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## Arsenic (+3 Oxidation State) Methyltransferase and the Methylation of Arsenicals

David J. Thomas<sup>\*,1</sup>, Jiaxin Li<sup>†</sup>, Stephen B. Waters<sup>†</sup>, Weibing Xing<sup>†</sup>, Blakely M. Adair<sup>\*</sup>, Zuzana Drobna<sup>‡</sup>, Vicenta Devesa<sup>§,2</sup>, and Miroslav Styblo<sup>‡,§</sup>

<sup>\*</sup> Pharmacokinetics Branch, Experimental Toxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711

<sup>†</sup> Curriculum in Toxicology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

<sup>‡</sup> Department of Nutrition, Schools of Public Health and of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

<sup>§</sup> Center for Environmental Medicine, Asthma, and Lung Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

### Abstract

Metabolic conversion of inorganic arsenic into methylated products is a multistep process that yields mono-, di-, and trimethylated arsenicals. In recent years, it has become apparent that formation of methylated metabolites of inorganic arsenic is not necessarily a detoxification process. Intermediates and products formed in this pathway may be more reactive and toxic than inorganic arsenic. Like all metabolic pathways, understanding the pathway for arsenic methylation involves identification of each individual step in the process and the characterization of the molecules which participate in each step. Among several arsenic methyltransferases that have been identified, arsenic (+3 oxidation state) methyltransferase is the one best characterized at the genetic and functional levels. This review focuses on phylogenetic relationships in the deuterostomal lineage for this enzyme and on the relation between genotype for arsenic (+3 oxidation state) methyltransferase and phenotype for conversion of inorganic arsenic to methylated metabolites. Two conceptual models for function of arsenic (+3 oxidation state) methyltransferase which posit different roles for cellular reductants in the conversion of inorganic arsenic to methylated metabolites are compared. Although each model accurately represents some aspects of enzyme's role in the pathway for arsenic methylation, neither model is a fully satisfactory representation of all the steps in this metabolic pathway. Additional information on the structure and function of the enzyme will be needed to develop a more comprehensive model for this pathway.

### Keywords

arsenic; methylation; arsenic (+3 oxidation state) methyltransferase

<sup>1</sup> To whom correspondence should be addressed at Pharmacokinetics Branch, Experimental Toxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711. E-mail: thomas.david@epa.gov.

<sup>2</sup> Current address: Institute of Agrochemistry and Food Technology, Burjassot, Valencia, Spain.

<sup>3</sup> W. Xing et al., unpublished results.

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## Introduction

Elucidation of the pathway for conversion of inorganic arsenic (iAs) to methylated metabolites began in the 19th century with studies in microorganisms and led to identification of trimethylarsine (TMA) as a volatile metabolite (1). Implicit in identification of this highly methylated arsenical as a metabolite was the possibility that other methylated arsenicals might be formed as intermediates in this pathway. Furthermore, widespread recognition that humans who ingested iAs often exhibited a strong garlic-like odor on expired breath led to a common understanding that humans must also form methylated arsenicals. Following Braman and Foreback (2) who reported the presence of iAs, methyl arsenic (MA), and dimethyl arsenic (DMA) in human urine, Crecelius (3) provided the first definitive report of the presence of these arsenicals in the urine of a volunteer who ingested wine or water containing inorganic arsenite (iAs<sup>III</sup>) or inorganic arsenate (iAs<sup>V</sup>). This result was consistent with the hypothesis that there is a metabolic pathway leading from iAs to these methylated products. Studies in humans and other species have provided a general understanding of the characteristics of the distribution, metabolism, retention, and excretion of arsenicals (4,5). In particular, these studies show that many animal species convert iAs into MAs and DMAs, which are excreted in urine. Determining the basis for this conversion of iAs into methylated species depended on development of strategies to isolate, purify, and characterize the molecules that are involved in each step of the pathway that leads from iAs to its methylated metabolites and development of sufficiently sensitive and specific analytical methods to identify intermediates and products formed in the methylation pathway.

Although the details of the pathway for arsenic (As) methylation will be discussed in the course of this review, it is sufficient to say that it has commonly been assumed that the pathway leading from iAs to the methylated metabolites involves several distinct steps. In this process, oxidative methylation of arsenicals alternates with reductive processes, yielding a number of intermediates and products. Some steps in this pathway have been reported to be strictly chemical reactions; others are enzymatically catalyzed. Many details of this pathway and the nature of each constituent steps remain uncertain (for a review of some of these controversies, see Ref. 6). However, work to date has identified one methyltransferase that is clearly a participant in this pathway. Arsenic (+3 oxidation state) methyltransferase (AS3MT)<sup>1</sup> catalyzes conversion of iAs to methylated products. This review examines the occurrence of *AS3MT* homologs in genomes of species ranging from sea urchins to humans and the association of occurrence of *AS3MT* and the As methylation phenotype; that is, the capacity to convert iAs to methylated products that can be detected in tissues and excreta. Finally, consideration of two conceptual models for As methylation by AS3MT suggests that elements of either model may be required to describe fully the role of cellular reductants in the catalytic function of this enzyme.

## Phylogenetic Relations for AS3MT

### Sequence Homologies in AS3MT

The AS3MT protein was the first As methyltransferase to be sufficiently purified to permit cloning of the corresponding gene (7,8). Sequence information on rat AS3MT has provided a starting point to seek a broader perspective on its structure and function as an As methyltransferase, using genomic data to investigate the occurrence of homologous genes across a wide range of species. Inspection of predicted products for these *AS3MT* homologs

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<sup>1</sup>This protein and its gene were initially termed *cyt19* by us and other investigators, following its original identification in GenBank with a methyltransferase of unknown function (NCB accession NP065602 and NP 05133). Subsequently, we have followed the recommendations of the Human Genome Nomenclature Committee (<http://www.gene.ucl.ac.uk/cgi-bin/nomenclature/searchgenes.pl>) for the systematic naming of genes and proteins and refer to protein or gene as arsenic (+3 oxidation state) methyltransferase (AS3MT).

identified proteins encoded in genomes of purple sea urchin (*Strongylocentrotus purpuratus*), sea squirt (*Ciona intestinalis*), rainbow trout (*Oncorhynchus mykiss*), chicken (*Gallus gallus*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), cow (*Bos taurus*), chimpanzee (*Pan troglodytes*), and human (*Homo sapiens*). Alignments of predicted protein products of these *AS3MT* homologs identified several common features (Table 1). For all species except *Pan*, *AS3MT* ranged from 348 residues (*Ciona*) to 382 residues (*Gallus*). All *AS3MT* sequences contained multiple cysteine residues ranging from five in the truncated *Pan* sequence to 15 in the *Gallus* sequence. Among all predicted *AS3MT* sequences, there were five fully conserved cysteines. Based on the 375 residue sequence of *Homo AS3MT*, these are cysteines in positions 32, 61, 85, 156, and 206. Predicted *AS3MT* proteins contained three sequence motifs common to many *S*-adenosylmethionine (AdoMet)-dependent methyltransferases that catalyze methylation of proteins and small molecules (9). Interactions between AdoMet and these conserved amino acid motifs are critical for methyl group transfer to substrate (10). Motif I, with a consensus sequence of (V/I/L)(L/V)(D/I)LG(G/C)G(T/P)G with an invariant G in position 5 occurred as the sequence (I/V/L)LDLGSGSG in *AS3MT*. This sequence in *AS3MT* conformed to the rule of hh (D/E)hGXGXG, where h was a hydrophobic amino acid, and X was any amino acid. Like other AdoMet-dependent methyltransferases, the predicted products of *AS3MT* in these species contained a conserved aspartate at 20 residues C-terminal to Motif I. This aspartate occurred in the common postmotif I in other methyltransferases (hhXh(D/E)). Motif II was found 65 residues C-terminal to Motif I in all *AS3MT* sequences. The Motif II consensus sequence ((P/G)(Q/T)(F/Y/A)DA(I/V/Y)(F/I)(C/ V/L) with an invariant aspartate at position 4 occurred as the sequence (E/N/T)(S/A)(Y/H/M/F)DI(V/I)(I/V)S in *AS3MT*. Motif III occurred 20 residues C-terminal to Motif II in the *AS3MT* sequences as VL(K/N)(H/Y/E/P/D)GGE(L/M/ F)YF. The consensus sequence for Motif III (LL(R/ K)PGG(R/I/L)(L/I)(L/F/I/V)(I/L)) has at least one of the two glycine residues conserved in most methyltransferases.

### Origins and Functions of *AS3MT*

The *arsM* gene has been identified as bacterial and archaeal homologs of *AS3MT* (11). ArsM protein of *Rhodospseudomonas palustris* catalyzes the formation of methylated arsenicals from iAs<sup>III</sup>. *AS3MT* homologs occur widely in genomes of many deuterostomes, ranging from relatively simple species (*S. purpuratus*, *C. intestinalis*) to *H. sapiens*. Because *AS3MT* homologs have not been identified in the genomes of two protostomes, *Drosophila melanogaster* or *Caenorhabditis elegans*, it may have arisen in the deuterostomal lineage after the division of bilaterian animals into deuterostomal and protostomal superphyla about 656 to 575 million years before present (12). Notably, a recent study found no methylation of iAs in *D. melanogaster* larvae or adults (13), consistent with the absence of *AS3MT* from its genome. Identification of *AS3MT* homologs in the genomes of *Strongylocentrotus* and *Ciona* suggests that this gene arose before the division of chordates and nonchordates. Although about 60% of identified *Ciona* genes have homologs in protostomes, about 16% of *Ciona* genes lack homologs among protostomes (14). Alternatively, the absence of *AS3MT* in *Drosophila* and *Caenorhabditis* genomes could be an example of lineage-specific loss of a gene present in some ancestral genome (15).

Evidence of iAs methylation in protostomes, including the polychaetes, *Nereis diversicolor* and *N. viriens*, may provide some insight into phylogenetic relations for *AS3MT*. For these species, tetramethylarsinonium ion and arsenobetaine are the predominant arsenicals in *Nereis* tissues after exposure to arsenate; however, a small amount of the tissue arsenic is present as MAs (16). In another polychaete, *Arenicola marina*, most tissue arsenic is present as inorganic species with small percentages (~5%) present as arsenobetaine and DMAs (17). These patterns, especially the predominance of the tetramethylated form, are not consistent with the pattern seen in species in which *AS3MT* methylates arsenicals. For instance, tetramethylarsinonium ion is a minor constituent (<1%) in urine of rats treated with arsenite

(18). Differences in metabolic profiles in polychaetes and in species in which AS3MT is expressed suggest that the pathway for arsenic methylation in polychaetes may involve other enzymes, not AS3MT.

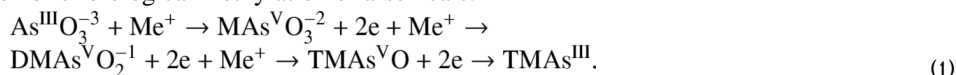
At least three explanations can be offered for the persistence of *AS3MT* homologs in the genomes of organisms representing the deuterostomal lineage. First, capacity to methylate inorganic As could be a mechanism for the detoxification of this metalloid. However, because AS3MT-catalyzed methylation of iAs yields products that are more reactive and toxic than the parent compound (19), the persistence of *AS3MT* would be dependent on the balance of the advantageous and deleterious effects of methylation of arsenicals. Second, methylation of iAs by AS3MT could yield a metabolite that is essential for normal cellular function. Studies in experimental species (rat, hamster, minipig, goat, and chicken) suggest arsenic to be an essential nutrient (20). For example, consumption of diets with low concentrations (ng per g range) of iAs have been associated with lower growth rates and decreased reproductive success in these species. Additional studies have demonstrated interactions between dietary deficiencies of iAs, methionine, and pyridoxine (21). Although the molecular bases for these actions of arsenic have not been determined, the salutary effects of iAs in diet might be due to an uncharacterized function of a methylated metabolite of iAs. If this conjecture is correct, then *AS3MT* could be vital for the production of this methylated species. Third, persistence of *AS3MT* may reflect a function of the protein that is not related to its capacity to catalyze methylation of arsenicals. For example, AS3MT could catalyze the methylation of other metalloids, such as selenium (Se) or tellurium (Te), or of another class of substrates. In this situation, catalysis of the methylation of arsenicals by AS3MT would be simply fortuitous.

### Genotype-Phenotype Correlations for Arsenic Methylation and AS3MT

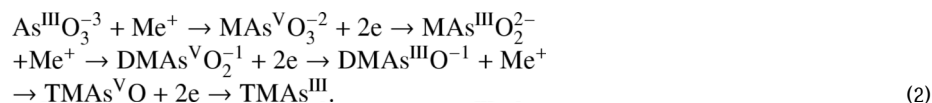
The As methylation phenotype is defined as the capacity to convert arsenicals to methylated forms that are present in tissues and/or excreted in urine. An absolute dependence of As methylation phenotype on AS3MT genotype can be demonstrated in cultured cells. The heterologous expression of rat *AS3MT* in a simian virus 40 (SV40)-transformed human urothelial (UROtsa) cell line confers the capacity to methylate iAs on cells that otherwise do not express *AS3MT* and that have a null phenotype for iAs methylation (22). Conversely, silencing of AS3MT expression by RNA interference reduces the capacity for methylation of iAs in human hepatoma (HepG2) cells (23). Among animal species examined in this study, homologous proteins with essential characteristics of AS3MT can be deduced from genomic data. For all species, except *Strongylocentrotus* and *Ciona* (for which there are no data on As phenotype), there is an association between the *AS3MT* genotype and the As methylation phenotype (Table 2). These data on patterns of metabolites of iAs found in tissue and excreta of species ranging from *Oncorhynchus* to *Homo* indicate that methylated metabolites are formed in all of these species, except *Pan*. As previously suggested, the predicted 205 amino acid product of *AS3MT* in *Pan* may be catalytically inactive (25), and the expression of the truncated protein could account for null phenotype for As methylation reported in this species (26). As a caveat, data in Table 2 on the pattern of methylated arsenicals should be regarded as provisional and incomplete. Earlier studies in *Bos* and *Oncorhynchus* used analytical techniques that may not resolve fully mono- and dimethylated arsenicals. The absence of trimethylarsine oxide (TMAO) as a metabolite in most species may also reflect limitations of analytical methods used in earlier studies. Similarly, an early study (27) of iAs metabolism in the green sea urchin *Strongylocentrotus droebachiensis*, which might provide evidence of iAs methylation in echinoderms, should be interpreted with caution. A systematic survey with adequate analytical methods would be needed to ascertain the possible significance of interspecies difference in the patterns of metabolites and the relation between this phenotypic variation and genotypic differences among species.

## Conceptual Models for Arsenic Methylation by AS3MT

Two conceptual models are currently available to describe the steps involved in the enzymatically catalyzed methylation of arsenicals by AS3MT. The first model grows out of the work of Frederick Challenger and his colleagues in the first half of the twentieth century. These studies were summarized in two comprehensive reviews (27,28) that outlined a scheme in which the oxidative methylation of arsenicals containing As<sup>III</sup> alternated with the reduction of arsenicals containing As<sup>V</sup> to trivalency. William Cullen *et al.* (29) summarized this general scheme for biological methylation of arsenicals:

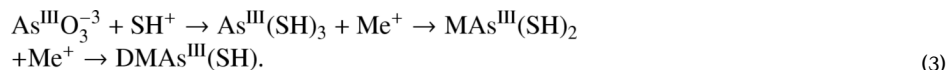


Here, metabolites are produced in sequence as monomethylated, dimethylated, and trimethylated arsenical species. Neither the methyl donor (Me<sup>+</sup>) nor the source of reducing equivalents is identified. The stated order of reactions is oxidative methylation followed by reduction; therefore, methylated arsenicals containing As<sup>III</sup> should be found as intermediates in reactions. A more detailed sequence explicitly shows this reaction order. The substrate for the oxidative methylation reaction contains As<sup>III</sup>, and reduction of the oxidized arsenical precedes oxidative methylation.



Although more detailed, this model specifies neither the source of methyl groups nor of reducing equivalents used in these reactions.

The second conceptual model takes a different approach to the reactions involved in the methylation of arsenicals (30). Here, thiol-containing complexes of arsenicals containing As<sup>III</sup> are substrates for sequential reactions that transfer methyl groups to the arsenical. This general scheme can be summarized as follows:



In this model, methylation occurs by a nonoxidative mechanism and thiol-arsenical complexes are obligatory substrates for methylation reactions.

The validity and utility of the two conceptual models can be evaluated in reactions in which AS3MT is the enzymatic catalyst. Studies with recombinant rat AS3MT (31) and human recombinant AS3MT (30) have examined in some detail the steps involved in the methylation of arsenicals catalyzed by this enzyme. Figure 1 provides more detailed descriptions of the conceptual models under consideration. Model A follows the work of Cullen *et al.* (29) and experimental results obtained with recombinant rat AS3MT, and Model B is based on the work of Hayakawa *et al.* (30). In both models, AdoMet is the methyl group donor. In Model A, three linked reactions (1, 2, and 3) involve oxidative methylation followed by reduction. The source of reducing equivalents needed to reduce As<sup>V</sup> to As<sup>III</sup> is not specified. The reactions are linked by common metabolites (MAs<sup>III</sup> and DMAs<sup>III</sup>) that are both products for a first reaction and substrates for a second reaction. In Model B, complexation of inorganic As<sup>III</sup> by the thiol-containing tripeptide glutathione (GSH) produces a substrate that acquires, in turn, two methyl groups. Here, reactions 1 and 2 overlap as the product of the first methylation step is the substrate for the second methylation reaction. The possibility of a third methylation reaction is not considered in Model B. The presence of MAs<sup>III</sup> and DMAs<sup>III</sup> in reactions is attributed

to decomposition of the GSH-arsenical complexes, and the presence of  $\text{MAs}^{\text{V}}$  and  $\text{DMAs}^{\text{V}}$  is attributed to the oxidation of the methylated arsenicals containing  $\text{As}^{\text{III}}$ .

## Catalytic Functions for AS3MT

### Activity in the Presence of Various Reductants and the Role of GSH

AS3MT was initially purified from the cytosolic fraction of the liver of adult male Fischer 344 rats, using pH-dependent fractionation, chromatofocusing, and *S*-adenosylhomocysteine-affinity chromatography (7). This procedure purified several thousand-fold a ~42 kDa protein that catalyzed the conversion of  $\text{iAs}^{\text{III}}$  to  $\text{MAs}$  and  $\text{DMAs}$  in reactions that depended on AdoMet as the methyl-group donor. Notably, the success of this scheme for purification of AS3MT from liver cytosol depended on the presence of 1 mM dithiothreitol (DTT) and 5 mM GSH in buffers used in each fractionation and chromatographic step. In the absence of either reductant, As methylating activity was quickly lost during purification. Hence, in initial studies, assays of the As methylating activity of AS3MT were conducted with both reductants in reaction mixtures.

Initial studies investigated the patterns of methylation of arsenicals by recombinant rat AS3MT in reaction mixtures in which different reductants were present (31). Because studies with nonphysiological reductants like DTT provided little information about the role of reductants in catalysis by AS3MT, primary emphasis has been placed on the role of potential physiological reductants in support of the enzyme's function. This approach was also strongly influenced by evidence that AS3MT might function as an  $\text{As}^{\text{V}}$  reductase. In this assay system, formation of  $\text{MAs}$  and  $\text{DMAs}$  proceeded in the presence of AS3MT, a reductant, AdoMet, and an arsenical substrate. This suggested that reduction of pentavalent arsenicals formed in the assay mixture could be catalyzed by AS3MT. Other studies with prokaryotic and eukaryotic  $\text{As}^{\text{V}}$  reductases indicated that three physiological reductants (thioredoxin, Trx; glutaredoxin, Grx; dihydrolipoic acid, DHLA) could provide reducing equivalents needed to produce  $\text{As}^{\text{III}}$  (32). Notably, each of these reductants contains a dithiol pair that is oxidized to a disulfide during reduction of an oxidized substrate. The regeneration of the dithiol pair in each of these reductants is accomplished by enzymatically catalyzed transfer of reducing equivalents from nicotinamide adenine dinucleotide phosphate (NADPH). For Trx and DHLA, Trx reductase (TR) catalyzes the reduction; for Grx, GSH and GSH reductase (GR) are required.

The capacity of different reductants to support catalysis of As methylation by AS3MT was determined in the absence or presence of 5 mM GSH (Fig. 2). Notably, in the absence of another reductant, addition of 5 mM GSH did not support the catalytic activity of AS3MT. Although addition of GSH had little effect on the rate of As methylation in the presence of 1 mM DTT, it sharply stimulated the methylation rate in the presence of 1 mM Tris (2-carboxylethyl) phosphine (TCEP). Compared with these nonphysiological reductants, the highest rates of methylation were found in assays containing a Trx/TR/NADPH-coupled system. Here, addition of GSH had little effect on an already high rate of As methylation. The activity of Grx as a reductant depended on addition of GSH to the reaction mixture, reflecting its role in a Grx/GSH/GR/NADPH-coupled system. DHLA also functioned as reductant when added in a DHLA/TR/NADPH-coupled system. Furthermore, the presence of methylated arsenicals containing either  $\text{As}^{\text{V}}$  or  $\text{As}^{\text{III}}$  in reaction mixtures was consistent with the formation and conversion of these species as the reaction progressed. Several conclusions can be drawn from these studies. First, among physiological reductants, addition of Trx to reactions at concentrations approximating those found in cells (33) yielded the highest catalytic activity for AS3MT. Second, for both physiological and nonphysiological reductants, addition of GSH stimulated the overall rate for As methylation. Third, under these assay conditions, addition of GSH resulted in little, if any, conversion of  $\text{iAs}$  to methylated metabolites.

In the course of identifying a trimethylated arsenical as a product of AS3MT-catalyzed reactions, the role of GSH in catalysis was further investigated (34). Although TMAO was rarely detected in reaction mixtures that contained 5 mM GSH, it accounted for nearly half of the methylated products present in reaction mixtures that contained AS3MT, AdoMet, iAs<sup>III</sup>, and TCEP but no GSH (Fig. 3). Notably, in the absence of GSH, a physiological reductant (Trx/TR/NADPH) also supported the formation of TMAO in AS3MT-catalyzed reactions. Increasing the concentration of GSH in reaction mixtures affected the pattern and extent of As methylation, suppressing formation of TMAO but increasing overall production of methylated products. This suggested that the third methylation reaction was inhibited by GSH. When DMAs<sup>III</sup> or dimethylthioarsenite-GSH (a 1:1 molar complex of DMAs<sup>III</sup> and GSH) was used as substrate for AS3MT-catalyzed reactions (final concentration of 1  $\mu$ M), TMAO was formed (Fig. 4). When 5 mM GSH was added to assay mixtures containing 1  $\mu$ M DMAs<sup>III</sup>, TMAO was not formed. This suggested that interactions between AS3MT and GSH that modulate the enzyme's activity do not involve a specific role for an arsenical-GSH complex but rather reflect a direct interaction between the enzyme and GSH.

### GSH-Dependent Activity

Model B proposes that As-GSH complexes are both the substrates for, and products of, reactions catalyzed by AS3MT. Thus, although the methylation of arsenicals requires As<sup>III</sup>, it must be present in a GSH-containing complex. Furthermore, methylation does not oxidize As<sup>III</sup> but yields a complex containing a methylated arsenical and GSH. This model requires scission of an As<sup>III</sup>-S bond between the cysteinyl residue of GSH and As<sup>III</sup>. For studies of the role of GSH in AS3MT-catalyzed methylation, Hayakawa *et al.* (30) used reaction mixtures containing 50  $\mu$ g of recombinant human AS3MT, 0.2  $\mu$ M iAs<sup>III</sup>, and 1 mM AdoMet in 25 mM phosphate buffer, pH 7. GSH concentration ranged from 0 to 5 mM and reactions were incubated for 3 hrs at 37°C. Under these conditions, methylated products were detected only in the presence of at least 2 mM GSH. The investigators found a positive relation between the percentage of concentration of iAs<sup>III</sup> present in an iAs<sup>III</sup>-GSH complex (at a 1:3 molar ratio of iAs<sup>III</sup> to GSH) and the extent of AS3MT-catalyzed methylation (Fig. 5). A MAs<sup>III</sup>-GSH complex (at a 1:2 molar ratio of MAs<sup>III</sup> to GSH) added to reaction mixtures was rapidly oxidized to MAs<sup>V</sup> and was not converted to DMAs. However, the presence of 5 mM GSH in reaction mixtures permitted the MAs<sup>III</sup>-GSH complex to be converted to DMAs by AS3MT. In the absence of GSH, MAs<sup>V</sup> was not a substrate for methylation by AS3MT. However, addition of 5 mM GSH to the reaction mixture permitted formation of a small amount of MAs<sup>III</sup>-GSH complex that was converted to DMAs.

Recent work with recombinant human AS3MT has examined the role of reductants, including the Trx/TR/NADPH-coupled system and GSH.<sup>2</sup> Here, reaction mixtures contained 3 to 5  $\mu$ g of recombinant human AS3MT, 3  $\mu$ M iAs<sup>III</sup>, and 1 mM AdoMet in 100 mM Tris, 100 mM phosphate buffer, pH 7.4. Concentrations of Trx, TR, NADPH, and GSH varied in reaction mixtures and are indicated in figure legends. As shown in Figure 6, AS3MT catalyzed conversion of iAs to MAs and DMAs in the presence of the complete Trx/TR/NADPH-coupled system; however, omission of Trx from reaction mixtures abolished activity. In the presence of 1 mM GSH, conversion of iAs to MAs and DMAs was stimulated. Again, omission of Trx from reaction mixtures containing GSH sharply reduced activity. A small amount of MAs was detected in these reaction mixtures, suggesting a minimal effect of GSH on AS3MT's catalytic activity. Examination of the concentration dependence of the effect of GSH on the activity of AS3MT in reaction mixtures containing the Trx/TR/ NADPH-coupled system showed that GSH stimulated the rate of conversion of iAs to MAs and DMAs (Fig. 7).

<sup>2</sup>Xing W, Adair BW, Thomas DJ. Unpublished results, 2006.

## Merits and Limitations of the Conceptual Models for Arsenic Methylation by AS3MT

The first conceptual model involving alternating steps of oxidative methylation and reduction of arsenicals can serve as a framework for understanding the role of cellular reductants in catalytic activity of AS3MT. However, two general questions must be addressed in evaluating the function of these cellular reductants. First, how do these reductants act to promote the catalytic activity of the enzyme? Second, how does the function of these reductants complement the actions of GSH? The use of cellular reductants by AS3MT is reminiscent of the role of these reductants in the activity of several well-characterized As<sup>V</sup> reductases. These enzymes use Trx/TR/NADPH- or Grx/GSH/GR/NADPH-coupled systems as the source of reducing equivalents for conversion of As<sup>V</sup> to As<sup>III</sup> (32). Because AS3MT catalyzes each step in a reaction sequence that leads from iAs to TMAs, it is conceivable that this protein is multifunctional, catalyzing both oxidative catalysis and reduction of As<sup>V</sup>. However, it should be noted that AS3MT activity is absolutely dependent on the presence of the complete Trx/TR/NADPH-coupled system; omission of Trx abolishes activity. This suggests that Trx is not only needed to reduce methylated intermediates containing As<sup>V</sup> (e.g., MAs<sup>V</sup>) but is also required for AS3MT to catalyze the reaction in which iAs<sup>III</sup> is present as the substrate. This suggests that Trx could be required for the reduction of some cysteine residue in AS3MT that is critical for its function as a methyltransferase.

The second conceptual model is dependent on interactions between GSH, arsenicals, and AS3MT. Speculation about the role of GSH in As methylation was first summarized by Thompson (35), who integrated much of the evidence on the reduction of pentavalent arsenicals by GSH and the role of GSH complexes in the enzymatically catalyzed methylation of As. Although GSH can reduce As<sup>V</sup> to As<sup>III</sup> in cellular environments and forms stable complexes with As<sup>III</sup> (36–38), good quantitative estimates are lacking the extent of reduction by this pathway or the persistence of these complexes in cellular environments. Although DMAs<sup>III</sup> present in a GSH complex could be converted to TMAO, it is not certain if the complex was the substrate for methylation. It is entirely possible that DMAs<sup>III</sup> could be transferred from the GSH complex to a higher affinity binding site on AS3MT. Hence, the GSH complex would serve only to maintain arsenic in a reduced state before its interaction with the enzyme. Data on the effects of GSH on iAs methylation in assay systems using crude cytosol or in tissue slices (39–41) provide little insight into the molecular processes that underlie the effects of GSH on enzymatically catalyzed methylation. It is possible that GSH functions to maintain the correct mix of reduced and of oxidized cysteine residues in AS3MT. The presence of multiple cysteines in this protein raises the distinct possibility that formation of intramolecular or intermolecular disulfide bonds affect the structure and function of the enzyme.

## Conclusions and Future Directions

Similarities in the sequences of the predicted protein products of *AS3MT* homologs found in genomes of a variety of deuterostomes argue that this ubiquitous protein serves a function that is conserved among these organisms. If the conserved function of these AS3MT homologs is catalysis of As methylation, then there has been a persistent selective pressure for the capacity of the protein to catalyze this reaction. Further understanding of the function of AS3MT depends on physicochemical studies probing its interactions with substrates and with critical reductants. Site-directed mutagenesis offers the possibility of identifying specific amino acid residues required for its catalytic activity. Altered expression of AS3MT in cultured cells using RNA interference could be used to examine effects of altered AS3MT expression on the As methylation phenotype. AS3MT gene knockouts can be used to examine the As methylation phenotype and the toxicological consequences of altered capacity to methylate arsenicals.

Examining evidence on the catalytic activity of AS3MT in relation to the two conceptual models for As methylation supports the conclusion that its activity does not strictly depend on



the presence of GSH. Methylation occurs in reaction mixtures that contain only the Trx/TR/ NADPH-coupled system as a source of reducing equivalents. Because different laboratories assay As methylation rates under different experimental conditions (e.g., enzyme concentration, buffer pH, analytical methods), quantitative comparisons of data obtained in different laboratories are problematic. However, rates of conversion of iAs to methylated metabolites using recombinant human AS3MT are much higher in reaction mixtures containing Trx/TR/ NADPH than in reaction mixtures containing GSH as the sole reductant. Using data from the work of Hayakawa *et al.* (fig. 5 in Ref. 30), a methylation rate of 0.1 pmols of methylated substrate per  $\mu\text{g}$  of protein per hr was calculated for reaction mixtures containing  $0.2 \mu\text{M}$  iAs<sup>III</sup> as substrate and 5 mM GSH as the reductant. By comparison, a methylation rate of 0.8 pmols of methylated substrate per  $\mu\text{g}$  of protein per hr was calculated for reaction mixtures containing  $0.3 \mu\text{M}$  iAs<sup>III</sup> as substrate and 5  $\mu\text{M}$  Trx, 1  $\mu\text{M}$  TR, and 300  $\mu\text{M}$  NADPH as the source of reducing equivalents.<sup>3</sup> In general, it can be suggested that the presence of GSH in reaction mixtures stimulates rates of reactions catalyzed by AS3MT that are supported by other physiological reductants. The nature of the interaction between physiological reductants and AS3MT that underlie the catalytic functions of the enzyme is unknown. Do the physiological reductants serve as a source of reducing equivalents needed to reduce As<sup>V</sup> to As<sup>III</sup>, or do the reductants help maintain the critical cysteine residues in the reduced state? Characterizing these interactions as well as interactions between AS3MT and GSH is an outstanding research problem that will require considerable ingenuity to solve. Studies of the catalytic function of common polymorphisms of AS3MT may provide cogent insights into the relation between enzyme structure and function (42).

A broader research topic grows out of this research on interactions between arsenicals, reductants, and AS3MT in reaction mixtures in which all elements present in the system are known and quantified. Cells have redundant systems for reduction, including GSH, Trx, Grx, and DHLA. The availability of each reductant is tightly regulated by biosynthetic control (e.g., GSH synthesis regulated by activity of glutamate-cysteine ligase; Ref 43), and there is evidence of interactions between reductants that affect their reactivity and concentration (e.g., glutathionylation of Trx; Ref. 44). Because AS3MT promiscuously uses each of these physiological reductants to support catalysis, its activity in cells must be determined at least in part by concentrations of several reductants. Availability of the reductants could contribute to the observed dose-dependency of the rate of methylation of iAs in cultured primary human hepatocytes (45). Similarly, differences in rates of methylation of iAs in cultured cells derived from different donor species or in intact organisms of different species could reflect interspecies differences in the concentrations of the reductants needed to support the catalytic activity of AS3MT. Integrating information on the regulation of AS3MT by reductants poses a considerable challenge in elucidating the control of As methylation at the cellular and systemic levels.

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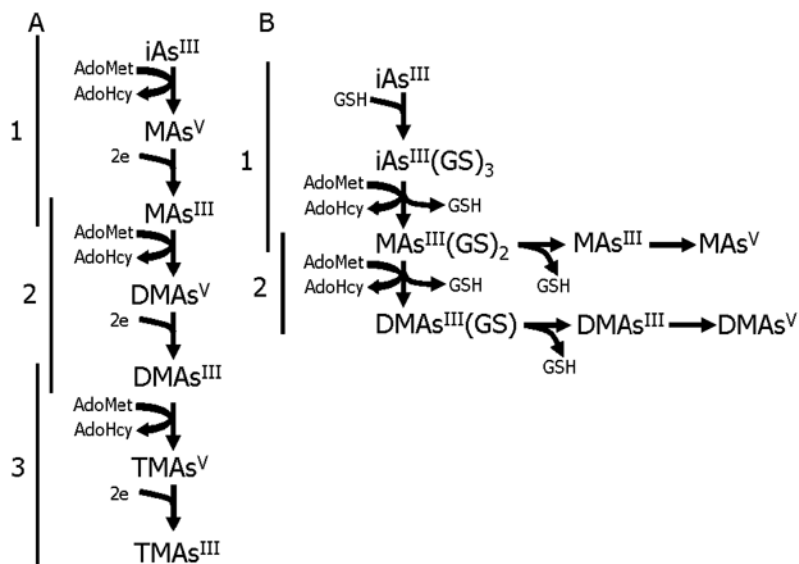
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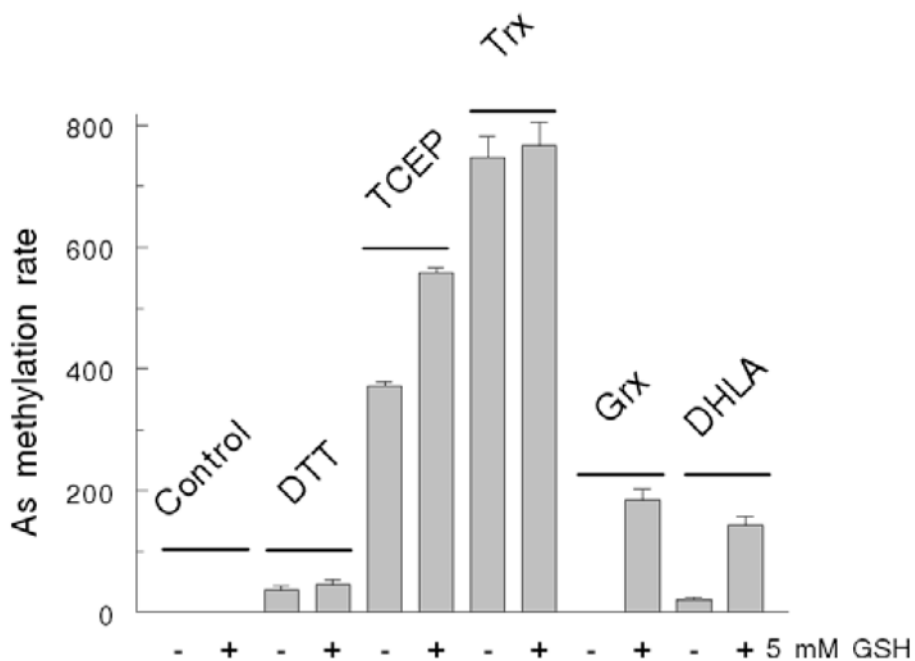
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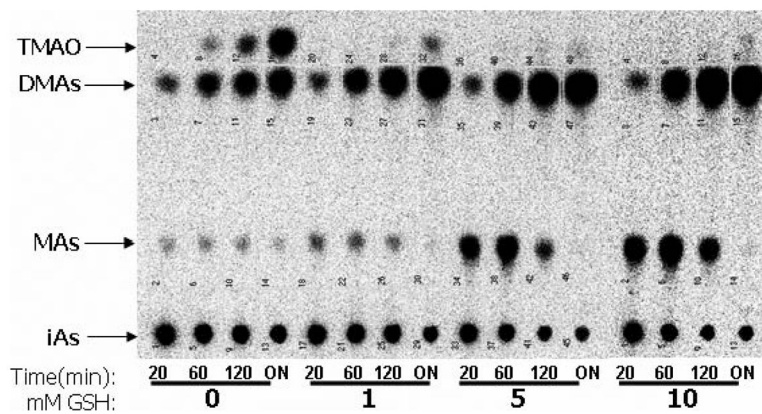
**Figure 1.**

Conceptual models for methylation of inorganic arsenic catalyzed by AS3MT. In Model A, oxidative methylation of trivalent arsenicals alternates with reduction of pentavalent arsenicals. In this pathway, arsenite ( $iAs^{III}$ ) is converted to methylarsonic acid ( $MAs^V$ ), which is reduced to methylarsonous acid ( $MAs^{III}$ ). This arsenical is converted to dimethylarsinic acid ( $DMAs^V$ ), which is reduced to dimethylarsinous acid ( $DMAs^{III}$ ). This dimethylated species is converted to trimethylarsine oxide ( $TMs^V$ ), which is reduced to trimethylarsine ( $TMs^{III}$ ). Model B involves the sequential addition of methyl groups to trivalent arsenicals that are complexed to glutathione (GSH). In each model, *S*-adenosylmethionine (AdoMet) is the methyl group donor; *S*-adenosylhomocysteine (AdoHcy) is produced by removal of a methyl group from AdoMet.



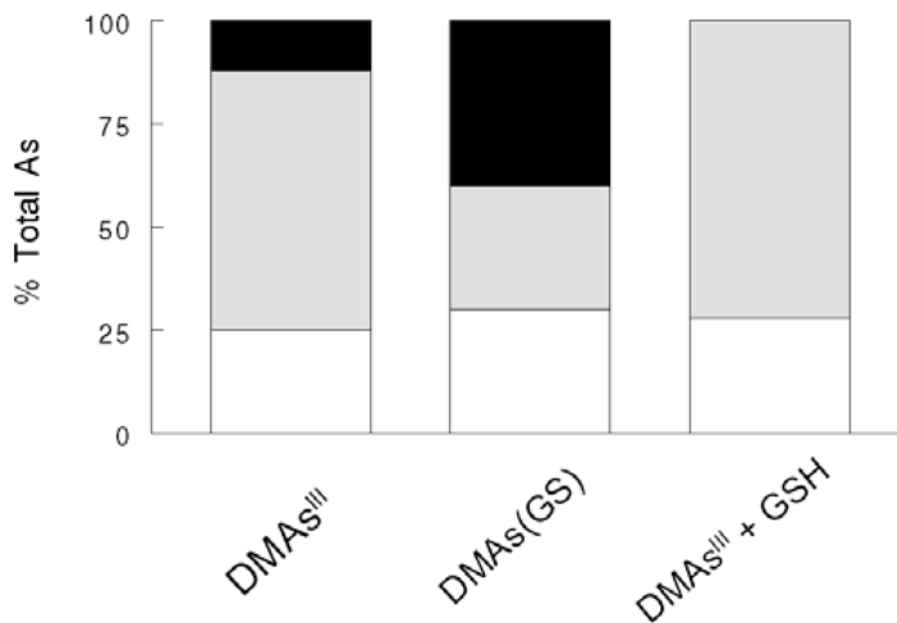
**Figure 2.**

Effect of glutathione (GSH) on capacity of reductants to support the methylation of inorganic arsenic by recombinant rat AS3MT. Reaction mixtures (50  $\mu$ l) containing 5  $\mu$ g of recombinant rat AS3MT, 1 mM AdoMet, and 1  $\mu$ M [ $^{73}$ As]-iAs<sup>III</sup> in 100 mM Tris/100 mM Na phosphate buffer, pH 7.4, with or without reductant, were incubated at 37°C for 20 mins. Reactions were run in the presence or absence of 5 mM GSH. Reductants were 1 mM dithiothreitol (DTT), 1 mM Tris(2-carboxyethyl) phosphine (TCEP), 10  $\mu$ M *Escherichia coli* thioredoxin (Trx) with 3  $\mu$ M rat liver Trx reductase and 300  $\mu$ M NADPH, 1  $\mu$ M *E. coli* glutaredoxin 1 (Grx) with 15 nM yeast GR, 300  $\mu$ M NADPH, 100  $\mu$ M dihydrolipoic acid (DHLA) with 3  $\mu$ M rat liver Trx reductase, and 300  $\mu$ M NADPH. Methylation rate calculated as nmols of arsenite methylated per  $\mu$ g of protein per 20 mins. (Upper) Mean and (lower) standard deviation shown ( $n = 3$ ).



**Figure 3.**

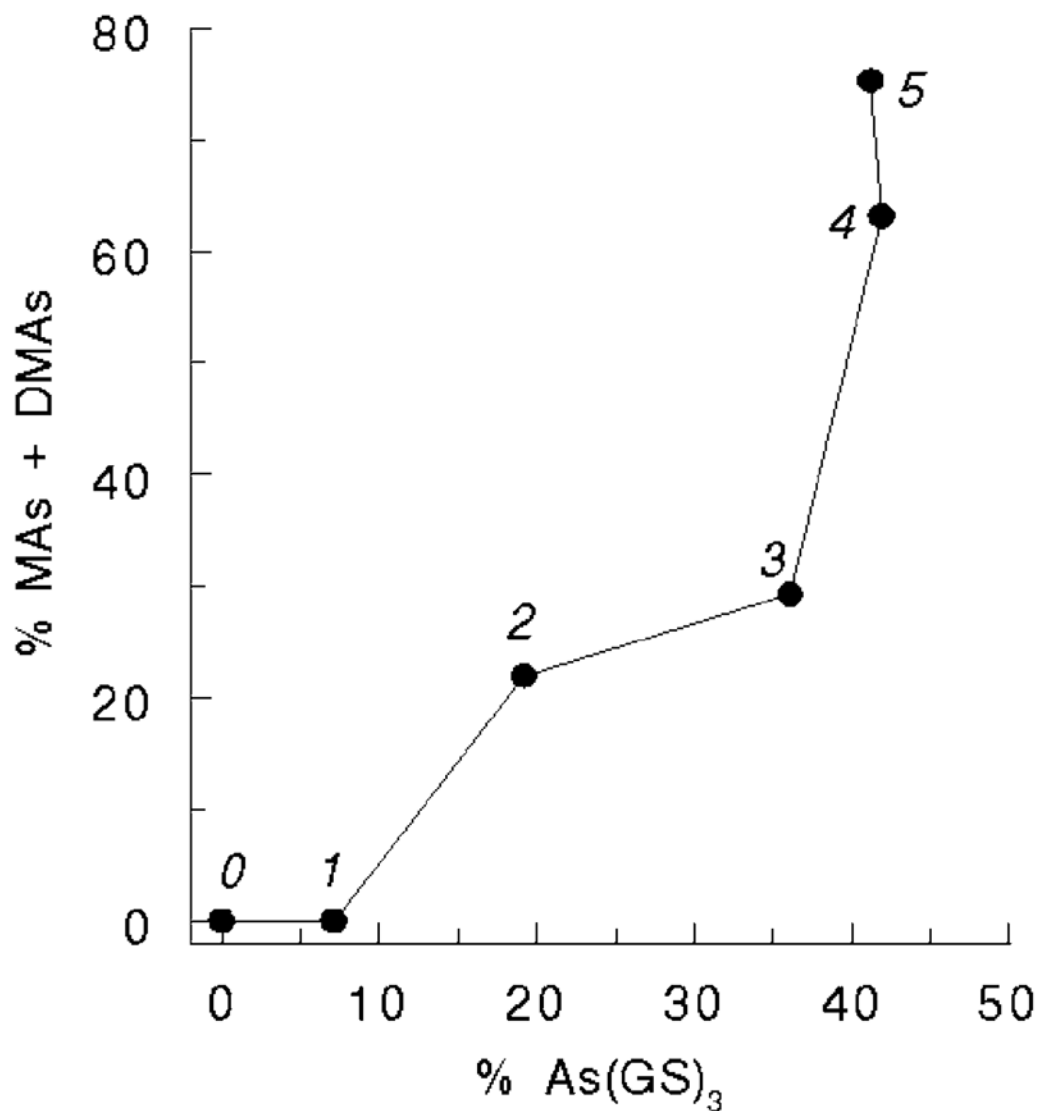
Effect of the concentration of glutathione (GSH) on the pattern of metabolites produced by recombinant rat AS3MT. Reaction mixtures (50  $\mu$ l) containing 5  $\mu$ g of recombinant rat AS3MT, 1 mM AdoMet, 1 mM Tris(2-carboxyethyl) phosphine, and 1  $\mu$ M [ $^{73}$ As]-iAs<sup>III</sup> in 100 mM tris/100 mM Na phosphate buffer, pH 7.4, were incubated at 37°C from 20 mins to overnight (16 hrs) in the presence of 0–10 mM of GSH. Reaction products separated by thin-layer chromatography as previously described (34). Positions for migration of inorganic arsenic (iAs), methylarsonic (MAs), dimethylarsinic (DMAs), and trimethylarsine oxide (TMA) are indicated. Arrow shows direction of chromatography.



**Figure 4.**

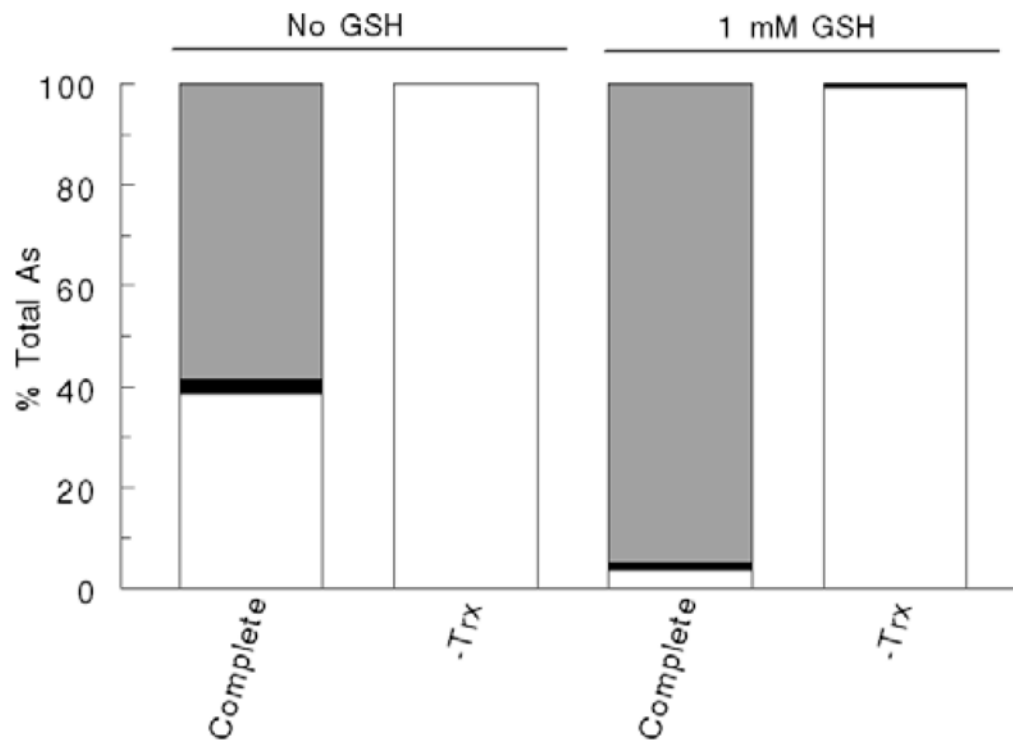
Effect of glutathione (GSH) on rat recombinant AS3MT-catalyzed methylation of dimethylarsenicals. Reaction mixtures (50  $\mu$ l) contained 5  $\mu$ g of rat recombinant AS3MT, 1 mM AdoMet, and 1 mM TCEP. The substrates were 1  $\mu$ M dimethylarsinous acid, iodide salt (DMAs<sup>III</sup>), 1  $\mu$ M of an equimolar complex of dimethylarsinous acid with GSH (DMAs<sup>III</sup>GS), or 1  $\mu$ M DMAs<sup>III</sup> and 5 mM GSH. Reactions were incubated at 37°C for 120 mins and oxidation states of arsenicals in reaction mixtures were determined by pH selective hydride generation-atomic absorption spectrophotometry. Percentages of arsenic present as (White) DMAs<sup>III</sup>, (Gray) DMAs<sup>V</sup>, or (Black) TMAO are shown.



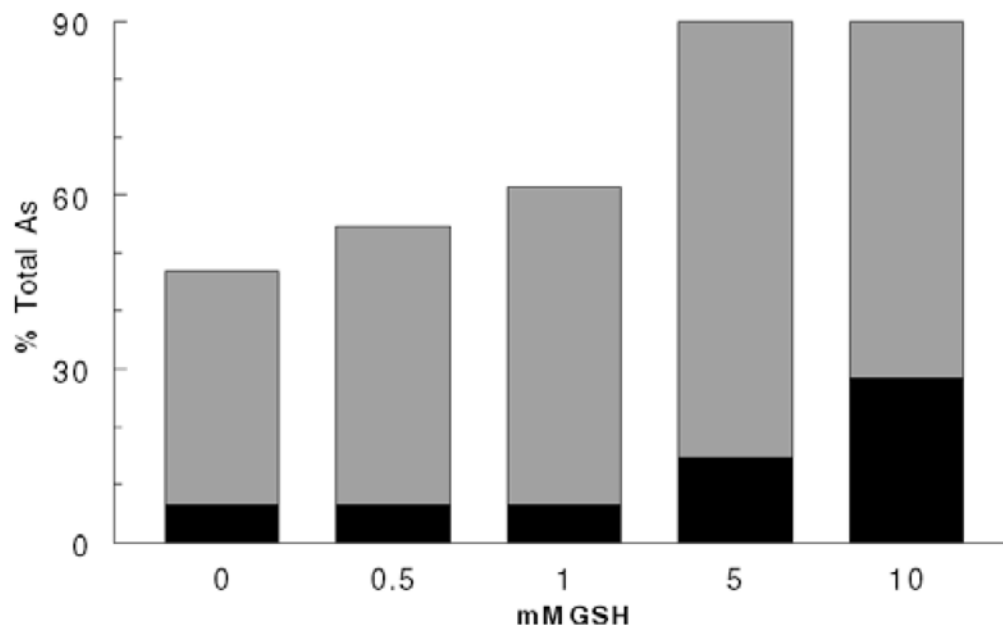


**Figure 5.**

Glutathione (GSH)-dependent conversion of arsenite to methylated arsenicals by recombinant human AS3MT. Relationship between the percentage of  $\text{iAs}^{\text{III}}$  converted to methylated (MAS) or dimethylated (DMAs) species and the percentage of  $\text{iAs}^{\text{III}}$  present in a triglutathione complex ( $\text{As(GS)}_3$ ) as GSH concentration increases from 0 to 5 mM. Reaction mixtures (100  $\mu\text{l}$ ) containing 50  $\mu\text{g}$  of recombinant rat AS3MT, 1 mM AdoMet, and 0.2  $\mu\text{M}$   $\text{iAs}^{\text{III}}$  in 25 mM phosphate buffer, pH 7.0, were incubated at 37°C for 3 hrs. (Redrawn from data in fig. 5 of Ref. 30).



**Figure 6.** Effects of glutathione (GSH) and a thioredoxin (Trx)-coupled reduction system on the activity of recombinant human AS3MT. Reaction mixtures (80  $\mu$ l) containing 3  $\mu$ g of recombinant human AS3MT, 1 mM AdoMet, and 3  $\mu$ M [ $^{73}$ As]-iAs<sup>III</sup> in 100 mM Tris/100 mM Na phosphate buffer, pH 7.4, were incubated at 37°C from 60 mins in the presence or absence of 1 mM GSH. Complete reaction mixtures contained 3  $\mu$ M *E. coli* thioredoxin (Trx) with 1  $\mu$ M rat liver Trx reductase and 300  $\mu$ M NADPH; Trx was omitted from some reaction mixtures (-Trx). Percentages of arsenic present as (Open) inorganic arsenic, (Black) methylarsonic, and (Gray) dimethylarsinic species are shown.



**Figure 7.**

Glutathione (GSH) concentration dependency of the activity of recombinant human AS3MT. Reaction mixtures (80  $\mu$ l) containing 4  $\mu$ g of recombinant human AS3MT, 1 mM AdoMet, and 3  $\mu$ M [ $^{73}$ As]-iAs<sup>III</sup>, 0.26  $\mu$ M *E. coli* thioredoxin (Trx) with 0.5  $\mu$ M rat liver Trx reductase and 300  $\mu$ M NADPH in 100 mM tris/100 mM Na phosphate buffer, pH 7.4, were incubated at 37 $^{\circ}$  C from 60 mins in the presence of 0–10 mM GSH. Percentages of arsenic present as (Black) methylarsonic and (Gray) dimethylarsinic species are shown.

Table 1

Multiple-Sequence Alignments of Human AS3MT and Eight Animal Homologues Illustrate Conserved Motifs I, II, and III, Conserved Asp at I', and Five Conserved Cys Residues. Underlined sequence is UbiE methyltransferase domain. The sequences of seven species were from NCBI GenBank (accession numbers are given in parentheses following species names): Homo sapiens (Q9HBK9); Pan troglodytes (XP\_508007); Bos taurus (NP\_001030195); Rattus norvegicus (NP\_543166); Mus musculus (AAH13468); Gallus gallus (XP\_421735); Strongylocentrotus purpuratus (XP\_784275). The partial sequences of another two species were from TIGR Gene Index Databases by querying human AS3MT cDNA with TBLASTX program: Rainbow trout (TC70487); Ciona intestinalis (TC65300). The missed potential residues in the N-termini are indicated with question marks (?)

Table with 2 columns: Species and Sequence. Rows list species (H. sapiens, P. troglodytes, B. taurus, R. norvegicus, M. musculus, G. gallus, O. mykiss, C. intestinalis, S. purpuratus) and their corresponding amino acid sequences. The table is divided into sections for motifs I, II, III, and I', with underlined sequences representing the UbiE methyltransferase domain. Conservation marks like asterisks and question marks are used to denote specific residues.

**Table 2**  
 Arsenic-Containing Metabolites Identified in Tissues (T) or Excreta (E) Following Administration of Inorganic Arsenic

Species	IAs	MAs	DMAs	TMAO	Reference
<i>H. sapiens</i>	TE	TE	TE		46,47
<i>P. troglodytes</i>	TE				25
<i>B. taurus</i>	E	E			48
<i>R. norvegicus</i>	TE	TE	TE	E	18
<i>M. musculus</i>	TE	TE	TE	T	49
<i>G. gallus</i>	T		T		50,51
<i>O. mykiss</i>	TE	TE			52