led to the loss of zAs3mt function (Song *et al.*, 2010). By homology modeling of As3mt using a recently available structure of an algal ArsM without (PDB ID 3P7E) and with bound As^{III} (PDB ID 3QNH), we identified conserved two residues, C160 and C210, that we suggest are required for initial As^{III} binding.

Zebrafish is an emerging model organism in toxicological studies with advantages in ease of toxicant exposure, high embryo availability and amenability of genetic modification. Additionally, zebrafish genome has conserved genes closely related to arsenic metabolism in humans thus providing an ideal animal model for investigating mechanisms of pathogenesis induced by arsenic exposure. In this study, we used zebrafish model to understand further the biomethylation of arsenic both *in vivo* and *in vitro*.

The methylation of arsenic in humans is influenced by many factors. For example, the individual difference, such as Body Mass Index (BMI), is found to be associated with altered arsenic methylation (Gomez-Rubio *et al.*, 2011). Environmental factors, including the types of arsenic exposure and availability of micronutrients, for example selenium, may lead to variation in arsenic methylation. The presence of selenium will interfere with arsenic methylation given its general chemical properties similar to arsenic and potential to inhibit AS3MT (Song *et al.*, 2010). Epidemiological studies demonstrated that low serum selenium is associated with increased MMA in urine (Basu *et al.*, 2011). In the current study we found that selenium, in the form of selenite, is a potent inhibitor for As3mt function and is predicted to compete with As^{III} for its initial binding. Since trivalent arsenite is also a clinical approved drug for leukemia treatment (Antman. *et al.*, 2001), factors modulating As3mt function will be correlated to the interindividual difference in arsenic pathology and pharmacology. Detailed understanding of the enzymatic mechanisms of As3mt will promote application of zebrafish in the screening of efficient pharmacological intervention in the methylation process. The analogy of arsenic metabolism in zebrafish validates the use of this model in future arsenic toxicology and carcinogenesis studies.

Materials and Methods

Zebrafish husbandry and arsenic exposure

All studies involving zebrafish have been approved by Oakland University IACUC. Zebrafish ZF-1 line was maintained in an automatic 14:10 light:dark cycle at 28°C. Static waterborne arsenic exposure was carried out in adult zebrafish using 300 ppb or 5 ppm sodium arsenite for 120 hours. After treatment, zebrafish were rinsed for 1 hour and euthanized with 0.3 mM tricaine (MS-222 (Ethyl 3-aminobenzoate methanesulfonate), Sigma). Tissues were isolated and pooled (30 fish tissues used as one sample) and mass of each sample was determined. All tissue samples were homogenized in phosphate buffered saline (PBS buffer, pH 7.4) and adjusted to a final concentration of 100 mg wet tissue/mL buffer, and then centrifuged at

13,000 × g for 10 minutes. The resulting supernatants were filtered with a Microcon Ultracel YM-3 centrifugal filter (Millipore, MA) for speciation analysis by HPLC-ICP-MS (Hamdi *et al.*, 2009).

Chemicals—The MMA^{III} was obtained through Dr. Styblo's lab (North Carolina University). All other chemicals were obtained through commercial sources at analytical grade.

Cloning of zebrafish *as3mt* and site-directed mutagenesis

Zebrafish *as3mt* was cloned from a cDNA mixture synthesized from mRNA that has been extracted from whole zebrafish, and amplified using a pair of PCR primers with *Bgl*III and *Xho*I restriction sites (forward: 5'-GCAGATCTATGGCACCACGTCCAAAGCAGG-3' and reverse: 5'-GCCTCGAGTCTATAAAGATGTTGCCTTCAG-3'). The amplified *as3mt* was initially cloned into pGEMT-easy (Promega) and a second round of PCR is applied to add 6XHis tag, followed by subcloning into pMAL-cX2 expression vector (NEB) (Liu *et al.*, 2006). The resulting plasmid pMal-*as3mt* was transformed into *E. coli* BL21 (NEB) for overexpression and purification. The mutants of C165S and C210S were created by site-directed mutagenesis (Stratagene) using following primers: C165S forward: 5'-GATATTATCATA TCAAATTCTGTGGTGAATCTG-3'; reverse: 5'-CAGATTCACCACAGAATTTGATATGATAATATC-3'; C210S forward: 5'-CTTTATGGGGCGAGAGCCTCAGTGGAGCATTG-3'; reverse: 5'-CAATGCTCCACTGAGGCTCTCGCCCCATAAAG-3'. Both the WT gene and the mutants were verified by nucleotide sequencing.

Overexpression and purification of As3mt and mutants in *E. coli*

The *E. coli* strain BL21 carrying pMAL-*as3mt1* was grown in LB medium supplied with ampicillin at 37 °C to an OD₆₀₀ 0.6–1.0, followed by induction with 0.6 mM IPTG for 8 hours. The overnight culture was harvested by centrifugation and washed. The cells were resuspended in buffer A (50 mM MOPS, pH 7.5, 20% (wt/vol) glycerol, 0.5 M NaCl, 20 mM imidazole, and 10 mM β-ME) and lysed by a single pass through French-press at 20,000 psi. Membranes and unbroken cells were removed by ultracentrifugation. The supernatant was loaded at a flow rate of 0.5 ml/min onto a Ni(II)-NTA column (QIAGEN) preequilibrated with buffer A. After washing with the same buffer, protein was eluted with 60 ml of buffer B (50 mM MOPS, pH 7.5, 20% (wt/vol) glycerol, 0.5 M NaCl, 200 mM imidazole, and 10 mM β-ME). Fractions containing zAs3mt were pooled and loaded onto the Amylose column (NEB) preequilibrated with buffer C (200 mM NaCl, 20 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM sodium azide, 10 mM β-ME) and washed. The zAs3mt protein was eluted with buffer D (200 mM NaCl, 20 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM sodium azide, 10 mM β-ME, 10 mM maltose). The zAs3mt fractions were concentrated using a 30-kDa-cutoff Amicon Ultrafilter (Millipore). Protein concentrations were determined by Absorbance Assay (280 nm). The purity of As3mt1 was determined by SDS gel electrophoresis.

SDS-PAGE and Western Immunoblotting

Protein samples or zebrafish tissue samples (males and females) were processed at 100 °C for 5 minutes and loaded on 12% SDS-PAGE gel, followed by coomassie brilliant blue staining. A custom raised primary polyclonal antibody (Abmart) of zAs3mt was applied in the western-blotting at 1:1000 dilution. Band density was quantified using the ImageJ software after scanning. Mean value and standard errors were calculated using SigmaPlot 10.0.

Analysis of enzymatic reaction using purified As3mt

The *in vitro* reaction mixture contained 10 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 0.5 mM EDTA, 5 mM β -ME, 1 mM SAM, 1 mM GSH, 100 μ M sodium arsenite (As^{III}) and 1 μ M zAs3mt, unless other concentrations indicated. Reaction mixtures were incubated at 37 °C for indicated times and the methylation reaction was stopped by filtration with a cut off column. A negative control was performed in the absence of zAs3mt from the above mixture. Inhibitors were added at indicated concentrations at the beginning of the reaction.

Arsenic binding assay

Sodium arsenite at indicated concentrations was incubated with 2 μ M purified WT or mutant proteins. After 30 minutes the mixture was passed through spin columns (Micro Bio-Spin 6, Biorad) to remove the unbound free arsenic. The protein bound arsenic was eluted and quantified by inductively coupled plasma-mass spectrometer (ICP-MS, ELAN 9000, PerkinElmer).

Structural modeling of zAs3mt

The 0.63 Å resolution structure *Cyanidobacterium* ArsM (CmArsM, 1–323 residues, PDB ID 3P7E) (Barry P. Rosen, PNAS, 2012, in press) was used as a reference to construct a C-terminal truncated (1–299 residue) As3mt model. The two sequences shared 43.85% of identity and 285 residues were aligned.

Arsenic speciation by HPLC-ICP-MS

The speciation of samples from cell lysate, enzymatic reaction mixtures or the zebrafish tissues was achieved using ICP-MS coupled with HPLC (Series 2000, PerkinElmer) in the front end. For arsenic samples generated from the enzymatic reaction the reaction was halted by removing As3mt from the reaction samples by centrifugation using a 10-kDa-cutoff Amicon Ultrafilter (Millipore). The filtrate was then separated and analyzed by HPLC-ICP-MS using a reverse-phase C18 column (Jupiter 300) eluted isocratically with a mobile phase (3 mM malonic acid, 5 mM tetrabutylammonium hydroxide, and 5% methanol, pH 5.6, with a flow rate of 1.0 ml/min). Arsenic standards were purchased from commercial suppliers.

Results

Zebrafish methylate inorganic As^{III} to MMA^{II} , MMA^{V} and DMA^{V} under arsenic exposure

The arsenic biomethylation profile was examined in zebrafish (males and females) and compared under arsenic exposure. Two arsenic concentrations, 300 ppb and 5 ppm, were applied for 120 hours to model two different levels of non-lethal doses. The pattern of arsenic methylation was determined and compared in different tissues. Our results showed that, compared with acute exposure, little methylated arsenic can be detected following sublethal exposure, with As^{III} as the dominant species in most tissues (Fig. 1). When 5 ppm was applied a significant amount of methylated arsenic species appeared. DMA^{V} was found in brain, gill, and liver while MMA^{V} was detected in all tissues at a relatively low level. The intestine showed a significant amount of MMA^{III} , and several unknown components, possibly involved the arsenic methylation through microbial activity. An unknown peak not corresponding to any arsenic standards that eluted after DMA^{V} was detected in almost all tissues and is hypothesized to be some type of As-GSH conjugate or a metabolite of an uncharacterized pathway. In all tissues the inorganic form of As^{III} persisted in many tissues as the dominant species, indicating the biomethylation in these tissues cannot be efficiently processed. Factors accounting for differences of arsenic retention patterns in specific tissues may be collectively determined by the combined activity of biomethylation, uptake and efflux. We noticed that skin displayed a minimal arsenic accumulation but with high ratio of

biomethylation. Since the arsenic transporters that facilitate As^{III} uptake were identified in skin (Hamdi *et al.*, 2009), we predict skin could have either efficient extrusion systems or is able to metabolize arsenic via unknown mechanisms. During all As^{III} exposures the other important inorganic arsenical, As^{V} , was not detected in any tissues. Our previous studies show that no As^{V} is detected when zebrafish were exposed to As^{V} , indicating efficient reducing systems exist in zebrafish (Beene *et al.*, 2011).

The As3mt is extensively expressed in zebrafish tissues

The expression of As3mt was ubiquitous in zebrafish tissues (Fig. 2). In contrast to mammalian AS3MT which is mainly expressed in liver, zebrafish As3mt is a broadly expressed protein detected in brain, eye, gill, intestine, liver, muscle and skin. Quantitative analysis showed that zAs3mt is mostly expressed in liver, muscle and intestine. As a result, arsenic methylation occurred in many tissues and methylated arsenic products were detected (Fig. 1). However, in tissues such as muscle with substantial As3mt expression, no methylated species were detected. It is possible that the activity of this enzyme is sensitive to tissue microenvironments, such as an absence of essential cofactors.

Purified As3mt catalyzed step-wise methylation of As^{III} , producing DMA^{V} as the end product and MMA^{III} and MMA^{V} as intermediates

His-tagged and MBP-tagged As3mt were purified as soluble recombinant proteins at approximately 80 kDa following Nickle-column and Maltose column purification (Fig. 3A). The enzymatic activity was assayed in buffer D, which contains SAM and β -ME, with an enzyme:substrate molar ratio of 1:100. The reaction was monitored in a time course fashion in order to detect step-wise formation and release of intermediates and products. The presence of MMA^{V} and MMA^{III} were detected in the first round of methylation, followed by a second round of methylation to DMA^{V} (Fig. 3). Our results showed that DMA^{V} is the final product and MMA^{V} and MMA^{III} are plausible intermediates. These *in vitro* results are consistent with our observations *in vivo*. DMA^{V} is also the dominant biomethylation product detected in mammals and the final product for most AS3MT homologues, showing zebrafish and mammals showed similar arsenic biomethylation pathways. MMA^{III} is rarely observed in tissues, possibly due to the rate of second round of methylation following MMA^{III} formation is faster than first round. To our knowledge, this is the first experimental evidence showing the existence of MMA^{III} intermediate in an *in vitro* system using a purified arsenic methyltransferase.

Increased substrate concentration inhibits As3mt function

As AS3MT catalyzes multiple steps in arsenic methylation it is anticipated that the methylation pattern might be different according to varied substrate concentrations. To address this question *in vitro*, the effect of substrate doses on zAs3mt activity was investigated by applying various concentrations of As^{III} concentrations in the enzymatic reaction mixture. As shown in Fig. 4, with an As^{III} concentration range from 25 μM to 200 μM the As3mt activity declined. As3mt completely lost methylation ability at an As^{III} concentration of 200 μM .

Selenite is an inhibitor for As3mt

Selenium has been used to antagonize arsenic toxicity. In this study we found that selenium, in the form of selenite, inhibited AS3MT activity efficiently. Addition of sodium selenite at 1:10 molar ratio with As^{III} in the reaction mixture completely abolished the arsenic methylation (Fig. 5).

Mutation of two cysteines (C160 and C210) lead to a loss of As^{III}mt function and arsenic binding

In this study we directly tested if two cysteines (Fig. 6A) are involved in As^{III} binding. The C165S and C210S mutants of As^{III}mt were created and ectopically expressed in *E. coli*. The arsenic methylation activity of these mutants was studied and compared with the wild type (WT) As^{III}mt. The cysteine mutation in these sites completely abolished the function of zAs^{III}mt and no methylated arsenic species were produced (Fig. 6B). To further determine if these cysteines are required for substrate As^{III} binding a direct assay was performed to investigate the properties of C165S and C210S in arsenic binding. Our results showed that WT As^{III}mt binds As^{III} substrates and saturated at an approximately 1:1 stoichiometry ratio, suggesting one As^{III} binding site in the enzyme. However, both C160S and C210S mutants lost capability to bind As^{III}, again indicating these two residues are required for substrate binding (Fig. 6C). In support of this hypothesis, a structure model was constructed based on a thermophilic alga *Cyanidioschyzon* sp. 5508 ArsM. As suggested in As^{III}mt structure, the cysteine C160 and C210 are in close proximity and form a reasonable As^{III} binding site (Fig. 6D).

Discussion

Due to the abundance of arsenic in the environment and diversity of mechanisms through which arsenic causes toxicity, it is important to understand the regulation of chemical metabolism, distribution, and bodily clearance of arsenic. The methylation of arsenic is one of the key variables and thus detailed understanding of mechanism of arsenic methyltransferases is crucial. Despite the possibility of other enzymes that catalyze arsenic biomethylation, such as N-6 adenine-specific DNA methyltransferase (N6AMT1), which was recently found to methylate MMA^{III} to DMA^V (Ren *et al.*, 2011), AS3MT is considered the major enzyme in arsenic biomethylation. This is consistently supported by many independent clinical studies which link AS3MT variants with altered profiles and ratios of arsenic methylated species (Valenzuela *et al.*, 2009; Engstrom *et al.*, 2011). It is clear that AS3MT transforms arsenic into less toxic DMA^V which is more easily cleared from the body. However, it remains an open question whether AS3MT is responsible for the formation of more toxic intermediates such as MMA^{III}. This study showed zAs^{III}mt transforms As^{III} to DMA^V similar to mammalian AS3MT homologues (Fig 3). The absence of significant MMA^{III} in zebrafish tissue after arsenic exposure can be explained by through MMA^{III} not being rate limiting in the second round of under these exposure conditions.

The unique nature of arsenic speciation may influence the pathogenesis of certain diseases such as cancer. The carcinogenic potential of arsenic is related to the sum of total arsenicals, including endogenously generated organic species. Therefore, function of AS3MT is a key factor in arsenic-induced carcinogenesis by affecting arsenic metabolism and bodily retention. This assumption is corroborated by a correlation between arsenic methylation and cancer incidence in epidemiological studies carried out in arsenic contaminated areas (Kwong *et al.*, 2010; Wnek *et al.*, 2010). Moreover, it has been shown by independent reports that AS3MT polymorphisms influence arsenic metabolism and individual cancer risk (Agusa *et al.*, 2009; Kojima *et al.*, 2009; Valenzuela *et al.*, 2009). Analysis of arsenic speciation in urine shows that the concentration of monomethylated species is highly correlated with increased cancer risk (Chung *et al.*, 2009).

Possible carcinogenic mechanisms include arsenic induced oxidative and DNA damage (Kojima *et al.*, 2009) and pathogenesis via the generation of reactive oxygen species (ROS) (Jomova *et al.*, 2011). Although all forms of inorganic arsenic as well as certain organic forms have been shown to generate ROS, the magnitude of arsenic induced oxidative stress is highly speciation dependent (Hughes, 2009). Arsenic also deregulates oncogenic signaling

pathways and this is also likely to be speciation dependent. Given arsenic biomethylation via AS3MT activity produces intermediates with different capacity in elevating oxidative stress and ROS, it is reasonable to assume AS3MT activity may link to overall arsenic-induced carcinogenesis via controlling OS during arsenic exposure. Thus, the biologic action of AS3MT is predicted to play important roles in arsenic induced oxidative stress, DNA damage and ultimately, carcinogenesis. It follows that factors that modify AS3MT activity will affect overall arsenic-induced carcinogenesis. Therefore it is critical to elucidate the fundamental mechanisms of arsenic-induced methylation by AS3MT, and investigate the factors that may modify the enzymatic activities.

The function of AS3MT in arsenic methylation raises the question of how AS3MT produces methylated trivalent species and under which conditions the more toxic intermediates exist. It has been difficult to measure the activity of AS3MT in real-time. Some trivalent arsenic, such as MMA^{III}, can be detected in human urine; however, this plausible intermediate has not been observed using purified enzyme. Possible reasons for the lack of such results are: 1) most studies of methylation activity using purified human or rodent AS3MT were performed with high enzyme:substrate ratios, which fosters a complete reaction, but not detectable quantities of intermediates, i.e., the trivalent methylated species (Song *et al.*, 2011); 2) Until recently, no three dimensional structure was available, which precluded structure-function analysis and elucidation of the catalytic mechanism. In our studies, using a purified zebrafish As3mt, we studied the time course of arsenic methylation, and demonstrated the existence of trivalent and pentavalent arsenic intermediates MMA^{III} and MMA^V, which were formed in the first round of the cycle. The reaction ends up with DMA^V and the third round of methylation is not achieved, therefore TMA gas is not detected (data not shown). These *in vitro* results are highly consistent with the arsenic distribution *in vivo*, with all these intermediates and end products detected under As^{III} exposure. In rodents, the dominant product of As^{III} biomethylation is DMA^V, with a small portion of MMA^V, which has been demonstrated in tissue culture and knockout mice (Drobna *et al.*, 2004). The arsenic cellular retention is related to both biomethylation and membrane transport of substrates and products. For example, in human hepatocytes, DMA^V is readily extruded while MMA^V is mostly retained (Drobna *et al.*, 2004). Our results in tissues of As^{III} exposed zebrafish indicated that MMA^V and DMA^V could be detected in many tissues with different patterns which resemble those in rodents and humans. Interestingly, an uncharacterized arsenic compound which is not consistent with any known arsenic methylated compounds was identified in all tissues samples. Further studies are required to determine whether this arsenic species resulted from a different metabolic pathway other than biomethylation.

The AS3MT catalytic mechanisms were previously studied using biochemical approaches (Song *et al.*, 2010) but the detailed mechanisms such as As^{III} binding, and the continuous catalysis of two cycle of reactions have not been elucidated in AS3MT. By site-directed mutagenesis, function of conserved and non-conserved cysteines were investigated in human AS3MT where a cysteine, C72, is shown to be critical for the reaction and hypothesized to be involved in the initial substrate binding (Song *et al.*, 2011). However, in our study using As3mt, we suggest C165 and C210 form substrate binding site. Single mutation of these cysteines disabled the As3mt function as well as binding ability to arsenic. Therefore the C165 and C210 may together form a coordinating site for arsenic binding. The coordinated arrangement of these two cysteines was indicated in the structure model of As3mt in this study (Fig. 6D). In this model, these two cysteines are close to each other and form a pocket for arsenic binding. Single mutation of C65S produced indistinguishable levels of As^{III} binding to WT (data not shown), indicating its importance comes from a role in catalytic activity following binding. Results using CmArsM orthologue show that C74 (C65 in zebrafish As3mt) is required only for the second round of methylation but not the first round

of methylation, while both Cys174 and Cys224 are involved in the entire reaction. Taken together our results are consistent with the information derived from CmArsM structure, showing that As^{III} is bound by Cys160 and C210 (Cys174 and Cys224 in CmArsM) while Cys65 (Cys72 in CmArsM) moves toward the other two As^{III}-binding cysteine residues when SAM is bound. The model also discloses a SAM binding domain and a C-terminal domain with unknown function. More studies are required to investigate the in depth coordination of the two rounds of reaction.

The function of AS3MT is affected by several factors, such as divalent metals zinc, copper, mercury and metalloid selenite (Song *et al.*, 2010). Through different mechanisms these compounds all react with coordinated thiols. We report here that selenite is an efficient inhibitor for zAs3mt function. Given both its similar molecular structure to As^{III} and ability to bind vicinal thiols, it is postulated that Se^{IV} is competing for same binding sites as arsenic, similar to the binding of cysteines in zinc finger structure (Larabee *et al.*, 2002). We also showed that elevated substrate concentration completely abolished the As3mt activity in the MMA or DMA production. Currently the basis is not clear and there are several possibilities: 1) Increased substrate will affect the initial binding and change the appropriate position of substrate and cofactors. 2) Increased substrate is toxic for the enzyme by modifying the overall enzyme conformation. More experiments are required to investigate the precise mechanisms of substrate inhibition in zAs3mt.

The *in vitro* activity of As3mt and other mammalian AS3MT is relatively low, which requires a higher enzyme:substrate ratio. Possible explanations for the reduced activity are that there are unknown cofactors absent from the *in vitro* system, such as endogenous reducing systems. The elevated As^{III} retention in tissues indicates that the *in vivo* activity of AS3MT is also limited. From an evolutionary perspective, As3mt function requires SAM, which requires ATP during synthesis. On the other hand, it is reasonable that zebrafish or mammals do not require a highly efficient enzyme to metabolize commonly occurring low levels of environmental arsenic. An additional detoxification pathway, such as MRP-mediated GSH conjugate efflux can provide an alternative detoxification pathway (Liu *et al.*, 2001).

Currently there are no studies on the regulation of As3mt and further studies are needed to understand the regulation of As3mt expression along with the role of As3mt in long-term chronic arsenic exposure. The zebrafish model can be well adapted to these purposes by the availability of *as3mt* transgenesis. Along with the advantage in conducting high throughput chemical exposure studies and dose responsive toxicity studies with large number of sample applied. Zebrafish can be an ideal animal model for translational research not only to understand the role of As3mt in overall arsenic-induced carcinogenesis but also to facilitate therapeutic drug discovery for treatment and prevention for arsenic related malignancy via pharmacological intervention (Lam *et al.*, 2006; Amatruda and Patton, 2008; Nguyen *et al.*, 2011).

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Abbreviations

SAM	S-adenosylmethionine
MMA ^{III}	monomethylarsonous acid

MMA^V	monomethylarsonic acid
DMA^V	dimethylarsinic acid
β-ME	β-mercaptoethanol
ICP-MS	Inductively-coupled plasma spectrometry
GSH	Glutathione

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Highlights

- Zebrafish methylated As^{III} to MMA^{III} , MMA^{V} and DMA^{V} .
- A zebrafish arsenic methyltransferase (As3mt) was purified in *E. coli*.
- As3mt catalyzed biomethylation of As^{III} to DMA^{V} and produced toxic intermediates.
- As3mt activity is inhibited by elevated substrate concentrations and selenite.
- C160 and C165 are predicted as As^{III} binding sites.

Figure 1. Arsenic methylation in zebrafish tissues

Adult zebrafish were exposed to sodium arsenite at 300 ppb or 5 ppm for 120 hours in static water. Different tissues were isolated for speciation assays using HPLC-ICP-MS. Each sample contains a pool of thirty tissues. Species analyzed in the assay include As^{III} , As^{V} , MMA^{III} , MMA^{V} , and DMA^{V} . The upper plots represent 5 ppm and the lower plots represent 300 ppb As^{III} treated samples.

Figure 2. Expression of As3mt in different zebrafish tissues determined by western-blotting Zebrafish tissue was isolated and homogenized. All samples were suspended in PBS buffer at final concentration of 200 mg wet tissue/mL. Western blotting was performed using a custom raised antibody (Abmart). To quantify the expression of As3mt in different tissues, a commercial antibody that reacts with pan-actin (alpha-actin and beta-actin) was applied as a control. Lower panel: Quantification of As3mt protein expression from western-blotting analysis. Three replicates were obtained from western-blotting and quantified by ImageJ after scanning. Mean value and standard errors were derived with SigmaPlot 10.0.

Figure 3. Purification of As3mt protein and time-course of As3mt catalysis

Left A: Double tagged As3mt was purified as a soluble protein. SDS-PAGE showed the purity of As3mt after elution from Ni(II)-NTA column and Maltose column (1. Protein ladder; 2. After Ni(II)-NTA column; 3. After maltose column). **Right B:** Time-course reaction of As3mt catalyzed arsenite methylation from As^{III} to DMA^V, producing MMA^V and MMA^{III} as initial intermediates. The ratio of E:S is 1:100 (1 μ M: 100 μ M). Reaction time frame is indicated. **Peaks indicated in the figure: 1. As^{III} 2. MMA^{III} 3. DMA^V, 4. MMA^V 5. As^V.**

Figure 4. Inhibition of As3mt by increased substrate concentrations

Sodium arsenite was added at indicated concentration (25, 50, 100, 200 μM) and incubated with 1 μM of purified As3mt and 100 μM of sodium arsenite. After reaction was finished (2 hours) arsenic speciation was determined by HPLC-ICP-MS.

Figure 5. Inhibition of As³mt by selenite
Sodium selenite was added at a final concentration of 2 μ M causing abolished As^{III} methylation catalyzed by As³mt.

Fig. 6A

zAs3mt	1	-----MADAARDRVTISVYNDVREYYGKTLQKSDLEKNACVPSAMPVSAYTRKVLIA
hsAs3MT	1	-----MALRLD--AEIQPDVQTYTGQVLRPSADLQINECVTNRPVYKHEALQ
CmArsM	1	MPCSCASGCQKSKNGGTPSTRDHVADYYGKTLQSSADLKTSACKLAA-AVESHRRKLLA
zAs3mt	53	EIHPPVYAKYYFCCLVMPCC--LEGCRVLDLGGCGSRDCYHLSQLVGEKGHVTGIDMTFA
hsAs3MT	49	NVHEEVALYYFCCLVPEH--LENCLLDLGGCGSRDCYHLSQLVGEKGHVTGIDMTKE
CmArsM	60	DIADVEVLEKYYFCCLSTDPADGSLGATVLDLGGCGGRDVYLAERLVGEHGRVIEVDMLDN
zAs3mt	111	QLEVARNYIDYHMQIFG--QKNPMVMFQGYIEALVEA---GDEKSYDIIISICVNLIS
hsAs3MT	107	QMEVAKYIDYHMEKFG--QASNVTFIHGYIEKLEA---GKNEHQDIVSICVNLIV
CmArsM	120	QLEVARKYVEYHAEKFFGSPSRNVRFLEGTIENLITAEPEGVPDSSEVDIVISICVNLIS
zAs3mt	166	PDKSSYLREAYCYLKGGLYFSDVYSARPEHFRANKLWGCISGALWEDLIRLAE
hsAs3MT	162	PDRQOVLQAYRVLRKHGGLYFSDVYTSLELPEEIRTHKRLWGCISGALYKSLAVLAQ
CmArsM	180	TNGLAIFREIHRVLRDGGELYFSDVYADRRISEAAQDPILWGCISGALYEDFRRLVA
zAs3mt	226	EKGFCPPRLVASLITVGMTELESILGDYKFVSATIRLFKIQKGLEKPKCLWYNGQITD
hsAs3MT	222	KIGFCPPRLVIAWLIHQKLELVIGDCRFVSATIRLFHSHITGPTRCQVWYNGGHTC
CmArsM	240	EAGFRDVRIVSVGPDIDSDPQLRKLVPDVFQVYSCITFCFKVATLEATREDYQGSATYIGC
zAs3mt	286	SESEFFDAQVAFRYDKWEVDGVAMILNRSRSESTFQDPQGVNT--ASGGGCAKP-
hsAs3MT	282	HRELMFDANFTFEGEIVEVDEETAILNRSRAQDFLRPIEENL--PSGGGSADEL
CmArsM	300	IGEEKLDRFFTFPRKPVVRDNTAEIRSRHLQDFSVSAEQQHMGLFKANDSVALLH
zAs3mt	343	NAISVNPPELVQQHGSASVSPSTGGCCACQPSCON----- 377
hsAs3MT	340	KDITDPRRLAEE--SDSRKRCVPDAAGE----- 367
CmArsM	360	APISHOVEQLVCEWKKESTGTICSAQASANGASCATGRRC 400

Fig. 6B

Fig 6C

Fig. 6D

Figure 6. Cys165 and Cys210 are predicted As^{III} binding sites

A). Sequence alignment of hsAS3MT, zAs3mt and CmArsM. Three conserved cysteines were indicated in the figure (**boxed**). **B)** Mutants of C160S and C210S were unable to methylate As^{III}. Upper panel: cytosol from *E. coli* expressing mutant C160S and C210S with IPTG induction. Lower panel: Arsenic speciation in the culture medium of WT, negative control, mutant C160S and C210S, respectively after IPTG induction. Inside the figure showed the As3mt expression in *E. coli* after IPTG induction. **C).** C160S and C210S

were unable to bind As^{III}. Upper panel, WT, C160S and C210S were purified as recombinant proteins. Lower panel, binding assay of zAs3mt WT, C160S and C210S indicate that mutation of cysteines at 160 and 210 disabled the As^{III} binding (plotted with SigmaPlot 10.0 with a hyperbolic curve). **D**). Structure model of zebrafish As3mt. The 0.63 Å CmArsM (1–323 residues) structure was used to construct As3mt model (1–299 residues). Cys160 and Cys210 were labeled in yellow color. Two addition functional domains, SAM binding domain and C-terminal domain were indicated in the structure.