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Research paper

New insights into the mechanism of arsenite methylation with the recombinant human arsenic (+3) methyltransferase (hAS3MT)

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ABSTRACT

The catalytic mechanism of the recombinant human arsenic (+3) methyltransferase (hAS3MT) was studied using kinetics, initial velocity and spectroscopy. The production and the distribution of methylated arsenicals changed with various concentrations of arsenite/*S*-adenosyl-L-methionine (SAM)/thiols, enzyme contents, and incubation times. These results suggest a sequential methylation of arsenite to monomethylated arsenicals (MMA) and dimethylated arsenicals (DMA). In addition, competition exists between the two reactions. hAS3MT showed the greatest activity at pH 8.5 with glutathione (GSH) as the reductant. This might indicate that a balance between the deprotonation and protonation of sulfhydryl groups is required. Initial velocity studies illuminate an ordered sequence for the binding of SAM and arsenite to the hAS3MT; while GSH should probably be placed either as the first reactant or as a reactant combining with the enzyme only after products have been released. The interactions between substrate/cofactors and the hAS3MT were first monitored by UV-visible and circular dichroism spectroscopy. It revealed that arsenite and SAM combined with the hAS3MT before reaction started; whereas, no interactions between GSH and the hAS3MT were detected. Integrating the results from kinetics, initial velocity and spectroscopy studies, an ordered mechanism are originally attained, with the SAM as the first reactant that adds to the hAS3MT and arsenite as the second one. Arsenite is successively methylated reductively, rather than a stepwise oxidative methylation. GSH should combine with the hAS3MT after the methylation to reduce the disulfide bond formed during the catalytic cycle in the hAS3MT to resume the active form of the enzyme.

1. Introduction

Inorganic arsenic (iAs) can produce both acute and chronic toxicity. Chronic exposure to arsenicals in occupational and environmental settings is known to cause cancers, cardiovascular diseases, skin and neurological system lesions [1–3]. In many species, including humans, inorganic arsenic is methylated enzymatically, resulting in urinary excretion of monomethylated (MMA) and dimethylated (DMA) arsenicals [4–6]. The conversion of iAs into these methylated metabolites is catalyzed by an enzyme called arsenic (+3 oxidation state) methyltransferase (AS3MT). There are two different proposed mechanisms at the present time. Cullen and his co-workers summarized a metabolic pathway incorporating oxidative methylation and the cycling reduction of pentavalent arsenicals to trivalent forms, $iAs^{5+} + 2e \rightarrow iAs^{3+} + CH_3^+ \rightarrow MMA^{5+} + 2e \rightarrow MMA^{3+} + CH_3^+ \rightarrow DMA^{5+} + 2e \rightarrow DMA^{3+} + CH_3^+$. In this scheme, because the preferred substrate for stepwise oxidative methylation must contain trivalent arsenic, the reduction of pentavalent arsenicals to trivalent states is a prerequisite. Then a reduction step has to be interposed between each of the oxidative methylation reactions [7]. The other scheme has been reported by Hayakawa et al [8] as successive methylation with simultaneous reduction in the presence of glutathione (GSH), $iAs^{5+} + 2e \rightarrow iAs^{3+} + GSH \rightarrow ATG^{3+}$ (arsenic triglutathione) $+ CH_3^+ \rightarrow MADG^{3+}$ (monomethylarsonic diglutathione) $\rightarrow MMA^{3+} \rightarrow MMA^{5+}$ and $MADG^{3+} + CH_3^+ \rightarrow DAMG^{3+}$ (dimethylarsinic glutathione) $\rightarrow DMA^{3+} \rightarrow DMA^{5+}$. In this scheme, the arsenical substrate must be initially complexed by GSH to preserve arsenicals in the trivalent oxidation state. Pentavalent

Abbreviations: iAs, inorganic arsenic; MMA, monomethylated arsenicals; DMA, dimethylated arsenicals; AS3MT, arsenic (+3) methyltransferase; BSA, bovine serum albumin; SAM, *S*-adenosyl-L-methionine; ATG, arsenic triglutathione; MADG, monomethylarsonic diglutathione; DAMG, dimethylarsinic glutathione; ME, 2-mercaptoethanol; HPLC-ICP-MS, high performance liquid chromatography-inductively coupled plasma-mass; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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arsenicals are present as end products of metabolism rather than intermediates.

Methylation of iAs have been commonly considered as a detoxification mechanism since the toxicity of methylated arsenicals is much lower than that of iAs [9–11]. However, recent research has shown that methylated arsenicals containing As^{3+} (MMA^{3+}, DMA^{3+}) are more cytotoxic, genotoxic [12,13] and potent enzyme inhibitors [14–16] than either arsenite (iAs^{3+}) or arsenate. Then the methylation of iAs can be properly considered as a bioactivation process for the activation of iAs to more reactive and toxic species. In both reaction schemes above, tri- and pentavalent methylated compounds are involved. Nevertheless, according to the first pathway, the pentavalent metabolites produced by the methyltransferase are reduced to the more toxic trivalent forms before further methylation, whereas, according to the second mechanism, the trivalent arsenicals produced by the enzyme undergo spontaneous oxidation to the pentavalent forms.

At the present time, there are many data about the methylation of iAs from different systems, such as in vitro studies with crude cytosol preparations [17], partially purified enzyme [18], purified enzyme [19], recombinant AS3MT [20] or cultured cells [21,22], and in vivo studies with different animals [23–26]. However, only a limited number of different experiments could be carried out in each work. And the use of crude extracts for the elucidation of enzyme mechanisms often results in misunderstandings since there are many interfering and antagonizing activities. In addition, previous investigations mainly focused on elucidating the pathway of iAs methylation. The roles of the AS3MT in the methylation process, such as the interactions between the substrate/cofactors and the AS3MT, the binding sequence and the conformational change of the enzyme are still unproved. It is necessary to take a system study with purified enzyme to better elucidate the mechanism of AS3MT. Therefore, we completely studied the methylation of iAs^{3+} by a purified recombinant hAS3MT with respect to incubation time, enzyme contents, wide substrate/cofactor concentration range, initial velocity and other factors. Furthermore, UV-visible (UV–vis) and circular dichroism (CD) spectroscopy were first used to monitor the interactions between the substrate/cofactor and the hAS3MT. Based on the results, we obtained some new insights into the mechanism of iAs methylation with hAS3MT.

2. Materials and methods

Caution: iAs [27] is classified as human carcinogens and appropriate safety measure should be taken when handling with sodium arsenite, arsenate and their metabolites.

2.1. Materials

Unless otherwise noted, all reagents are analytical grade or better. Arsenicals were bought from J&K Chemical Ltd. Bovine serum albumin (BSA), *S*-adenosyl-L-methionine (SAM), *dithiothreitol* (DTT), cysteine (CySH), β-mercaptoethanol (ME), meso-2,3-dimercaptosuccinic acid (DMSA) and *glutathione* (GSH) were all bought from Sigma. All solutions were prepared using Milli-Q deionized water. The phosphate buffer (PBS) was prepared from Na_2HPO_4 and NaH_2PO_4.

Stock solutions containing 1000 mg As/L each of the following species were prepared in Milli-Q deionized water: arsenite and arsenate prepared from $NaAsO_2$ (iAs^{3+}) and $Na_2HAsO_4{\cdot}7H2O$ (iAs^{5+}), respectively; methylarsonate prepared from disodium methylarsonate (MMA); dimethylarsinate prepared from dimethylarsinic acid (DMA) (J&K Chemical Ltd.). All the stock solutions were stored at 4 °C in the dark. Working solutions of standards were prepared fresh daily with the stock solutions.

2.2. UV–vis, and CD spectroscopy

All spectra were recorded for the purified hAS3MT at 37 °C. UV–vis spectra were measured on a Perkin Elmer Lambda-35 spectrophotometer. CD (190–260 nm) spectra were recorded by a JASCO-J810 spectropolarimeter (Jasco Co., Japan) in a cell with 1 mm light length. The scanning rate was set at 50 nm/min and the spectra were the average of five readings. The secondary structure parameters of the hAS3MT were computed using Jwsse32 software with reference of CD-Yang. Jwr [28]. The spectra titrations of hAS3MT in PBS (25 mM, pH 7.0) were performed by using a fixed protein concentration (1.5 μM) to which iAs^{3+}, SAM, and GSH fresh solutions were added, respectively. The mixtures were incubated for 15 min before being measured.

2.3. Assay of hAS3MT activity

The preparation of the purified recombinant hAS3MT was carried out as described previously [20]. The purity of the hAS3MT was confirmed by SDS-PAGE, and a single band yielded as stained with Coomassie brilliant blue. The protein concentration was determined by the Bradford assay based on a BSA standard curve.

The enzymatic methylation of iAs^{3+} was assayed under standard conditions (100 μl) which contained hAS3MT (11 μg), GSH (7 mM), SAM (1 mM), and iAs^{3+} (1 μM) in PBS (25 mM, pH 7.0). In some cases, various amounts of hAS3MT, GSH (CySH, DTT, ME, DMSA), SAM, and iAs^{3+} were used. Because the thiols at high concentration can markedly acidify the buffer, the pH of the stock solutions of thiols was set to 7.0. In the experiments, in which the effect of varying the pH on methylation of iAs^{3+} was examined, incubations at pH from 5.8 to 8.0 were performed in the presence of PBS (25 mM), while those at pH from 8.5 to 10.0 were done in the presence of Tris–HCl buffer (25 mM). All the reaction mixtures were incubated in capped tubes at 37 °C for the desired time.

2.4. HPLC-ICP-MS analysis of methylated products

Following incubation at 37 °C, reactions were terminated by boiling for 5 min. Because trivalent arsenicals are known to bind to proteins and thiols [7,8,23], the samples were analyzed with H_2O_2 treatment at a final concentration of 3% to convert all arsenic metabolites to pentavalent forms [8]. Before analysis, the samples were centrifugated at 12,000 rpm for 10 min and filtered through a pore membrane (0.22 μm). Then an aliquot of reaction mixtures (20 μl) was separated on an anion-exchange column (PRP-X100 250 mm × 4.6 mm i.d., 5 μm, Hamilton) using $(NH_4)_2HPO_4$ (15 mM, adjusted to pH 6.0 with H_3PO_4) as the mobile phase at a flow rate of 1.2 ml/min. Separated arsenicals were directly detected with an Elan 9000 ICP-MS. The amounts of each arsenical were calculated with the working curves prepared using 5, 10, 20, 40, 80, and 160 ppb As with standard arsenic species [29]. Methylation rates were calculated as mole equivalents of methyl groups transferred from SAM to iAs^{3+} (i.e., 1 pmol CH_3 per 1 pmol MMA or 2 pmol CH_3 per 1 pmol DMA) [22].

3. Results

3.1. Effect of H_2O_2 treatment on the recovery of arsenic metabolites

Fig. 1A is the elution profiles of standard arsenicals on a PRP-X100 column by HPLC-ICP-MS. As shown in Fig. 1C, H_2O_2 treatment could totally recover the arsenic metabolites. The recovery of

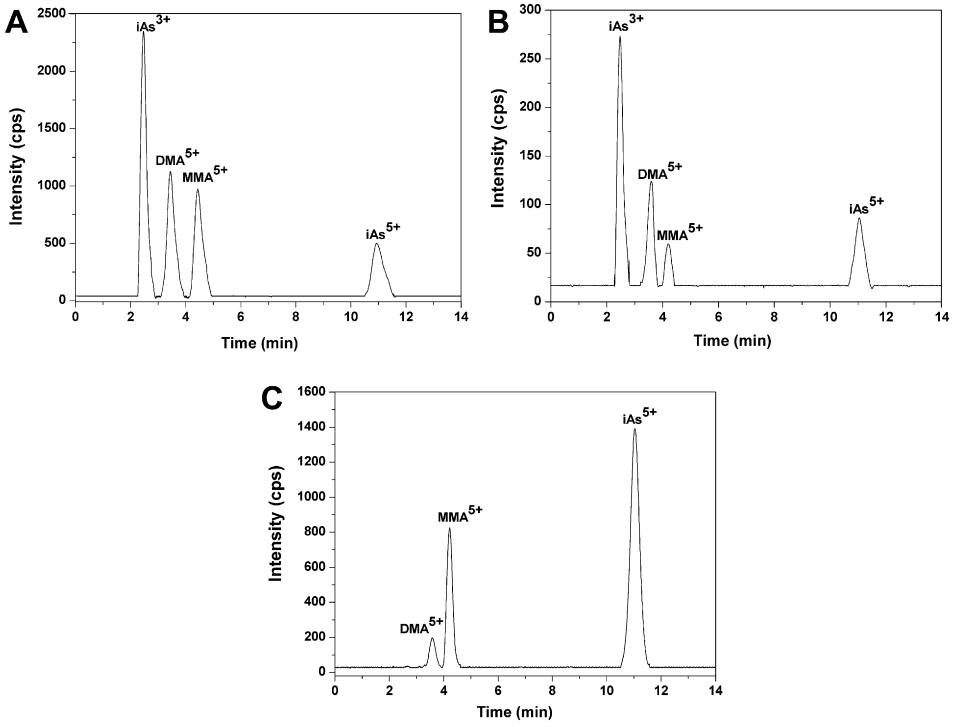


Fig. 1. Elution profiles of arsenicals on a PRP-X100 column by HPLC-ICP-MS. (A) A 20 μL of authentic arsenicals (20 ppb), inorganic arsenic (iAs^{3+}), dimethylarsinic acid (DMA^{5+}), monomethylarsonic acid (MMA^{5+}), and inorganic arsenic (iAs^{5+}). Analysis presented in (B) was performed without prior H_2O_2 treatment and analysis presented in (C) was performed with prior H_2O_2 treatment. The reaction mixture (100 μL), containing 11 μg hAS3MT, 1 mM SAM, 7 mM GSH, 1 μM iAs^{3+}, in 25 mM PBS (pH 7.0), was incubated at 37 °C for 1.5 h.

arsenic species (sum of species/total arsenic injected) was 97.3%. However, only a little of arsenicals could be detected in the sample without H_2O_2 treatment (Fig. 1B). A little of iAs^{3+} and iAs^{5+} were also detected in the H_2O_2-untreated sample (Fig. 1B). One possible explanation is that the binding of iAs^{3+} to the protein or thiols is saturated. A little of iAs^{3+} was left in the sample and was converted to iAs^{5+}.

3.2. Effect of incubation time on the methylation of iAs^{3+}

Fig. 2 illustrates the time course of the iAs^{3+} methylation in the standard system (see Materials and methods section). Production of MMA was linear over the first hour and then slowed and reached a plateau, accounting for about 48% of the total arsenic in the reaction mixture. However, a 30 min lag period occurred before DMA was produced. After that, the pattern of DMA production resembled that of MMA, accounting for about 52% of total arsenic ultimately. Under the standard conditions, 1 μM iAs^{3+} can be totally transformed into MMA and DMA at about 7 h.

3.3. Effect of hAS3MT contents, iAs^{3+}, SAM, and thiol concentrations on the methylation of iAs^{3+}

The methylation of iAs^{3+} was examined in the standard assay system that contained hAS3MT up to 30 μg (Fig. 3). The production of MMA increased as hAS3MT contents increased from 1 to 10 μg. At higher hAS3MT contents, from 10 to 30 μg, MMA production decreased. No DMA formed with low contents of hAS3MT (1 and 2.5 μg). After that, production of DMA increased as hAS3MT contents increased. In the standard system, 1 μM iAs^{3+} could be totally transformed into MMA and DMA by 30 μg hAS3MT after 2 h incubation.

A wide substrate concentration range was studied in the standard assay that contained iAs^{3+} up to 1000 μM (Fig. 4). Maximal conversion of iAs^{3+} to MMA occurred at about 100 μM iAs^{3+}. Higher substrate concentrations inhibited MMA yields. The production of DMA increased as iAs^{3+} concentration increased from only 0.5 to 8.3 μM, and then quickly decreased to zero. The ratio of DMA to MMA (D/M) decreased as substrate concentration increased. As shown in Fig. 5, the influence of SAM concentration on the pattern and extent of the methylation of iAs^{3+} was examined in the

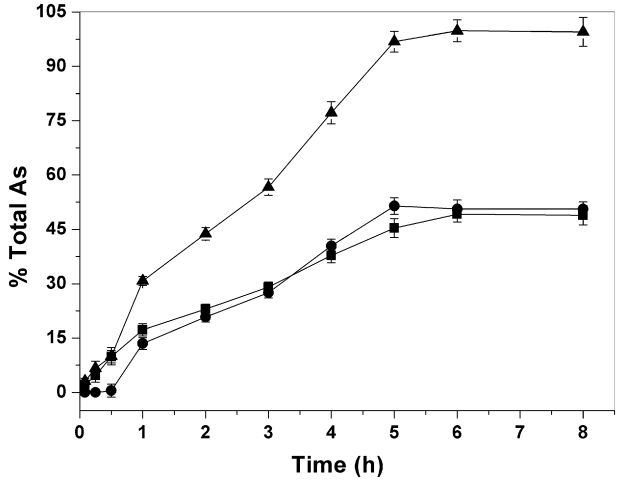


Fig. 2. Time courses for mono- and dimethylation of iAs^{3+} by hAS3MT. (■) MMA, (●) DMA, (▲) MMA + DMA. The reaction mixture (100 μl) containing hAS3MT (11 μg), SAM (1 mM), GSH (7 mM), iAs^{3+} (1 μM) in PBS (25 mM, pH 7.0) was incubated at 37 °C for different time with H_2O_2 treatment before analyzed by HPLC-ICP-MS. Data are presented as the means ± SD of three separate experiments.

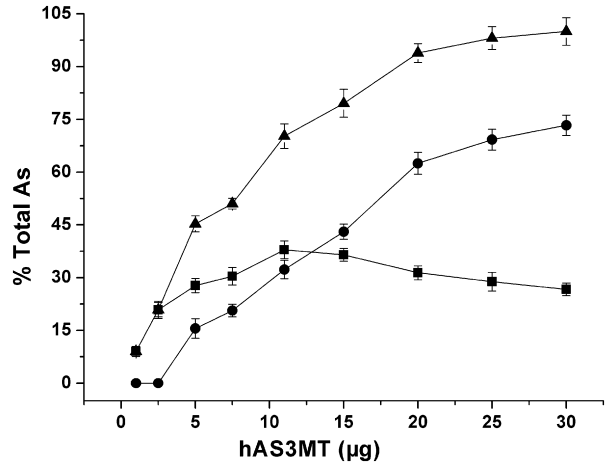


Fig. 3. Methylation of iAs^{3+} as a function of hAS3MT content. (■) MMA, (●) DMA, (▲) MMA + DMA. The reaction mixture (100 μl) containing hAS3MT (1, 2.5, 5, 7.5, 10, 20, 25, 30 μg), SAM (1 mM), GSH (7 mM), iAs^{3+} (1 μM) in PBS (25 mM, pH 7.0) was incubated at 37 °C for 2 h with H_2O_2 treatment before analyzed by HPLC-ICP-MS. Data are presented as the means ± SD of three separate experiments.

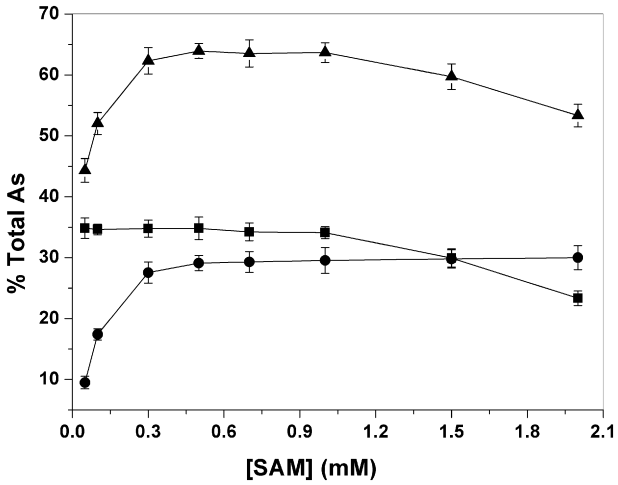


Fig. 5. Methylation of iAs^{3+} by hAS3MT as a function of SAM concentration. (■) MMA, (●) DMA, (▲) MMA + DMA. The reaction mixture (100 μl) containing hAS3MT (11 μg), SAM (0.05, 0.1, 0.3, 0.5, 0.7, 1, 1.5, 2 mM), GSH (7 mM), iAs^{3+} (1 μM) in PBS (25 mM, pH 7.0) was incubated at 37 °C for 2 h with H_2O_2 treatment before analyzed by HPLC-ICP-MS. Data are presented as the means ± SD of three separate experiments.

standard system containing up to 2 mM SAM. With the SAM concentration increasing, the yields of DMA increased rapidly to a plateau. In contrast, there was no obvious influence on the MMA yields from 0.05 to 1 mM SAM. Then a little of decrease was observed at higher SAM concentrations. Maximal conversion of iAs^{3+} to methylated products occurred at about 1 mM SAM, and the DMA/MMA ratio increased with SAM concentration increasing.

The roles of thiols (GSH, CySH, ME, DTT, and DMSA) in the methylation of iAs^{3+} were examined in the standard system. For the production of MMA, the hAS3MT activity was greatest at low concentrations of DTT, followed by CySH, GSH or ME (Fig. 6A). With increasing concentrations of these thiol and dithiol compounds, MMA yields first increased, and eventual inhibition was observed with all. In the case of DMA yields, initially, small amounts of these thiols (1 mM) were no production except DTT (Fig. 6B); larger amounts were strongly stimulatory; similarly, inhibition was observed at higher thiol concentrations. The total production of MMA and DMA with these thiols was shown in Fig. 6C. hAS3MT showed its greatest activity at about 7 mM GSH. CySH was the most effective thiol for the methylation of iAs^{3+}. In the standard condition with 10 mM CySH, 1 μM iAs^{3+} was almost totally transformed into methylated arsenicals after 2 h incubation. The relative contents of DMA to MMA are displayed in Table 1. The D/M increased as CySH concentration increased. The pattern of MMA and DMA production with ME resembled that with GSH, first increased as their concentration increased and then decreased. Similarly, the D/M first increased as DTT concentration increased and then decreased. In the presence of DMSA at concentrations of 2, 5, 10, 100, 500 μM, and 1, 3, 5 mM as the only thiol in the assay systems, neither MMA nor DMA could be detected (data not shown).

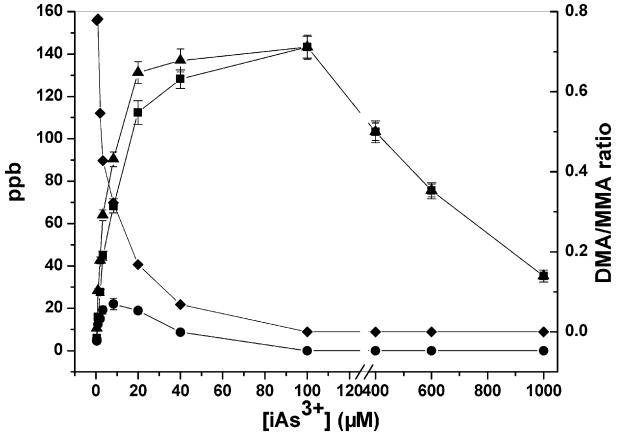


Fig. 4. Methylation of iAs^{3+} by hAS3MT as a function of substrate concentration. (■) MMA, (●) DMA, (▲) MMA + DMA, (◆) DMA/MMA, values for DMA/MMA are on the right axes. The reaction mixture (100 μl) containing hAS3MT(11 μg), SAM (1 mM), GSH (7 mM), iAs^{3+} (0.5, 1, 2, 3.3, 8.3, 20, 40, 100, 400, 600, 1000 μM) in PBS (25 mM, pH 7.0) was incubated at 37 °C for 2 h with H_2O_2 treatment before analyzed by HPLC-ICP-MS. Data are presented as the means ± SD of three separate experiments.

3.4. Effect of pH on the methylation of iAs^{3+}

The pH dependence of the iAs^{3+} methylation was examined over the pH range from 5.8 to 10.0 (Fig. 7). Here, hAS3MT (4.5 μg) in PBS (25 mM, pH 7.0, 10 μl) containing iAs^{3+} (10 μM), GSH (70 mM), and SAM (10 mM), was diluted into the buffer (90 μl) at the desired final pH. Substantial production was found between pH 7.0 and 10.0 with peak activity at about pH 8.5. D/M increased as pH increased from 5.8 to 8.5 and then plateaued from 8.5 to 10.0. As well, DTT was used as the thiol to study its effect on methylation of iAs^{3+} at different pH. In the reactions containing various amounts of DTT, hAS3MT showed maximal activity at pH 8.0 (data not shown).

3.5. Initial velocity studies

Detailed initial velocity studies were carried out by varying the concentration of one reactant in the presence of different fixed concentrations of the second reactant, while the concentrations of the other reactants were held constant. When the initial velocity of the reaction was studied by varying the iAs^{3+} concentration ($[iAs^{3+}]$) at different fixed concentrations of SAM ([SAM]), the double reciprocal plots intersected on the ordinate (Fig. 8A); whereas, when the data were plotted as $(\text{velocity})^{-1}$ versus $[SAM]^{-1}$ at different fixed $[iAs^{3+}]$, the lines intersected on the abscissa (Fig. 8B). The slopes of the lines obtained in Fig. 8B, when plotted against $[iAs^{3+}]^{-1}$, gave a line which passed through the origin (Fig. 8C). By contrast, parallel lines were obtained when

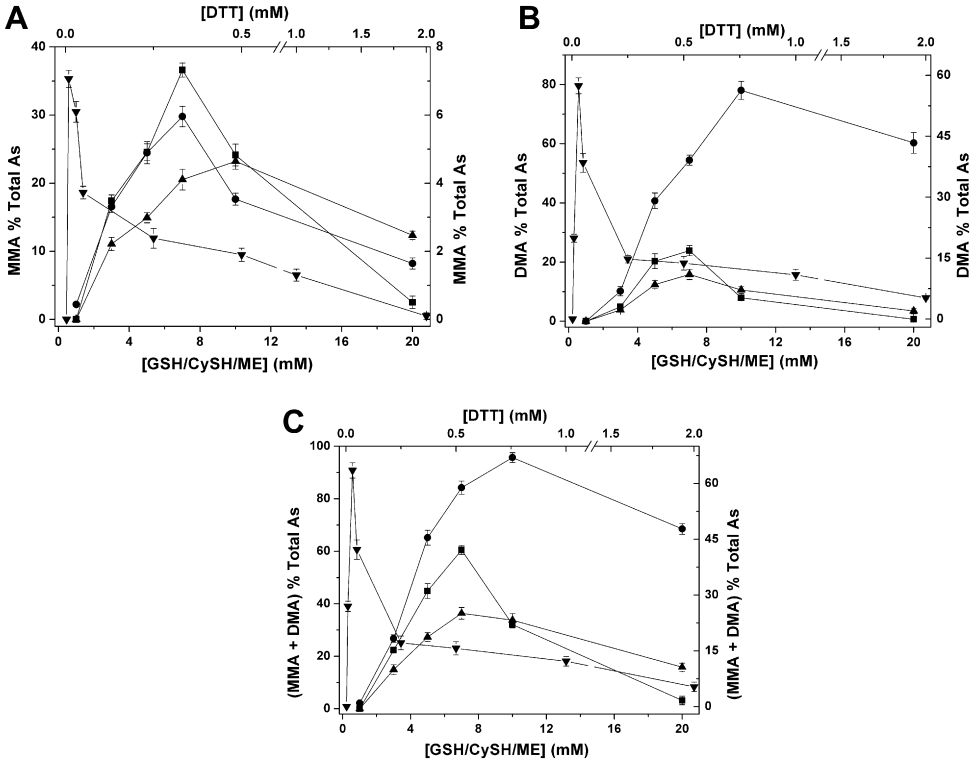


Fig. 6. Methylation of iAs^{3+} by hAS3MT as a function of thiol concentration. (A) MMA, (B) DMA, (C) MMA + DMA. (■) GSH, (●) CySH, (▲) ME, (▼) DTT. The reaction mixture (100 μl) containing hAS3MT (11 μg), SAM (1 mM), GSH (or CySH, ME) (1, 3, 5, 7, 10, 20 mM) or DTT (0.003, 0.009, 0.03, 0.05, 0.25, 0.5, 1, 2 mM), iAs^{3+} (1 μM) in PBS (25 mM, pH 7.0) was incubated at 37 °C for 2 h with H_2O_2 treatment before analyzed by HPLC-ICP-MS. The values for GSH, CySH, and ME are on the bottom and left axes. The values for DTT are on the top and right axes. Data are presented as the means ± SD of three separate experiments.

[iAs^{3+}] was varied at different fixed GSH concentrations ([GSH]) (Fig. 9A). Similarly, it also showed parallel lines when studying the effect of [SAM] on the methylation of iAs^{3+} at different fixed [GSH] (Fig. 9B).

3.6. Interactions of iAs^{3+}/SAM/GSH with hAS3MT

The interactions of iAs^{3+}/SAM/GSH with hAS3MT were determined by UV–vis and CD spectroscopy. Each sample had a control in which only corresponding concentration of iAs^{3+}, SAM, or GSH was added. Fig. 10A shows the changes in the UV–vis spectra of the hAS3MT when first, iAs^{3+} (2 μM) and then SAM (1 mM) was added, respectively. The variations of the UV–vis spectra of the hAS3MT resulted from the opposite order are shown in Fig. 10B. When iAs^{3+} (2 μM) was added, it yielded hyperchromism and then hypochromism was observed with the addition of SAM (1 mM). Interestingly, the opposite order gave the reverse results, first hypochromism and then hyperchromism. The CD spectra of the hAS3MT in the solutions in which iAs^{3+} (2 μM) and SAM (1 mM) were added in the two orders above are shown in Fig. 11A and B, respectively. When iAs^{3+} (2 μM) was added, the intensities of the CD spectra decreased. However, the secondary structures of the hAS3MT (calculated from the CD spectra) did not change significantly (about 7.6% β-pleated sheet increased, 6.2% random decreased and little change in α-helix and turn) compared with that of hAS3MT alone. The successive addition of SAM induced the intensities of the CD spectra further decreased. The peak at 208 nm had a blue shift. Also the secondary structures of the hAS3MT changed a lot (15% α-helix increased, 6.7% turn decreased, 9.2% random decreased and little change in β-pleated sheet) compared with the secondary structures when only iAs^{3+} had been added. When SAM was added firstly, the intensities of the CD spectra had a large decrease with about 3 nm blue shift. The α-helix increased about 33%, turn decreased 8.5%, random decreased 23.2%, and β-pleated sheet changed a little compared with that of hAS3MT alone. Then the addition of iAs^{3+} increased the intensities of the CD spectra and made the peak at about 217 nm retoured. The secondary structures of the hAS3MT further changed with 21% α-helix decreased, 6.9% β-pleated sheet increased, 6.2% turn decreased and 27% random increased compared with the

Table 1
The ratio of DMA to MMA at different thiol concentrations.[a]

	Concentration of thiol compounds (mM)												
	0.003	0.009	0.03	0.05	0.25	0.5	1	2	3	5	7	10	20
DTT	×	2.9	8.1	10.3	8.3	7.2	6.8	NM[b]					
GSH							×[b]		0.3	0.8	0.7	0.4	ND
ME							×		0.4	0.8	0.7	0.5	0.3
CySH							ND[b]		0.6	1.7	1.8	4.6	7.3

[a] The reaction mixture (100 μl) containing hAS3MT (11 μg), SAM (1 mM), iAs^{3+} (1 μM), various concentrations of thiols and PBS (25 mM, pH 7.0) was incubated at 37 °C for 2 h. Samples were analyzed with H_2O_2 treatment before analyzed.
[b] ×: no methylated arsenicals; ND: no DMA; NM: no MMA.

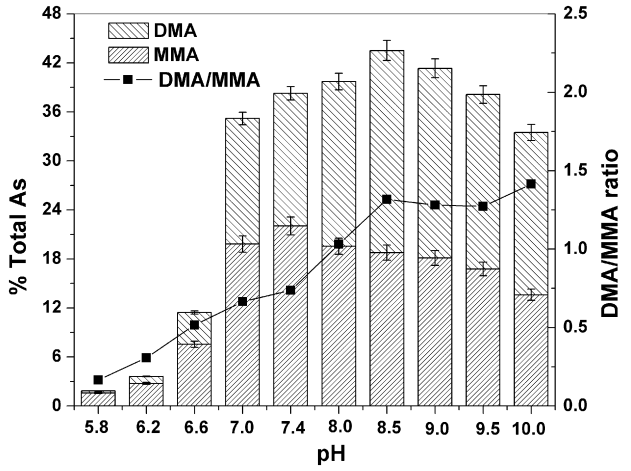


Fig. 7. Methylation of iAs^{3+} by hAS3MT as a function of pH. MMA and DMA production. The values for DMA/MMA (■) are on the right axes. The reaction mixture (100 μl) containing hAS3MT (4.5 μg), SAM (1 mM), GSH (7 mM), iAs^{3+} (1 μM) in buffer with different pH was incubated at 37 °C for 2 h with H_2O_2 treatment before analyzed by HPLC-ICP-MS. Data are presented as the means ± SD of three separate experiments.

secondary structures when SAM had been added. However, these two orders above brought the consistent final changes of the secondary structures of the hAS3MT. Whereas, no changes were detected in the UV–vis and CD spectra of the hAS3MT when GSH was added (data not shown).

4. Discussion

Arsenic methylation is a common character of the metabolism of this metalloid in organisms ranging from microorganisms to humans [30–33]. This process is catalyzed by an enzyme called arsenic (+3) methyltransferase (AS3MT), which enzymatic activities were found to require reductants (e.g. thiols) and SAM as essential cofactors [21,34]. However, the mechanism of the AS3MT is still unclear. In this report, we have prepared and purified the recombinant hAS3MT to further study the methylation of iAs^{3+} to probe the enzyme mechanism.

The time course of appearance or disappearance of methylated arsenicals from the standard assay system described here monitored the sequential methylation of iAs^{3+} to MMA and MMA to DMA, belonging to the precursor-product relationship. The production of DMA is necessarily linked to the production of MMA since a 30 min lag period occurred before DMA was produced. So any factor affecting the yields of MMA will also alter the yields of DMA. This pattern is consistent with the results from the studies with a purified rat liver AS3MT [18] and the studies with rat liver cytosol reported by Thomas [17]. However, the pattern is different from the results obtained from the studies with rat or human cells, such as rat hepatocytes, human hepatocytes, human epidermal kerationcytes and human bronchial epithelial cells [13,35]. This may attribute to time-dependent changes in viability in primary cultures of the cells exposed to arsenicals.

In the standard assay system containing a wide range of iAs^{3+}, the inhibition of DMA production occurred at a lower iAs^{3+}

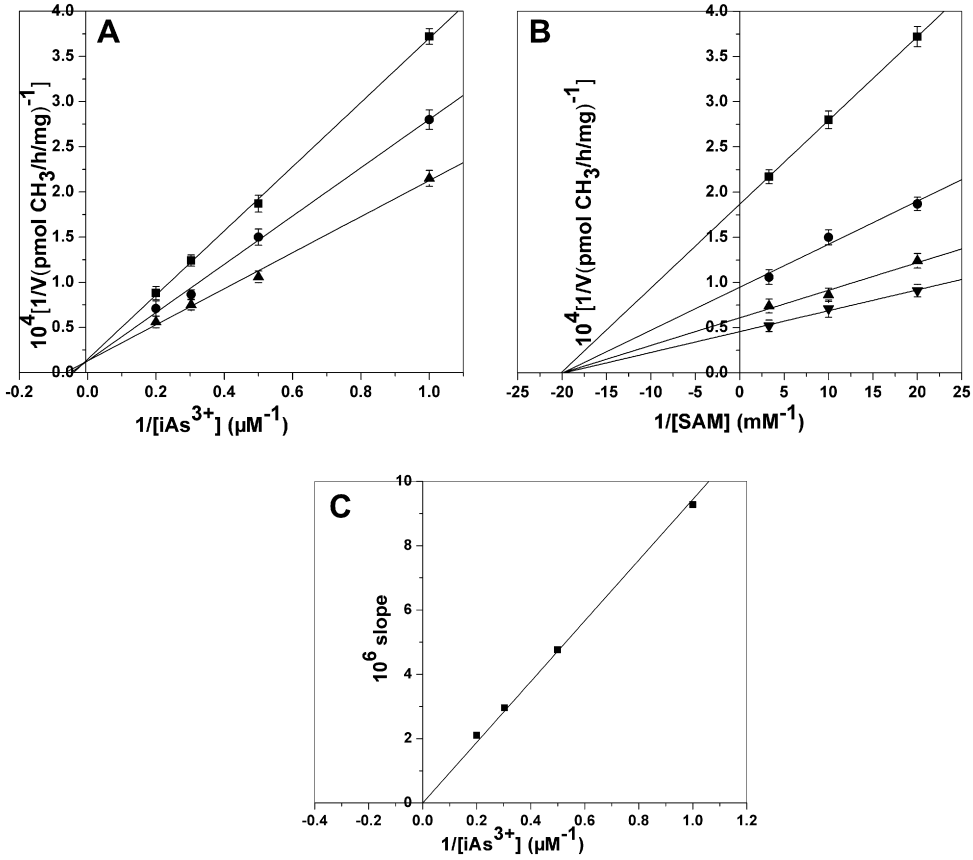


Fig. 8. (A) Effect of iAs^{3+} concentration on the rate of hAS3MT reaction at different fixed concentration of SAM and (B) Effect of SAM concentration on the rate of hAS3MT reaction at different fixed iAs^{3+} concentration. For (A), the concentrations of SAM were: (■) 0.05 mM, (●) 0.1 mM, (▲) 0.3 mM and for (B), the concentrations of iAs^{3+} were: (■) 1 μM, (●) 2 μM, (▲) 3.3 μM, (▼) 5 μM. The concentrations of the other components were constant: hAS3MT (11 μg), and GSH (7 mM) in PBS (25 mM, pH 7.0). (C) A secondary plot of the slopes of the lines obtained in B when plotted against $[iAs^{3+}]^{-1}$. The reaction was incubated at 37 °C for 30 min with H_2O_2 treatment before analyzed by HPLC-ICP-MS. Data are presented as the means ± SD of three experiments.

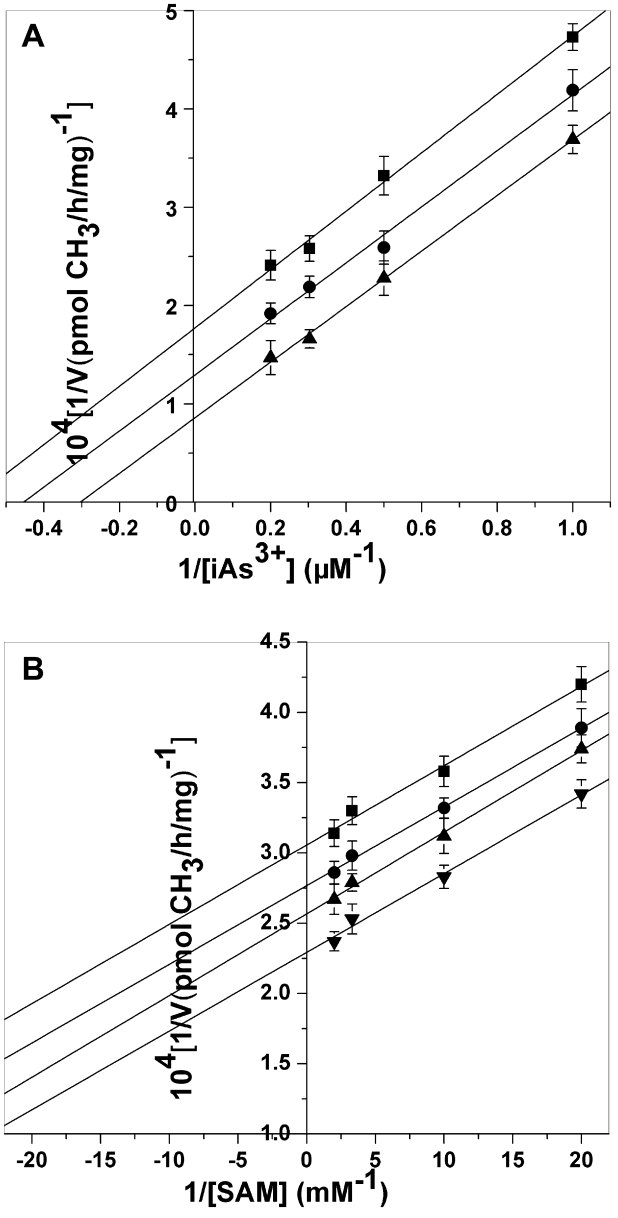


Fig. 9. (A) Effect of iAs^{3+} concentration on the rate of hAS3MT reaction at different fixed concentration of GSH and (B) Effect of SAM concentration on the rate of hAS3MT reaction at different fixed GSH concentration. For (A), the concentrations of GSH were: (■) 4 mM, (●) 5 mM, (▲) 7 mM, and the concentrations of the other components were constant: hAS3MT (11 μg), and SAM (1 mM) in PBS (25 mM, pH 7.0). For (B), the concentrations of GSH were: (■) 3 mM, (●) 4 mM, (▲) 5 mM, (▼) 7 mM, and the concentrations of the other components were constant: hAS3MT (11 μg), and iAs^{3+} (1 μM) in PBS (25 mM, pH 7.0). The reaction was incubated at 37 °C for 30 min with H_2O_2 treatment before analyzed by HPLC-ICP-MS. Data are presented as the means ± SD of three separate experiments.

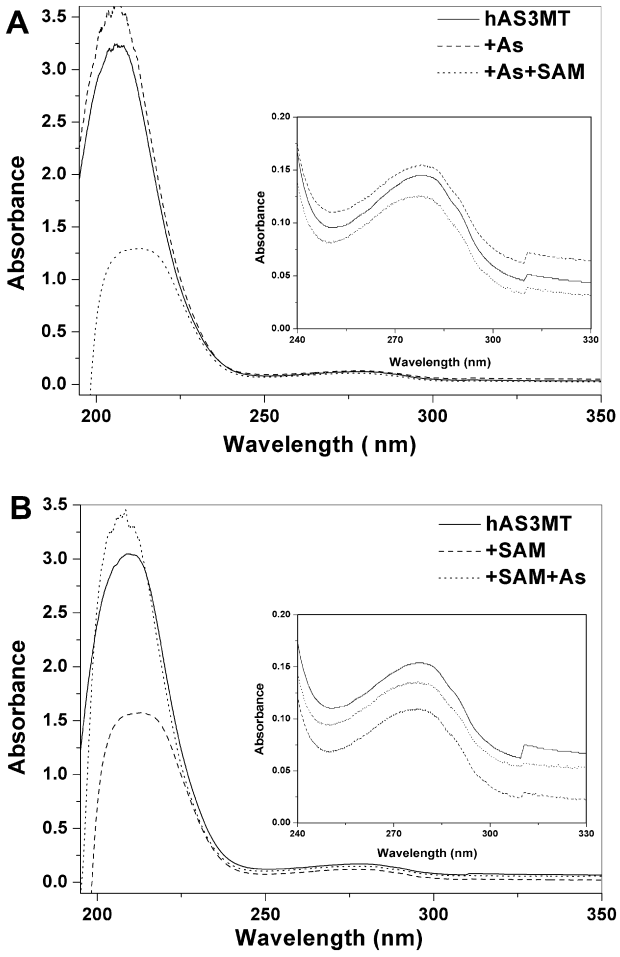


Fig. 10. UV–vis spectra of hAS3MT in the absence and presence of iAs^{3+} and SAM in PBS (25 mM, pH 7.0). (A) First, iAs^{3+} (2 μM) was added and then SAM (1 mM). (B) First, SAM (1 mM) was added and then iAs^{3+} (2 μM). Insets show the UV–vis absorption spectra at 280 nm. Plot is the representative of four independent measurements.

concentration compared with the inhibition of MMA, suggesting the inhibition of the conversion of MMA to DMA by iAs^{3+} is greater than that of iAs^{3+} to MMA. It also probably indicates that MMA first needs to be released from the hAS3MT, and then competes with iAs^{3+} to contact with the enzyme to be metabolized. Earlier reports also showed that iAs^{3+} inhibited the formation of DMA in cultured cells [13] and rat liver cytosol [17]. This is consistent with our results. No DMA was observed at lower contents of hAS3MT but then its yields increased as hAS3MT contents increased. Contrarily, MMA yields decreased at higher contents of hAS3MT. This appearance pattern of methylated arsenicals also indicates a sequential methylation. Otherwise, competitive combination with the hAS3MT between iAs^{3+} and MMA^{3+} might also be suggested. SAM serves as the methyl group donor for both reactions. With the SAM concentration increasing, the yields of DMA increased rapidly to a plateau. However, there was no obvious influence on the MMA yields. The production of total methylated arsenicals increased as the SAM concentration increased but was inhibited at higher concentrations of SAM. In the absence of SAM, methylated arsenicals were not detected in reaction mixtures (data not shown). These results show that the production of DMA is more dependent on the SAM concentration, and competition exists between the methylation of iAs^{3+} and MMA^{3+}. To sum up, the findings above are compatible with sequential methylation of iAs^{3+} and reveal the competitive methylation between iAs^{3+} and MMA^{3+}.

The studies with different thiols clearly indicate that enzymatic activities of the hAS3MT do not have an absolute requirement for GSH as a cofactor. However, previous studies showed that GSH was required for full activity of crude cytosol [36]. According to our data, these conclusions should be reestimated. The enzyme just appears to require a reducing environment which can be provided by thiols (such as GSH, CySH, ME, DTT) and even non-sulfhydryl reductant, e.g. TCEP or coupled system (such as Trx/TR/NADPH, DHLA/TR/NADPH) [37]. However, considering that methylation of iAs^{3+} mainly happens in liver [38] and GSH has a high concentration in this tissue, we chose GSH as the reductant in our standard assays.

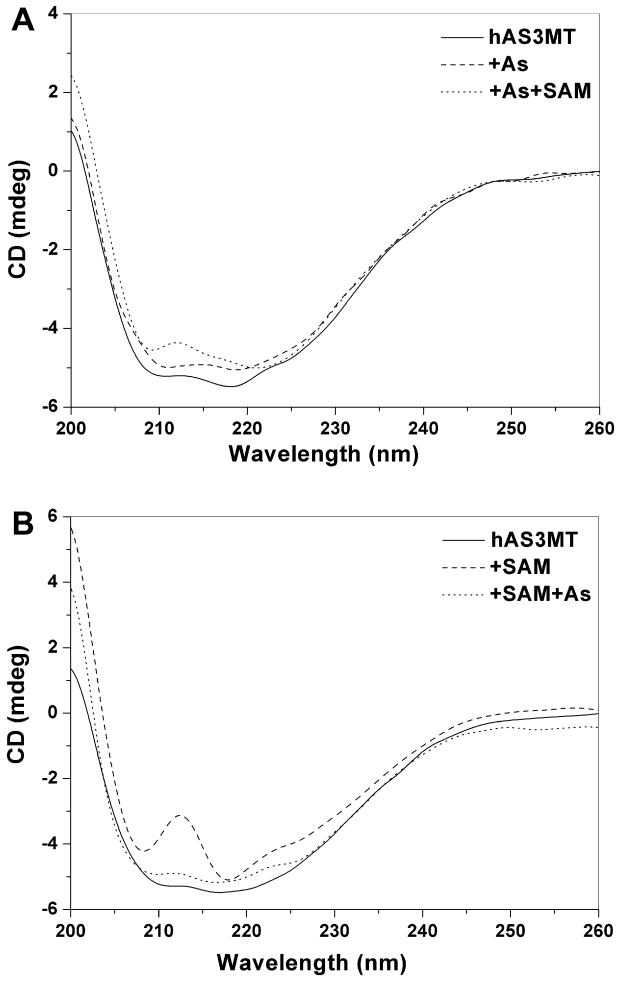


Fig. 11. CD spectra of hAS3MT in the absence and presence of iAs^{3+} and SAM in PBS (25 mM, pH 7.0). (A) First, iAs^{3+} (2 μM) was added and then SAM (1 mM). (B) First, SAM (1 mM) was added and then iAs^{3+} (2 μM). Plot is the representative of four independent measurements.

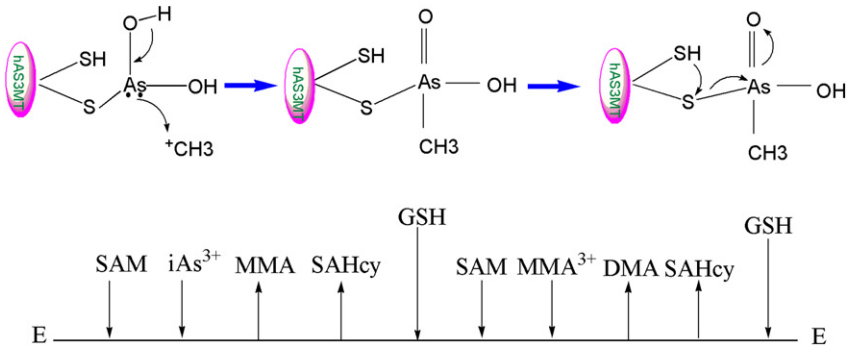
The inhibition of iAs^{3+} methylation was observed in all the conditions containing higher concentrations of GSH, CySH, ME, and DTT as the reductant, respectively. These data conflict with the results showing that GSH did not inhibit the methylation of iAs^{3+} at concentrations greater than 5.0 mM [17,19] but accord with the reports by Buchet [36]. This may attribute to that the actual GSH concentration in the assays [36] was far higher than 5.0 mM since crude extracts of rat liver used by Buchet contained a high concentration of this thiol. However, partially purified rat AS3MT solutions [19] contained low concentrations of GSH, and the GSH concentration assayed in that report was not high enough to inhibit the reaction. Otherwise, in the crude extracts of rat liver [17], more GSH was needed to protect the labile thiol groups of AS3MT to preserve the enzymatic activities in the extracts [39]. Earlier studies reported that methylated arsenicals did not produce by a recombinant rat AS3MT with 5 mM GSH alone [37], and 1 mM GSH could not support the activity of a recombinant hAS3MT in an hour [39]. This is inconsistent with our findings. The reasonable explanation may be that the incubation time (20 min) in the former assay conditions was too short to produce methylated arsenicals when GSH alone was used, while the concentration of GSH in the latter assay conditions was too low since reductant in this reaction might play multiple roles [39]. hAS3MT showed its maximal activity at a much lower concentration of DTT, while no methylated arsenicals were detected with DMSA as the reductant. These results indicate that the interactions of iAs^{3+} with thiols are a major factor in the inhibition of arsenite methylation since DTT usually forms an unstable 7-membered ring with iAs^{3+} and DMSA usually forms a stable 5-membered heterocyclic chelate structure [40]. So there was no free iAs^{3+} in the reaction mixtures containing DMSA or high concentrations of DTT. Thomas et al reported that AS3MT also displayed activity with non-sulfhydryl reductants [37]. This indicates that an As-thiol complex is not a requirement for iAs^{3+} as the substrate. To sum up, moderate reductant is needed to support the enzymatic activity of the hAS3MT. Higher concentrations of thiols may destroy the structure of the hAS3MT and chelate free iAs^{3+}, resulting in the inhibition of the methylation of iAs^{3+}. From our studies, GSH alone can support the activity of the hAS3MT and it is a rational reductant in our in vitro assays. CySH can strongly improve the production of DMA, which is the primary form excreted in urine [5] and is generally considered to be nontoxic iAs metabolite.

The optimum pH of the methylation of iAs^{3+} by hAS3MT was around 8.5 when GSH was used as the reductant and 8.0 when DTT was used. Previous report showed that the peak activity of a purified rat liver AS3MT was at about 9.5, which is similar to our findings [18]. The effective pH range was from 7.0 to 10.0 in our assay conditions. It is known that sulfhydryl group will need to be deprotonated in an alkaline environment (pH value no less than 7.0) for reductive activity. This is consistent with our results. At the optimum pH, the sulfhydryl groups in GSH and DTT are inclined to be deprotonated. These results illuminate that thiols really play a reductive role in the reactions. In addition, Cys156 and Cys206 in the hAS3MT were proved to be the active sites in our previous report [29]. They also need to be deprotonated in the course of the reaction with iAs^{3+}. The weak alkaline environment can facilitate the balance between the deprotonation and the protonation of these two active sites to expedite the reaction.

Our main findings above indicate that the enzyme reaction in our assays with the hAS3MT can reflect the essence in cells and tissues. So our data are rational. Otherwise, the effects of some cofactors are not completely consistent with the previous works, such as the roles of GSH. This just suggests the necessity to do an investigation with the purified hAS3MT. So, our results are the complementarity and verification of the previous works. Thus, it is reasonable to further study the mechanism of the hAS3MT.

Initial velocity analysis of the reaction sequence is an effective approach for understanding the enzyme mechanism. With the conditions under which hAS3MT showed the greatest activity and enzyme assays were within linear range, we carried out a series of experiments to study the reaction sequence. The lines obtained from the studies by varying the iAs^{3+} concentration ($[iAs^{3+}]$) at different fixed concentration of SAM ([SAM]), intersected on the ordinate. When the data plotted as $(\text{velocity})^{-1}$ versus $[\text{SAM}]^{-1}$ at different fixed $[iAs^{3+}]$, lines intersected on the abscissa. Otherwise, the slopes of the latter lines were plotted against $[iAs^{3+}]^{-1}$. A line which passed through the origin was obtained. These results illuminate an ordered mechanism for the binding of SAM and iAs^{3+}, with the addition of SAM being at the thermodynamic equilibrium. It thus follows that the addition of SAM occurs before that of iAs^{3+}, and SAM can not dissociate once iAs^{3+} has bound to the enzyme [41,42]. By contrast, parallel lines were observed in the double-reciprocal plots for pairs containing GSH and each of the other reactants. Accordingly, GSH should probably be placed either as the first reactant in the reaction scheme or as a reactant combining with the enzyme only after one or more products have been released [41,42].

In order to further probe the reaction sequence, we used UV–vis and CD spectroscopy to monitor the interactions between hAS3MT



Scheme 1. Scheme depicting the hypothetical mechanism for methylation of iAs^{3+} by hAS3MT. E: enzyme (hAS3MT); SAHcy: *S*-adenosylhomocysteine.

and iAs/SAM/GSH. In our studies, changes were observed for both UV–vis and CD spectra of the hAS3MT when either iAs^{3+} or SAM was added. This shows that iAs^{3+} and SAM alone can interact with the hAS3MT, bringing the conformational change of the enzyme. However, the changes arose from the addition of SAM were larger than that from the addition of iAs^{3+}. In previous reports, the kinetics of a wide variety of methyltransferase using SAM as methyl donor was studied. It showed that if the reactions took place through a sequential mechanism, either ordered [43–46] or random [47,48], a ternary complex of enzyme, SAM and methyl-acceptor substrate would be synthesized before the reaction started. With enzymes following an ordered binding mechanism, SAM was the first substrate to combine [43–46]. Accordingly, integrating the spectra and the initial velocity results above (ordered mechanism), SAM should be the first reactant binding to the hAS3MT in the methylation of iAs^{3+}. In contrast, no interactions between GSH and the hAS3MT were detected by UV–vis and CD spectra. Previous report speculated that an interaction between AS3MT and GSH underlied the stimulative effects of this thiol compound on the methylation of arsenicals at each level of biological complexity [49]. According to our spectra results, this interaction may happen during or at the end of the catalytic reaction. Further considering the data from the initial velocity studies, GSH should not be placed as the first reactant in the reaction scheme, but as a reactant combining with the enzyme only after one or more products have been released.

Lately, Cys157 and Cys207 have been proved to be the active sites of the recombinant mouse AS3MT. In the protein model, the topology supported the formation of an intramolecular disulfide bond by these two active-site cysteine residues during the catalytic cycle [50]. In our previous studies, we reported that Cys156 and Cys206 were the active sites of the hAS3MT [29]. As homologous protein, Cys156 and Cys206 should form an intramolecular disulfide during the reaction as well. Based on the result that GSH should be placed as a reactant after the products have been released, GSH just seems to reduce the disulfide bond to resume the active form of the enzyme. The intramolecular disulfide bond implies that the enzyme is oxidized at the end of the catalytic cycle by losing two electrons. In the reaction, methyl cation is reduced to methyl anion by getting two electrons. Considering the balance of gaining and losing electrons in redox reactions, the arsenical metabolites should be trivalent and then are transformed to pentavalency.

In conclusion, based on all the results and discussions above, we propose a mechanism (Scheme 1) which is essentially consistent with Hayawaba's proposal. However, As-thiol complex is not a requirement for iAs^{3+} as the substrate in our scheme. The hAS3MT first combines with SAM, and then iAs^{3+} adds to it, forming a ternary complex. After that, iAs^{3+} is successively methylated reductively, rather than a stepwise oxidative methylation. A disulfide bond forms in the enzyme at the end of the catalytic cycle. Then reductant (thiols or non-sulfhydryl reductants that can reduce disulfide bond) reacts with the disulfide bond to resume the active form of the enzyme.

Acknowledgements

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