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High-throughput screening-compatible assays of As(III) *S*-adenosylmethionine methyltransferase activity

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ABSTRACT

Arsenic is a naturally existing toxin and carcinogen. As(III) *S*-adenosylmethionine methyltransferases (AS3MT in mammals and ArsM in microbes) methylate As(III) three times in consecutive steps and play a central role in arsenic metabolism from bacteria to humans. Current assays for arsenic methylation are slow, laborious, and expensive. Here we report the development of two in vitro assays for AS3MT activity that are rapid, sensitive, convenient, and relatively inexpensive and can be adapted for high-throughput assays. The first assay measures As(III) binding by the quenching of the protein fluorescence of a single-tryptophan derivative of an AS3MT ortholog. The second assay utilizes time-resolved fluorescence resonance energy transfer to directly measure the conversion of the AS3MT substrate, *S*-adenosylmethionine, to *S*-adenosylhomocysteine catalyzed by AS3MT. These two assays are complementary, one measuring substrate binding and the other catalysis, making them useful tools for functional studies and future development of drugs to prevent arsenic-related diseases.

Arsenic is considered the most ubiquitous environmental toxin and carcinogen, and, consequently, the U.S. Environmental Protection Agency and Agency for Toxic Substances and Disease Registry rank arsenic at the top of the U.S. Priority List of Hazardous Substances (http://www.atsdr.cdc.gov/SPL/index.html). In humans exposure is a major contributor to arsenic-related diseases [1,2], including bladder, lung, and skin cancers [3]. The primary sources of dietary arsenic are food [4] and drinking water [5]. As a consequence of the environmental pervasiveness of arsenic, detoxifying systems are found in nearly every organism, from bacteria to humans [6]. A common means of microbial arsenic detoxification is by methylation catalyzed by As(III) *S*-adenosylmethionine (SAM)[1] methyltransferases, enzymes termed ArsM [7,8]. The enzyme was originally identified in mammals, in which it was called Cyt19 or AS3MT [9]. Methylation by AS3MT was originally considered a detoxification mechanism but is now thought to transform inorganic arsenic (As(III)) into the more toxic and carcinogenic trivalent methylated species methylarsenite (MAs(III)) and dimethylarsenite (DMAs(III)) [10,11].

Identification of inhibitors or activators of AS3MT will be useful for the development of drugs to prevent arsenic-related diseases. To date there is no convenient, rapid assay for AS3MT activity that can be adapted for high-throughput assays for drug development. Current assays of As(III) methyltransferase activity are slow and laborious, making them unsuited to high-throughput screening for inhibitors. Postreaction assays of AS3MT activity utilize radioactive substrates [12,13] or expensive instrumentation such as high-performance liquid chromatography (HPLC) coupled with hydride generation–atomic absorption spectroscopy [14], electrospray ionization tandem mass spectrometry detection, or inductively coupled plasma mass spectroscopy (ICP–MS) [15]. Moreover, these assays use high amounts of enzyme and substrates with long reaction times and are difficult to apply to detailed enzymological studies.

We have developed two new assays for analysis of AS3MT activity that are better, faster, and cheaper than current assays. Both assays utilize small amounts of enzyme and short reaction times. The first assay employs As(III)-induced fluorescence quenching of an engineered single-tryptophan derivative of an AS3MT ortholog from the eukaryotic alga *Chlamydomonas reinhardtii* termed either

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[1] *Abbreviations used:* SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; sinefungin, 5′-deoxy-5′-(1,4-diamino-4-carboxybutyl)adenosine; AS3MT, As(III) SAM methyltransferase; TR–FRET, time-resolved fluorescence resonance energy transfer; As(III), arsenite; MAs(III), methylarsenite; DMAs(III), dimethylarsenite; Rox(III), reduced roxarsone (3-nitro-4-hydroxyphenylarsonic acid); Nit(III), reduced nitarsone (*p*-nitrophenylarsonic acid); pASA(III), reduced *p*-arsanilic acid; PhAs(III), phenylarsenite or phenylarsine oxide; NEM, *N*-ethylmaleimide; MTS, methanethiosulfonate; MMTS, methylmethanethiosulfonate; MTSEA, ethylaminomethanethiosulfonate; MTSET, ethyl(trimethylammonium)methanethiosulfonate; MTSES, 2-sulfonatoethyl methanethiosulfonate; TCEP, tris(2-carboxyethyl)phosphine.

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CrArM or CrAS3MT. This real-time assay for substrate binding is based on the effect of As(III) on a single-tryptophan derivative of a thermophilic ortholog [16], but the current assay has the advantage that it works at room temperature in microtiter plates. The second assay utilizes time-resolved fluorescence resonance energy transfer (TR–FRET) between a Tb^{3+}-labeled cryptate coupled to an *S*-adenosylhomocysteine (SAH) antibody and a fluorescently labeled SAH to detect formation of the SAH product of the AS3MT reaction. This is the first rapid assay for detection and quantification of SAH generation within the first few minutes of reaction. These assays are complementary and provide the basis for future high-throughput screens for inhibitors and activators of AS3MT.

Materials and methods

Reagents

SAM was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). MTS reagents were purchased from Biotium, Inc. (Hayward, CA, USA). Other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). A stock solution of tris(2-carboxyethyl)phosphine (TCEP) was prepared at 0.5 M and adjusted to pH 7.0. MAs(V), 4-aminobenzenearsonic acid (*p*-arsanilic acid or pASA(V)), 4-hydroxy-3-nitrobenzenearsonic (roxarsone or Rox(V)), and (4-nitrophenyl)arsonic acid (nitarsone or Nit(V)) were reduced to trivalent MAs(III), pASA(III), Rox(III), and Nit(III) and adjusted to pH 6.5, as described [17]. The products of reduction were analyzed by HPLC coupled to ICP–MS, as described below. The products were simultaneously analyzed for both arsenic and sulfur, and no sulfur eluted with the arsenic, demonstrating that the reduction did not produce thioarsenicals.

DNA manipulation and mutagenesis

Wild-type CrAS3MT [18] was used as the starting point for construction of a single-tryptophan derivative. First, the codon for Trp332 was changed to a tyrosine codon by site-directed mutagenesis using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA). The forward and reverse primers were 5′-GGTGGGCGAGTCC TATCTGGCACCCCACTT-3′ and 5′-AAGTGGGGTGCCAGATAGGACTC GCCCACC-3′, respectively. A single-tryptophan derivative, Y72W, was constructed by site-directed mutagenesis of the W332Y CrAS3MT in which the codon for Tyr72 was changed to a tryptophan codon using primers 5′-CACAGAGGTGAAGGAGAAGTTCTGG GGCTGCGGAAAC-3′ and 5′-GTTTCCGCAGCCCCAGAACTTCTCCTTCA CCTCTGTG-3′. The Y72W/W332Y CrAS3MT construct has a single tryptophan residue near the As(III) binding site to serve as a fluorescence reporter of As(III) binding.

Purification of AS3MT enzymes

AS3MT enzymes with a C-terminal histidine tag were purified by Ni-NTA chromatography from cells of *Escherichia coli,* as described previously [19,20].

Assays of fluorescence quenching

Protein fluorescence measurements were assayed in 384-well microtiter plates with a total volume of 15 μl of a buffer consisting of 50 mM MOPS–KOH, pH 7.5, containing 0.15 M KCl and 5 μM Y72W CrAS3MT. Substrates were added to the assay buffer to initiate the reaction. Where indicated, purified CrAS3MT was preincubated with the thiol-modifying reagent methylmethanethiosulfonate (MMTS) for 15 min before the reaction with As(III) was initiated. Protein fluorescence was determined with a Synergy H4 Hybrid Multi-Mode microplate reader (BioTek Instruments, Winooski, VT, USA) with excitation and emission wavelengths set at 295 and 345 nm, respectively.

TR–FRET assay of AS3MT activity

AS3MT activity was assayed with an EPIgeneous Methyltransferase Assay kit (Cisbio Bioassays, Bedford, MA, USA) by measuring the conversion of SAM to SAH according to the manufacturer's directions. The reaction has two steps: (1) the enzymatic reaction, which converts SAM to SAH, and (2) the detection step that quantifies SAH production. An anti-SAH antibody was labeled with a terbium cryptate, which fluoresces at 620 nm when excited at 337 nm. When the antibody binds a proprietary SAH-d2-labeled tracer, there is energy transfer with emission at 665 nm. The SAH released by the enzymatic reaction competes with labeled SAH-d2, leading to a decrease in the homogeneous time-resolved fluorescence (HTRF) signal. The two steps were carried out sequentially in the same well of a low-volume 384-well microtiter plate, with a total volume of 20 μl (10 μl for the enzymatic step and 10 μl for the detection step) in a buffer consisting of 50 mM MOPS, pH 7.5, containing 0.15 M KCl, 10 μM SAM, 10 μM As(III), 20 μM TCEP, and inhibitors, as indicated. Purified CrAS3MT or hAS3MT at 1 μM, final concentration, was added to initiate the reaction. The reactions were carried out at either room temperature or 37 °C, as indicated, for 5 min in most assays or, when noted, as a time course. The reaction was terminated by addition of the proprietary detection reagent, followed by SAH-d2 and anti-SAH-Lumi4-Tb reagents. The plates were incubated for 1 h, and fluorescence was measured at both 665 and 620 nm with excitation at 337 nm in a Synergy H4 Hybrid Multi-Mode microplate reader. The HTRF was calculated from the ratio of emission at 665 and 620 nm. The concentration of SAH was calculated from a calibration curve of the HTRF with known concentrations of SAH.

The performance parameters, including the signal-to-background ratio, signal-to-noise ratio, and coefficient of variation, were determined at both 665 and 620 nm (Table 1). The robustness of the assay was evaluated from the processed coefficient of variation and the Z′ factor, a dimensionless statistical value of the quality of the assay, both of which were calculated from the 665 nm/620 nm HTRF ratio. The values for the statistical parameters meet the requirements for high-throughput screening assays [21].

HPLC–ICP–MS assay of AS3MT activity

Methylation of As(III) was assayed in a buffer consisting of 50 mM MOPS, pH 7.5, containing 0.15 M KCl, 0.5 mM SAM, 10 μM As(III), 1 mM TCEP [20]. Inhibitors were added as indicated. The reaction was initiated by addition of purified CrAS3MT, 1 μM

Table 1
Performance and robustness parameters.

	Performance parameters			Robustness parameters	
	Signal to background	Signal to noise	CV at each wavelength	CV from HTRF	Z′ factor
Wavelength					
620 nm	76.3 ± 12.4	23.8	0.06		
655 nm	18.8 ± 3.8	19.0	0.11		
No enzyme				0.100	
+CrAS3MT				0.033	0.78

The signal-to-background ratio, signal-to-noise ratio, and first coefficient of variation (CV) were measured and calculated at both wavelengths. The second CV and the Z′ factor were calculated from the 665 nm/620 nm HTRF ratio.