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Identification of Catalytic Residues in the As(III) *S*-Adenosylmethionine Methyltransferase

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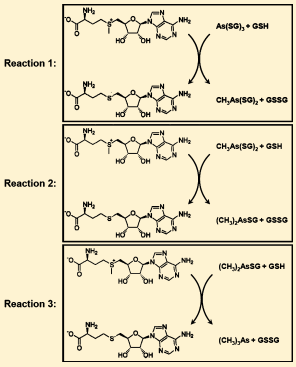
Supporting Information

ABSTRACT: The enzyme As(III) *S*-adenosylmethionine methyltransferase (EC 2.1.1.137) (ArsM or AS3MT) is found in members of every kingdom, from bacteria to humans. In these enzymes, there are three conserved cysteine residues at positions 72, 174, and 224 in the CmArsM orthologue from the thermophilic eukaryotic alga *Cyanidioschyzon* sp. 5508. Substitution of any of the three led to loss of As(III) methylation. In contrast, a C72A mutant still methylated trivalent methylarsenite [MAs(III)]. Protein fluorescence of a single-tryptophan mutant reported binding of As(III) or MAs(III). $As(GS)_3$ and $MAs(GS)_2$ bound significantly faster than As(III), suggesting that the glutathionylated arsenicals are preferred substrates for the enzyme. Protein fluorescence also reported binding of Sb(III), and the purified enzyme methylated and volatilized Sb(III). The results suggest that all three cysteine residues are necessary for the first step in the reaction, As(III) methylation, but that only Cys174 and Cys224 are required for the second step, methylation of MAs(III) to dimethylarsenite [DMAs(III)]. The rate-limiting step was identified as the conversion of DMAs(III) to trimethylarsine, and DMAs(III) accumulates as the principal product.



Arsenic is a ubiquitous environmental toxin, introduced primarily from geochemical sources. This Group 1 human carcinogen ranks first on the U.S. Center for Disease Control and Prevention's Toxic Substances and Disease Registry Comprehensive Environmental Response, Compensation, and Liability Act Priority (CERCLA) List of Hazardous Chemicals (http://www.atsdr.cdc.gov/spl/). Arsenic-detoxifying systems are found in nearly all organisms. Microorganisms can detoxify arsenic by methylation to mono-, di-, and trimethylated species catalyzed by As(III) *S*-adenosylmethionine methyltransferase (EC 2.1.1.137) (ArsM).[1,2] In mammals, this enzyme, termed AS3MT, is thought to contribute to arsenic carcinogenesis rather than detoxification.[3,4]

The pathway of arsenic methylation is not certain. One hypothesis proposed by Challenger[5] is that the enzyme catalyzes a series of alternating oxidative methylations and reductions, using *S*-adenosylmethionine as the methyl donor to produce the pentavalent species methylarsenate [MAs(V)], dimethylarsenate [DMAs(V)], and trimethylarsine oxide [TMAs(V)O] and the trivalent species MAs(III), DMAs(III), and TMAs(III). Most consistent with this hypothesis is the observation that mammals, including humans, excrete primarily DMAs(V) and to a lesser extent MAs(V) but little or no trivalent arsenicals.[6,7] More recently, Hayakawa and co-workers[8] proposed an alternate pathway in which the preferred substrates of the methyltransferase are the glutathione (GSH) conjugates $As(GS)_3$ and $MAs(GS)_2$. This pathway also involves a series of sequential oxidations and reductions; however, SAM is reduced to *S*-adenosylhomocysteine (SAH), and GSH is oxidized to GSSG rather than changes in the oxidation state of arsenic, which remains trivalent throughout the catalytic cycle. Pentavalent arsenicals would result from nonenzymatic oxidation of the trivalent species.

To differentiate between these two hypotheses, we analyzed the enzymatic properties of an orthologue of human AS3MT, CmArsM, from an environmental isolate of the thermoacidophilic eukaryotic red alga *Cyanidioschyzon merolae*.[1] Purified CmArsM (411 residues, including a histidine tag) exhibits a temperature optimum of 60−70 °C. All As(III) SAM methyltransferases identified to date have three conserved cysteines, at positions 72, 174, and 224 in CmArsM. Here we show that substitution of any of the three with alanine led to loss of As(III) methylation. In contrast, a C72A mutant still methylated MAs(III). CmArsM has a single tryptophan residue that was changed to tyrosine to produce a tryptophan-free derivative. In this background, Tyr70 was changed to a tryptophan, producing the single-tryptophan derivative Y70W. Purified Y70W reported binding of As(III) and MAs(III) with quenching of protein fluorescence. CmArsM also bound and methylated inorganic Sb(III), producing volatile antimony species. $As(GS)_3$ and $MAs(GS)_2$ bound substantially faster than the free metalloids, supporting the hypothesis that glutathionylated arsenicals are preferred substrates for the enzyme.[8] In the Y70W background, each of the three mutants, C72A, C174A, and C224A, exhibited fluorescence quenching with addition of As(III), suggesting that the mutants bind but do not methylate As(III). Moreover, the data indicate that all three cysteines are involved in As(III)

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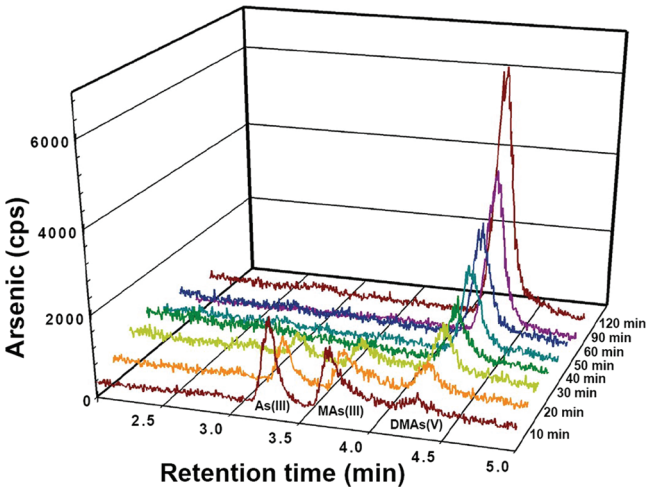

Figure 1. Time course of As(III) methylation by CmArsM. Methylation was assayed at 60 °C as described in Materials and Methods. The reaction mixture contained 2 μM ArsM in 50 mM MOPS and 0.5 M NaCl (pH 7.5) containing 10 μM sodium arsenite, 5 mM GSH, and 1 mM SAM. The samples were analyzed at the indicated times by reverse phase HPLC–ICP-MS.

methylation but that only Cys174 and Cys224 are necessary for MAs(III) methylation. We propose that, following the first round of methylation [As(III) to MAs(III)], the first product, MAs(III), undergoes a second round of methylation faster than it dissociates from the enzyme. The second product, DMAs(III), has a relatively low affinity for the enzyme compared with As(III) or MAs(III) and dissociates faster than it undergoes the third round of methylation to the third and final product, TMAs(III). Our hypothesis predicts that the principal product is DMAs(III) and that arsenic remains trivalent during the entire catalytic cycle.

MATERIALS AND METHODS

Reagents. Dimethylarsenate (cacodylate), potassium hexahydroxoantimonate [Sb(V)], potassium antimonyl tartrate [Sb(III)], and trimethylantimony dibromide [TMSb(V)] and other reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO) unless otherwise noted. The commercial herbicide Ortho Weed-B-Gone Crabgrass Killer Formula II, which contains calcium methylarsenate as its sole active ingredient, was used as the source of MAs(V). MAs(V) was reduced to MAs(III) with a mixture of metabisulfite and thiosulfate,[9] with the final pH adjusted to 6.5 with NaOH. The concentration of stock solutions of MAs(V) was determined by inductively coupled plasma mass spectroscopy (ICP-MS) using an ELAN DRC-e spectrometer (PerkinElmer, Waltham, MA). The glutathione conjugates of the metalloids were prepared by incubation of 1 mM free metalloid with a 4-fold molar excess of GSH for 5 h at 23 °C in degassed buffers under argon.

Preparation of Mutants. *Escherichia coli* strain JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ(*lacproAB*) F′ (*traD36 proAB+ lacIq lacZ*Δ*M15*)] was used for molecular cloning. Cloning and expression of the *arsM7* gene were described previously.[1] Mutations in the gene were generated by site-directed mutagenesis using a Quick Change mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotides used for mutagenesis are listed in Table 1 of the Supporting Information. The codon for Trp336 was mutated to a tyrosine codon, generating the tryptophan-free ArsM derivative W336Y. In this background, the codon for Tyr70 was replaced with a tryptophan

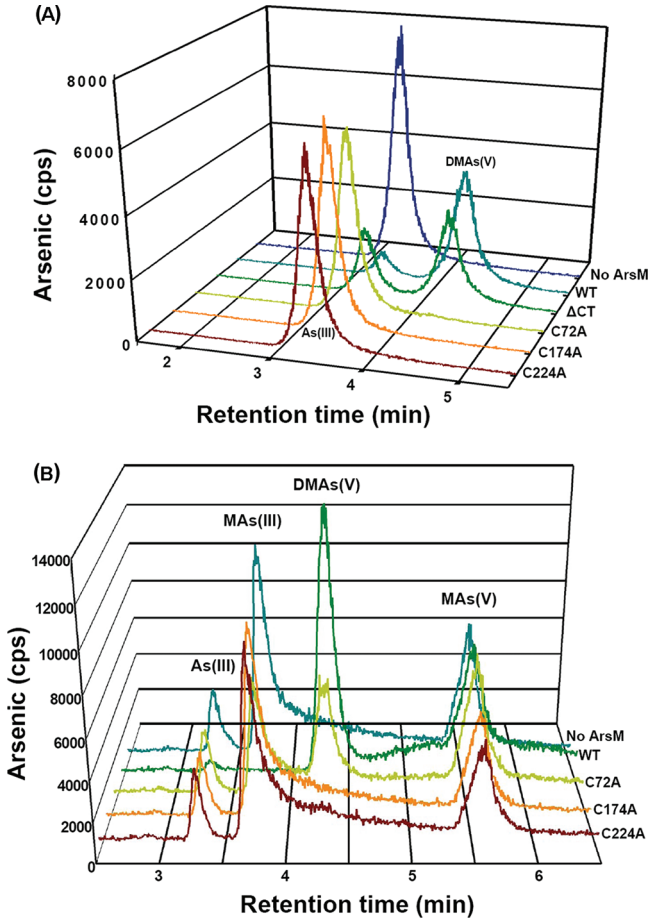

Figure 2. Methylation of As(III) and MAs(III) by wild-type and mutant CmArsM enzymes. Methylation of As(III) or MAs(III) was assessed after 2 h at 60 °C as described in Materials and Methods with either (A) As(III) or (B) MAs(III) at 10 μM, 5 mM GSH, and 1 mM SAM. The samples were analyzed by reverse phase HPLC–ICP-MS.

codon, generating the single-tryptophan derivative Y70W. Substitutions C72A, C174A, and C224A were introduced into the wild type and Y70W. A C174A/C224A double substitution was also introduced into Y70W. Each mutation was confirmed by sequencing of the complete gene.

Purification of ArsM. Wild-type and mutant ArsM enzymes with a C-terminal histidine tag were purified by Ni-NTA chromatography from cells of *E. coli* BL21(DE3) pET28*arsM*, as described previously.[1,2] Protein concentrations were determined from absorbance at 280 nm.[10]

Arsenic Methylation. Methylation of As(III) and MAs(III) was assayed using purified wild-type and mutant ArsMs. Assays were conducted in a buffer consisting of 50 mM MOPS and 0.5 M NaCl (pH 7.5) containing 5 mM GSH and 1 mM SAM. The protein was removed from the reaction mixture by centrifugation using a 3 kDa cutoff Amicon Ultrafilter (Millipore, Billerica, MA). The filtrate was speciated by high-pressure liquid chromatography (HPLC) (Series 2000, PerkinElmer) using a C18 reverse phase column eluted with a mobile phase consisting of 3 mM malonic acid, 5 mM tetrabutylammonium hydroxide, and 5% methanol (pH 5.9) with a flow rate of 1 mL/min, and arsenic content was determined by ICP-MS.

Antimony Methylation. Antimony methylation reactions were conducted in 1.5 mL tubes in 0.5 mL of a buffer consisting of 25 mM MOPS-KOH (pH 7.5) containing 125 mM

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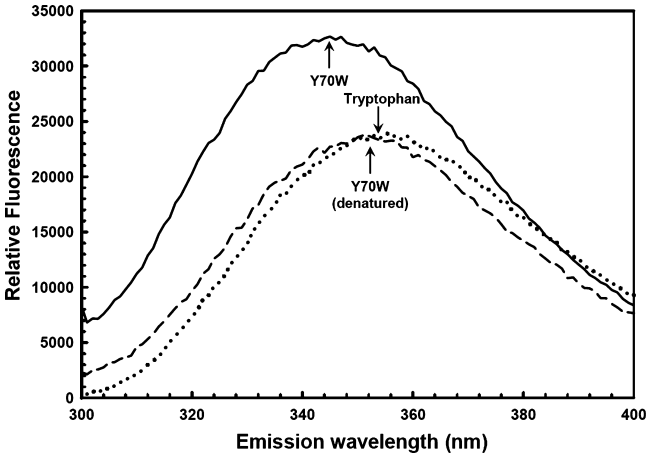

Figure 3. Emission spectra of native and denatured CmArsM Y70W. Samples were excited at 295 nm, and emission spectra were aquired at 23 °C as described in Materials and Methods. The emission spectra of 1 μM ArsM Y70W (—) and the same protein denatured with 6 M guanidine hydrochloride (···) were compared with that of 1 μM tryptophan (---).

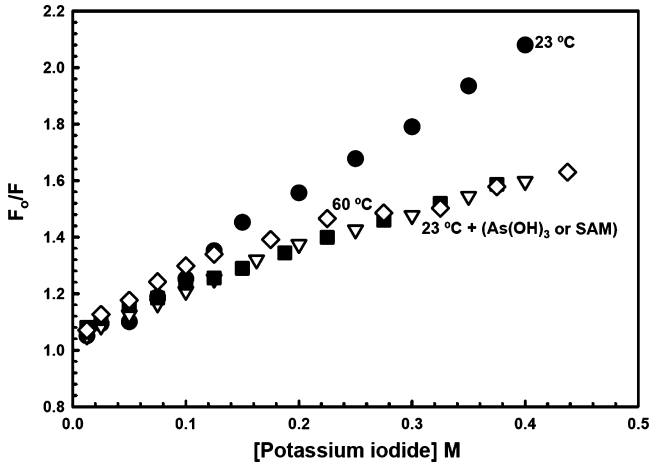

Figure 4. Iodide quenching of Y70W fluorescence. Quenching of the fluorescence of 2 μM CmArsM Y70W with sodium iodide was performed as described in Materials and Methods at 23 °C in the absence of substrates (●), after preincubation for 30 min with 50 μM As(III) (■) or 50 μM SAM (▽), or in the absence of substrates at 60 °C (◇), and the data were analyzed using the Stern−Volmer relationship.

NaCl, 25 μM Sb(III), 0.8 mM SAM, 2.5 mM tris(2-carboxyethyl)phosphine, and 10 μM ArsM at 37 °C overnight. For analysis of gas evolution, methylation reactions were performed in 4 mL in capped 20 mL vials in which two 2 cm nitrocellulose membrane filters (Millipore) were placed in the cap. The filters were impregnated with 0.15 mL of 6% H_2O_2 to oxidize the gases. After the reaction, the filters were digested with 0.2 mL of 70% HNO_3 at 70 °C for 20 min, diluted 20-fold with water, and analyzed by HPLC−ICP-MS. Antimony speciation was performed by the method of Muller et al.[11] Proteins were removed from the samples by centrifugation using a 3 kDa cutoff Amicon Ultrafilter. The filtrate was speciated by HPLC−ICP-MS using a Hamilton PRP-X100 anion exchange column eluted with a step gradient composed of 6 mL of 20 mM ethylenediaminetetraacetate (EDTA) (pH 4.5) and 13.5 mL of NH_4OH (pH 11) containing 1 mM EDTA at a flow rate of 1.5 mL/min.

Fluorescence Assays. Fluorescence measurements were performed on a temperature-controlled QuantaMaster UV−vis QM-4 steady state spectrofluorometer (Photon Technology International, Birmingham, NJ) at 23 or 60 °C, as noted. For steady state measurements, both emission and excitation monochromator slits were set at 1 nm. Samples were excited at 295 nm, with emission at 345 nm for time-based data acquisition. Spectra were corrected for background fluorescence and Raman scattering by subtracting buffer spectra. The buffer consisted of 50 mM MOPS and 0.5 M NaCl (pH 7.5). For quenching experiments, small amounts of a potassium iodide quenching solution were added from a 5 M stock solution, and fluorescence spectra were recorded after each addition. The iodide stock solution contained 0.2 mM $Na_2S_2O_3$ to prevent the formation of triiodide. Fluorescence intensities were corrected for volume changes. The quenching data obtained were analyzed using to the Stern−Volmer equation:[12] $F_0/F = 1 + K_D[Q]$, where F_0 and F are the relative fluorescence intensities in the absence and presence of quencher, respectively, [Q] is the quencher concentration, and K_D is the Stern−Volmer constant. For determination of relative affinities of As(III) and MAs(III), fluorescence spectra were acquired at various concentrations of arsenicals, and the affinity for each was calculated according to the method of Rosenthal[13] by plotting $(\Delta F/\Delta F_{max})/[L]$ as a function of $\Delta F/\Delta F_{max}$, where $\Delta F/\Delta F_{max}$ is the fractional change in fluorescence at 345 nm, ΔF is the quenching at a particular concentration of arsenical ligand, [L], and ΔF_{max} is the quenching at the highest (saturating) arsenical concentration.

RESULTS

Effect of Substitution of Conserved Residues Cys72, Cys174, and Cys224 on ArsM Activity. All ArsM and AS3MT proteins identified to date have three conserved cysteine residues, Cys72, Cys174, and Cys224 in CmArsM (Figure 1 of the Supporting Information). Cys44 of CmArsM is not conserved in other orthologues and so was not considered further in this study. ArsMs also have cysteine residues and pairs at the C-terminus (Cys340-Cys341 and Cys347-Cys348 in CmArsM), but these are not required for activity (vide infra)[1] and so were not considered further in this study. To explore the role of the three conserved cysteine residues in ArsM function, we individually altered each to alanine by site-directed mutagenesis to create C72A, C174A, and C224A derivatives. The wild type, a C-terminally truncated derivative that had been used for crystallization,[14] and three single-cysteine mutants were purified and examined for their ability to methylate inorganic As(III) and the trivalent methylated species MAs(III). While it is possible that the mutations have structural alterations that could affect activity, each mutant protein was produced in amounts comparable to that of the wild type and was soluble in the cytosol, indicating relatively normal folding. After 10 min at 60 °C, the wild-type enzyme methylated As(III) to MAs(III), which was converted to DMAs(V) at later times (Figure 1). Under these conditions, DMAs(III) is unstable and spontaneously oxidizes to DMAs(V), so it is not possible from these results to deduce which dimethylated species is directly produced by ArsM. The C-terminally truncated form also methylated As(III), but none of the three purified single-cysteine mutants was able to methylate As(III) after 2 h, consistent with a role in ArsM catalysis (Figure 2A). In contrast, methylation of MAs(III) was observed in the C72A mutant but not in the C174A or C224A

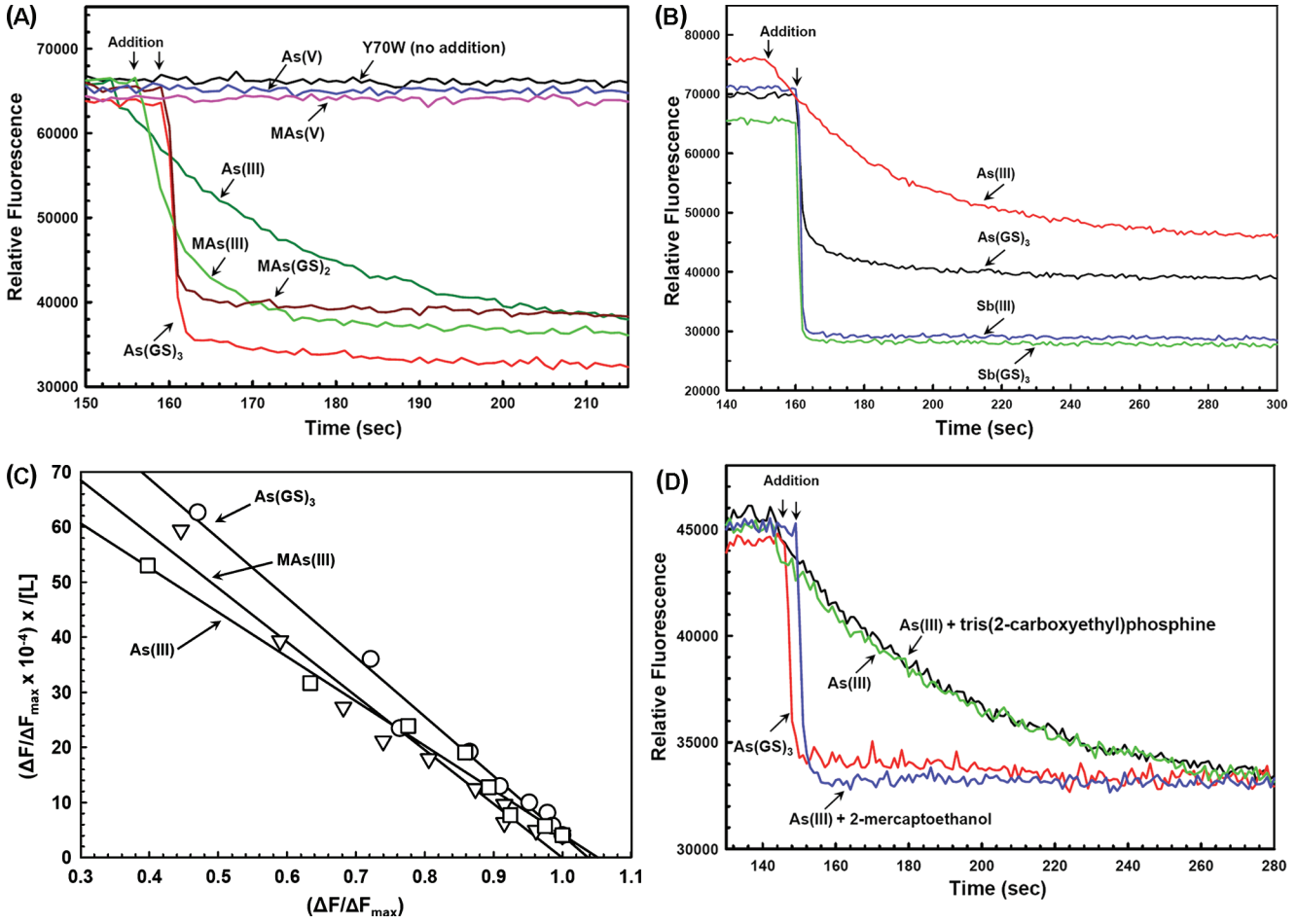

Figure 5. Quenching of CmArsM Y70W reveals metalloid binding. Protein fluorescence was assayed at 23 °C with excitation and emission wavelengths of 295 and 345 nm, respectively, as described in Materials and Methods. Free metalloids or their glutathione conjugates were added at a concentration of 25 μM to 2 μM CmArsM Y70W at the times indicated by the arrows. (A) Comparison of As(III) and MAs(III) and their glutathione conjugates. (B) Comparison of As(III) and Sb(III). (C) Scatchard analysis of the effect of binding of $As(GS)_3$ (○), $MAs(GS)_2$ (▽), or As(III) (□) on the fluorescence of 1 μM Y70W. (D) Comparison of the effect of the reductants. As(III) (1 mM) was preincubated in degassed buffer at 23 °C with 5 mM GSH, 10 mM 2-mercaptoethanol, or 5 mM tris(2-carboxyethyl)phosphine. All data were corrected for dilution effects.

mutant (Figure 2B). These results suggest that the three conserved cysteines are required for the first methylation step [As(III) to MAs(III)], while only two (Cys174 and Cys224) are required for the second methylation step [MAs(III) to DMAs(III)]. Note that the three conserved residues could provide three sulfur ligands for binding of As(III), while only two sulfur ligands are necessary for binding of MAs(III). Because DMAs(III) can bind to only a single thiol, it is likely that this product readily dissociates from the enzyme, so the third methylation [DMAs(III) to TMAs(III)] would occur slowly. Consistent with this prediction, dimethylated arsenic is the major species of purified CmArsM, with TMAs(III) produced only after long times.[1]

Fluorescence Properties of the Single-Tryptophan Derivative Y70W. Tryptophan fluorescence has proven to be of value in examining arsenic binding in arsenic detoxification proteins.[15−20] CmArsM has a single tryptophan at position 336, but the fluorescence of the wild-type protein does not respond to ligand binding (data not shown); therefore, Trp336 was changed to a tyrosine to create a tryptophan-free protein. Preliminary analysis of X-ray crystallographic data suggested that Tyr70, which is near conserved residue Cys72, is on the surface of the protein. For those reasons, in the tryptophan-free background, a single tryptophan was introduced by substitution of Tyr70 (Figure 1 of the Supporting Information), resulting in a Y70W derivative with wild-type catalytic activity (vide supra).

The emission maximum of a tryptophan residue reflects the polarity of its environment.[21] The observed emission maximum (λ_{max}) for ArsM Y70W of 345 nm indicates that the tryptophan residue is in a less solvent-exposed environment than free tryptophan (λ_{max} = 355 nm) (Figure 3). When ArsM was denatured with 6 M guanidine hydrochloride, the emission maximum red-shifted to 354 nm, indicating that the residue became more exposed to solvent. The intensity of the emission spectrum of CmArsM dramatically decreased when the temperature was increased from 30 to 60 °C (data not shown), probably because the quantum yield of tryptophan decreases with an increase in temperature.[22] The environment of Y70W was examined by the accessibility to the collisional fluorescence quencher iodide, which quenches the fluorescence of tryptophan residues lying on or near the surface of a protein.[12] Y70W fluorescence was quenched by iodide, indicating that Trp70 may be close to the CmArsM surface. Stern−Volmer plots were obtained for iodide quenching in the presence and absence of substrates (Figure 4). The K_{sv} value for ArsM was determined to be 2.67 in the absence of ligands at 23 °C. In the presence of As(III), the value of the Stern−Volmer constant was 1.4, and in the presence of SAM, it was 1.36.

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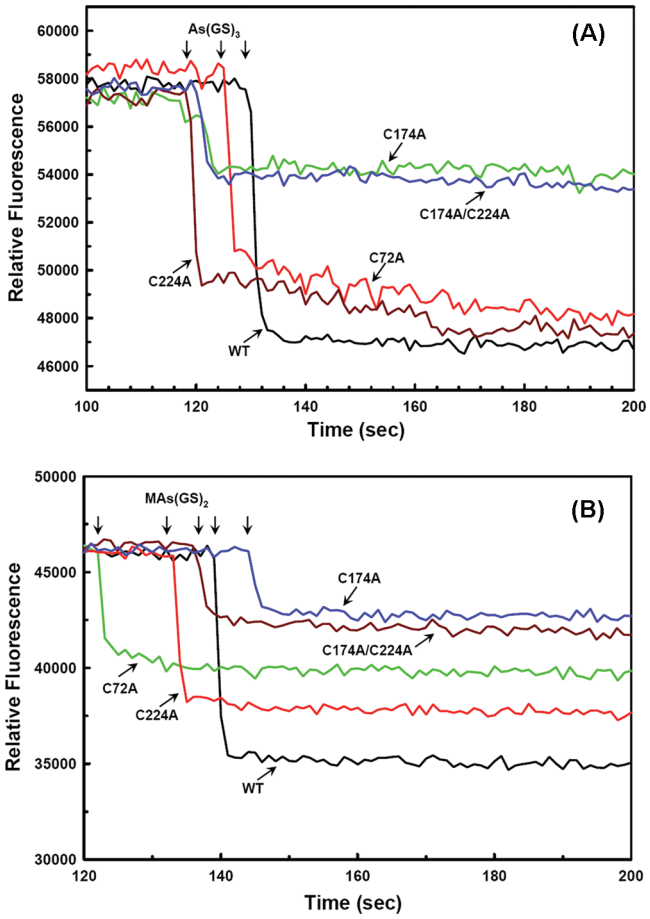

Figure 6. Effect of $As(GS)_3$ and $MAs(GS)_2$ on the fluorescence of Y70W and cysteine mutants. Protein fluorescence was assayed at 23 °C with excitation and emission wavelengths of 295 and 345 nm, respectively, as described in Materials and Methods. Each assay contained 2 μM Y70W or mutant enzyme in the Y70W background. $As(GS)_3$ (A) or $MAs(GS)_2$ (B) was added at a concentration of 25 μM.

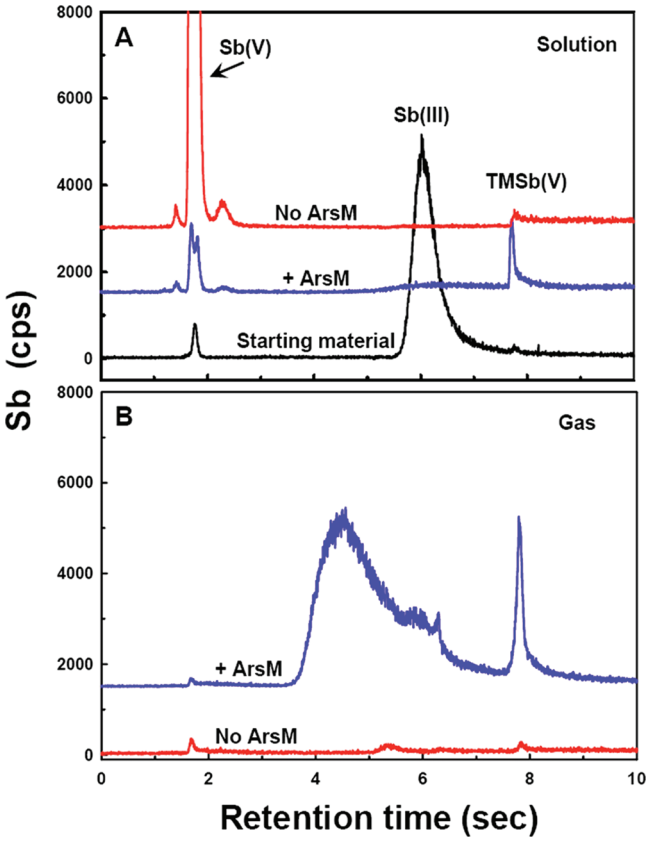

Figure 7. Antimony methylation and volatilization by CmArsM. Methylation of Sb(III) was assayed at 37 °C as described in Materials and Methods. Soluble (A) and volatile (B) products were analyzed by ion exchange HPLC–ICP-MS.

These results indicate that the tryptophan moves into a less solvent-exposed environment when either As(III) or SAM is bound. At 60 °C, the temperature optimum of CmArsM, the Stern–Volmer plots had a lower slope than at 23 °C and appeared to be biphasic (K_{sv1} of 2.5 and K_{sv2} of 0.89). The lower slope, again, reflects the decrease in the quantum yield and lifetime of tryptophan fluorescence at higher temperatures,[22] but it is not clear why it is biphasic.

CmArsR Y70W Exhibits Metalloid Binding. The effect of arsenicals on the time course of fluorescence quenching of purified Y70W was examined (Figure 5A). The pentavalent arsenicals inorganic As(V), MAs(V), and DMAs(V) had no effect. Addition of the trivalent arsenicals As(III) and MAs(III) decreased the magnitude of the fluorescence signal. MAs(III) quenching was faster than that of As(III). The effect of DMAs(III) could not be examined because of its rapid oxidation. Sb(III) quenched Y70W even faster than arsenicals (Figure 5B). Fluorescence spectra were recorded with addition of varying concentrations of arsenite. A maximum of 40% fluorescence quenching was observed with the highest concentration added (25 μM). Scatchard analysis yielded K_d values of 0.4 ± 0.04 μM for As(III), 0.31 ± 0.2 μM for $As(GS)_3$, and 0.34 ± 0.04 μM for MAs(III) (Figure 5C), indicating that ArsM binds the three with similar affinity. The *x*-intercept was nearly the same for each, indicating similar binding stoichiometry.

In vivo, As(III) is nearly entirely complexed with glutathione as the $As(GS)_3$ complex.[23] We have proposed that $As(GS)_3$ is the preferred substrate for the ArsD metallochaperone, which binds arsenite several orders of magnitude faster when presented as $As(GS)_3$.[20] It seemed reasonable therefore to examine the effect of the glutathione complexes of inorganic and methylated metalloids on Y70W fluorescence. The rate of quenching following addition of either $As(GS)_3$ or $MAs(GS)_2$ was considerably faster than that of the free forms (Figure 5A). Preincubation of As(III) with the nonphysiological monothiol 2-mercaptoethanol produced a similar result. In contrast, the strong non-thiol reductant tris(2-carboxyethyl)phosphine had no effect on quenching by As(III), indicating that the increase in the rate of quenching by GSH is not simply due to the need for a reductant (Figure 5D). GSH alone had no effect on Y70W fluorescence, and addition of As(III) and GSH together without preincubation produced a rate of quenching similar to that of As(III) alone (data not shown). $Sb(GS)_3$ also bound rapidly (Figure 5B). However, the rates of quenching with the glutathionylated metalloid complexes were too fast to accurately determine, so it was not possible to compare the rates of the glutathionylated species with each other or with that of free Sb(III). Stopped-flow fluorescence experiments will be required to resolve these questions. Although the absolute rates could not be determined, the fluorescence data suggest that the rate of binding of As(III) from $As(GS)_3$ or $MAs(GS)_2$ is faster than the rate from $As(OH)_3$ or $MAs(OH)_2$ (with the reasonable assumption that

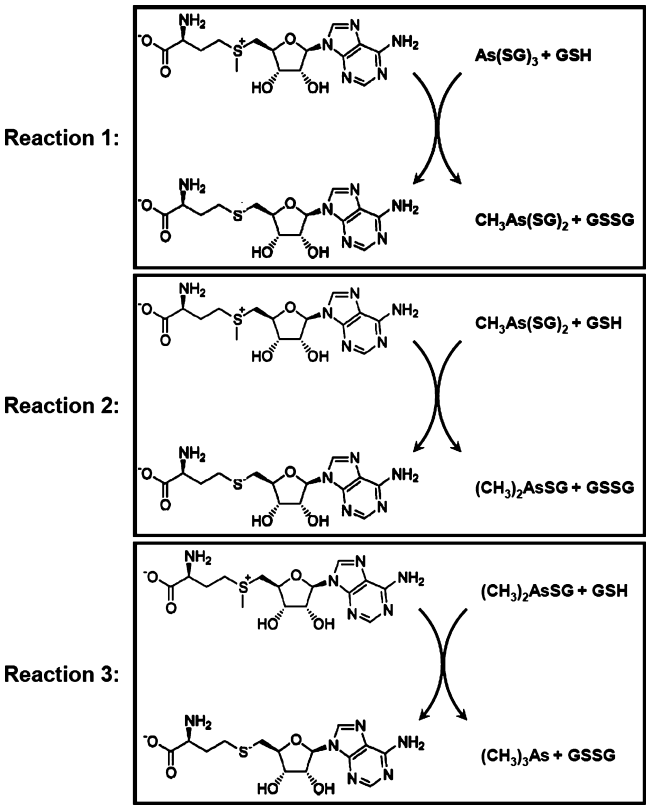

Figure 8. Proposed pathway of methylation by As(III) *S*-adenosylmethionine methyltransferases. As(III) SAM methyltransferases conduct three successive rounds of oxidative methylation, with the glutathione conjugates serving as metalloid donors to the enzyme. In each round, there is metalloid methylation, GSH oxidation, and SAM reduction. This is similar to the pathway proposed by Hayakawa[8] but differs from the proposed pathway of Challenger,[5] which involves oxidation and reduction of a metalloid during each round.

Trp70 fluorescence quenching is proportional to binding). As nearly all metalloid would be glutathionylated in the reducing environment of the cytosol, where GSH levels are millimolar, it is likely that these are the forms that the enzyme recognizes in vivo.

Effect of Substitution of Conserved Residues Cys72, Cys174, and Cys224 on As(III) Binding by Y70W ArsM. The C72A, C174A, C224A, and C174A/C224A substitutions were individually introduced into the Y70W background. Addition of $As(GS)_3$ to the C72A or C224A mutant resulted in fluorescence quenching similar to that of the Y70W protein (Figure 6A). C174A and the C174A/C224A double mutant exhibited less quenching than the other ArsMs, although the significance of this reduction is not clear. Similar results were obtained with $MAs(GS)_2$ (Figure 6B). These results suggest that the mutants still bind As(III) but that binding is not sufficient for methylation (Figure 2). Because addition of As(III) to the C174A mutant produces less quenching, Cys174 may have a role in binding different from the role of the other two. One possibility is that all three cysteines are required for catalysis, but Cys174 and either Cys72 or Cys224 are sufficient for As(III) binding.

Methylation of Antimony. As shown above, fluorescence quenching with inorganic Sb(III) was even faster than that with either MAs(III) or As(III) (Figure 5B). CmArsM was also found to methylate trivalent antimony (Figure 7). In the absence of enzyme, oxidation of Sb(III) to Sb(V) was observed. CmArsM converted Sb(III) to a number of products, one of which eluted at the same position as TMSb(V) (Figure 7A). Most of the inorganic Sb(III) disappeared from solution. Some remained bound to the enzyme (data not shown), and some was volatilized (Figure 7B). Although it was difficult to quantify the amount of antimony volatilized, a rough estimate is that it was less than 10% of the initial amount of Sb(III). In this assay, the gas was trapped on filters impregnated with H_2O_2 to oxidize the products to soluble species. Some of the gas was converted to a species that eluted from an ion exchange HPLC column at the same position as TMSb(V), but the majority was present in an unidentified peak. This is the first demonstration of enzymatic methylation of antimony.

DISCUSSION

Arsenic methylation is a widespread biotransformation process and is catalyzed by the enzyme As(III) *S*-adenosylmethyltransferase. In microorganisms, this enzyme, termed ArsM, clearly catalyzes detoxification of this toxic metalloid.[1,2] In human liver, arsenic methylation by the orthologous AS3MT has been proposed to transform inorganic arsenic into more carcinogenic species.[3] The enzyme methylates trivalent As(III) up to three times, producing mono-, di-, and trimethylated species. Neither the pathway nor the products of the enzymatic reaction are certain. The original postulate by Challenger[5] was that As(III) undergoes a series of oxidative methylations, with MAs(V), DMAs(V), and TMAs(V)O as the immediate products. These would then be reduced back to the trivalent form, which became the substrate of the subsequent methylation reaction. A simpler pathway in which arsenic remained trivalent throughout was proposed by Hayakawa et al.[8], with the products being MAs(III), DMAs(III), and TMAs(III). The substrates would be the glutathionylated forms of trivalent arsenic, $As(GS)_3$, $MAs(GS)_2$, and DMAs(GS), with the pentavalent species forming postreaction by oxidation. The methyl group of SAM would be transferred to trivalent arsenic. The glutathione moiety was proposed to be transferred to SAM, but this species presumably would react with another molecule of GSH to form GSSG and SAH.

To clarify the mechanism of an enzyme that may contribute to an increased incidence of cancer, we characterized a eukaryotic thermophilic orthologue of human AS3MT. The results are instructive and relevant to the mechanism of the human enzyme. Here we show that both $As(GS)_3$ and $MAs(GS)_2$ bind to ArsM faster than either $As(OH)_3$ or $MAs(OH)_2$, consistent with the glutathionylated forms being the preferred substrates of the enzyme. We propose a modified version of this pathway in which SAM is reduced to SAH, GSH is oxidized to GSSG, and arsenic is methylated in three steps (Figure 8). Thus, the overall reaction is an oxidative methylation, but the arsenic remains trivalent in each step. However, the arsenic atom in $As(GS)_3$ has three sulfur coordinations versus two for $MAs(GS)_2$ versus only a single arsenic−thiol bond in DMAsGS. How does ArsM conduct three reactions with three different substrates that have different chemical properties? ArsM has three highly conserved cysteine residues, Cys72, Cys174, and Cys224. Although results of mutagenesis should be interpreted carefully because amino acid substitutions can affect protein conformation, they are consistent with each of the three cysteine residues having a role in catalysis and being required to form a three-coordinate sulfur site for As(III) binding and methylation. Moreover, there is a clear difference between Cys72 and the other two. Cys72 is not required for binding or methylation of MAs(III), which we propose forms a two-coordinate site with Cys174 and Cys224.

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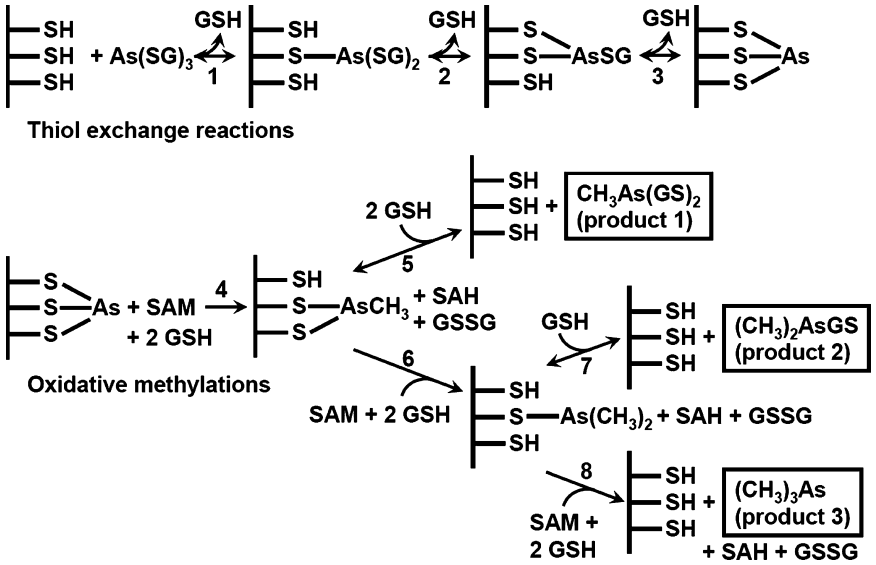

Figure 9. Proposed reaction scheme of As(III) *S*-adenosylmethionine methyltransferases. The overall scheme involves two types of reactions, thiol exchange reactions (top) and oxidative methylations (bottom). The reducing cytosol of nearly every cell contains millimolar concentrations of small molecule thiols, most frequently GSH. Even though trivalent arsenicals do not bind monothiols with high affinity, the high intracellular concentration of GSH would shift the equilibrium in favor of complexes $As(GS)_3$, $MAs(GS)_2$, and DMAsGS, the arsenic donors of the three successive reactions shown in Figure 8. Illustrated by $As(GS)_3$, As(III) is bound at the active site of the enzyme through three successive reversible exchanges between the thiols of glutathione and the thiolates of the three conserved cysteine residues, Cys72, Cys174, and Cys224, in CmArsM (reactions 1−3). While the order of binding is not known, Cys174 and Cys224 are required for binding of both As(III) and MAs(III), so we would propose that Cys72 is the last residue to participate in As(III) binding and is not involved in binding of MAs(III) or DMAs(III). After a metalloid is bound, a methyl group is transferred from SAM, which becomes reduced to SAH (reaction 4). The electrons for reduction of SAM are provided by two GSH molecules, which become oxidized to GSSG. The enzyme-bound form of the methylated trivalent arsenic can dissociate from the enzyme in the reverse of the initial thiol exchange (reaction 5), producing $MAs(GS)_2$, the first product. Alternatively, it can remain bound in the active site and undergo a second round of methylation (reaction 6). Exchange with GSH and dissociation (reaction 7) produce the second product, DMAs(GS). Alternatively, DMAs(III) can remain bound to the active site and undergo a third round of methylation (reaction 8), producing the final product, TMAs(III) gas. Exposure to oxygen results in nonenzymatic oxidation to the frequently observed pentavalent species. Reactions 4 and 6 are favored over reaction 5 or the reversal of reaction 3 because both As(III) and MAs(III) bind with higher affinity to the thiol pairs of the enzyme than to the monothiol GSH. In contrast, DMAs(III) can bind only to monothiols with low affinity, and the high concentration of GSH thiols relative to the enzyme cysteine thiols favors reaction 7 over reaction 8. These considerations explain why human urine most commonly contains 60−80% DMAs(V) and only 10−30% MAs(V)[6] and why so little TMAs(V)O or TMAs(III) gas is produced.

We postulate that arsenic from $As(GS)_3$ is transferred to the three cysteine thiolates in a series of three reversible thiol transfer reactions, forming the $E(S)_3As$ complex and three GSH molecules (Figure 9, reactions 1−3). The methyl group from SAM is transferred to the arsenic atom, displacing one of the three cysteine residues (which we predict is Cys72); SAM is reduced to SAH, and GSH is oxidized to GSSG (Figure 9, reaction 4). The methylated arsenic either undergoes a second round of methylation (Figure 9, reaction 5) or dissociates as $MAs(GS)_2$ in the reverse thiol exchange reactions with GSH (Figure 9, reaction 6). In liver, most of the arsenic is found in the form of the dimethylated species which suggests that the monomethylated product undergoes a second round of methylation faster than it dissociates from the enzyme, while the third methylation step is slower than the other two. From the results of fluorescence quenching with the Y70W mutant, MAs(III) appears to bind to CmArsM with a slightly higher affinity than As(III). MAs(III), the product of the first methylation step, is more likely to remain in the active site than it is to dissociate, while DMAs(III), which can bind to only a single cysteine residue, is more likely to dissociate (Figure 9, reaction 7) than to undergo the final round of methylation to TMAs(III) (Figure 9, reaction 8), resulting in a prevalence of DMAs(III) production, which spontaneously oxidizes to DMAs(V), the main urinary form of arsenic.[6,7]

Identification of TMAs(III) formation as the rate-limiting step in the reaction cycle has several implications. First, if production of DMAs(III) increases the carcinogenicity of arsenic,[3,4] then decreasing the barrier to TMAs(III) formation might reduce its carcinogenic potential. Second, transgenic rice expressing an *arsM* gene has been constructed with the goal of limiting arsenic in the food supply.[24] Increasing production of TMAs(III) gas, which is nontoxic,[25] might improve this process. How can this be accomplished? If the rate-limiting step results from dissociation of DMAs(III) from the enzyme (Figure 9, reaction 7) that is faster than its methylation to TMAs(III) (Figure 9, reaction 8), then altering the binding site in ArsM to increase its affinity for DMAs(III) might have the desired consequences. This requires knowledge of the structure of the binding site. CmArsM has been crystallized,[14] and determination of its structure is imperative.

■ ASSOCIATED CONTENT

Supporting Information

Multiple-sequence alignment of ArsM orthologues (Figure 1) and a list of mutagenic oligonucleotides (Table 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; GSH, reduced glutathione; GSSG, oxidized glutathione; MAs(III), methylarsenite; MAs(V), methylarsenate; DMAs(III), dimethylarsenite; DMAs(V), dimethylarsenate; TMAs(III), trimethylarsine; TMAs(V)O, trimethylarsine oxide; EDTA, ethylenediaminetetraacetate.

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