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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · APRIL 2004

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Endogenous Reductants Support the Catalytic Function of Recombinant Rat Cyt19, an Arsenic Methyltransferase

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Received October 23, 2003

The postulated scheme for the metabolism of inorganic As involves alternating steps of oxidative methylation and of reduction of As from the pentavalent to the trivalent oxidation state, producing methylated compounds containing As^{III} that are highly reactive and toxic. *S*-Adenosyl-L-methionine:As^{III} methyltransferase purified from rat liver catalyzes production of methyl and dimethyl arsenicals from inorganic As. This protein is encoded by the *cyt19* gene orthologous with *cyt19* genes in mouse and human. The reductants dithiothreitol or tris(2-carboxylethyl)-phosphine support catalysis by recombinant rat cyt19 (rrcyt19). Coupled systems containing an endogenous reductant (thioredoxin/thioredoxin reductase/NADPH, glutaredoxin/glutathione/glutathione reductase/NADPH, or lipoic acid/thioredoxin reductase/NADPH) support inorganic As methylation by rrcyt19. Although glutathione alone does not support rrcyt19's catalytic function, its addition to reaction mixtures containing other reductants increases the rate of As methylation. Aurothioglucose, an inhibitor of thioredoxin reductase, reduces the rate of As methylation by rrcyt19 in thioredoxin-supported reactions. Addition of guinea pig liver cytosol, a poor source of endogenous As methyltransferase activity, to reaction mixtures containing rrcyt19 shows that endogenous reductants in cytosol support the enzyme's activity. Methylated compounds containing either As^{III} or As^{V} are detected in reaction mixtures containing rrcyt19, suggesting that cycling of As between oxidation states is a component of the pathway producing methylated arsenicals. This enzyme may use endogenous reductants to reduce pentavalent arsenicals to trivalency as a prerequisite for utilization as substrates for methylation reactions. Thus, cyt19 appears to possess both As^{III} methyltransferase and As^{V} reductase activities.

Introduction

In humans and many other species, MAs[1] and DMAs are the predominant products of enzymatically catalyzed methylation of iAs. Hence, humans ingesting iAs in food or water excrete iAs, MAs, and DMAs in urine. Cullen and associates (*1*) summarized the pathway for the formation of methylated arsenicals from iAs as

$$As^{V}O_4^{3-} + 2e \rightarrow As^{III}O_3^{3-} + CH_3^{+} \rightarrow CH_3As^{V}O_3^{2-} + 2e \rightarrow CH_3As^{III}O_2^{2-} + CH_3^{+} \rightarrow (CH_3)_2As^{V}O_2^{-} + 2e \rightarrow (CH_3)_2As^{III}O^{-}$$

Here, the oxidative methylation of trivalent arsenicals alternates with reductive steps that convert pentavalent arsenicals to trivalency. Unique As methyltransferases have been purified from rabbit (*2*) and rat (*3*) liver. Because the amino acid sequence of the enzyme purified from rat liver is similar to the predicted translations of the *cyt19* genes encoding methyltransferases of unknown function in the mouse and human genomes, rat liver As methyltransferase is designated as cyt19. Rabbit liver As methyltransferase and cyt19 use AdoMet as the methyl group donor, prefer substrates containing As^{III}, and require the presence of a reductant for activity. Oxidation of mono- and dithiols has been linked to the reduction of As^{V} in inorganic and organic arsenicals (*1*, *4*−*6*). Both prokaryotic and eukaryotic As^{V} reductases have been characterized. A 72 kDa protein purified from human liver functions as an As^{V} reductase (*7*). PNP purified from rat and human liver (*8*−*10*) also catalyzes iAs^{V} reduction, although its function as an iAs^{V} reductase in cellular environments is uncertain (*11*). MAs^{V} reductases purified

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[1] Abbreviations: MAs, methyl arsenic; DMAs, dimethyl arsenic; iAs, inorganic arsenic; AdoMet, *S*-adenosyl-L-methionine; PNP, purine nucleoside phosphorylase; DHLA, dihydrolipoic acid; Trx, thioredoxin; TR, thioredoxin reductase; Grx, glutaredoxin; GR, glutathione reductase; DTT, dithiothreitol; LA, lipoic acid; TCEP, tris(2-carboxyethyl)-phosphine; ATG, aurothioglucose.

10.1021/tx0342161 CCC: $27.50
Published on Web 02/14/2004

from rabbit and human liver share sequence similarities with glutathione-S-transferase Ω (*12*, *13*). In in vitro assays, PNP requires a purine nucleoside and DHLA to reduce iAs^V (*10*). GSH is required for purified MAs^V reductase to reduce MAs^V (*10*, *12*). By comparison, bacterial and yeast iAs^V reductases use coupled reaction systems involving Trx/TR/NADPH or Grx/GSH/GSH reductase (GR)/NADPH to provide reducing equivalents (*14*).

Here, we examined the capacity of rat recombinant cyt19 (rrcyt19) to catalyze the formation of MAs and DMAs from iAs^{III}. Because cyt19 converts iAs^{III} to DMAs by way of a MAs intermediate in the absence of other enzymes (i.e., MAs^V reductase), we hypothesized that it catalyzes not only the oxidative methylation of As but also the reduction of MAs^V to MAs^{III}. We have examined the effects of exogenous and endogenous reductants on the catalytic function of cyt19. Both DTT and TCEP, a nonthiol reductant, are effective reductants. A Trx/TR/NADPH-coupled system also supports the methylation of iAs^{III} by rrcyt19. Addition of ATG, an inhibitor of TR (*15*), to reaction mixtures containing the Trx/TR/NADPH-coupled system decreases iAs^{III} methylation. The addition of GSH alone to reaction mixtures with rrcyt19 results in low rates of iAs^{III} methylation; however, the addition of GSH to reaction mixtures containing other reductants sharply stimulates the rate of methylation. Furthermore, the presence of GSH is an absolute requirement for activity in reaction mixtures containing either a DHLA/TR/NADPH-coupled system or a Grx1/GR/NADPH-coupled system. In reaction mixtures containing rrcyt19 and guinea pig liver cytosol, addition of ATG reduces, but does not eliminate, the methylation of iAs^{III}, suggesting that multiple endogenous reductants support cyt19's catalytic function in the cellular environment.

Experimental Procedures

Caution: *iAs is classified as a human carcinogen (16). Toxicities of methylated arsenicals have not been fully evaluated. All arsenicals should be handled as potentially highly toxic compounds.*

Arsenicals. [^{73}As]Arsenic (iAs^V) acid (estimated specific activity of 13.3 Ci/mg As) was obtained from Los Alamos Meson Production Facility (Los Alamos, NM). [^{73}As]arsenous acid (iAs^{III}) was produced from radiolabeled arsenic acid by reduction with metabisulfite−thiosulfate reagent (*17*). Arsenous acid, sodium salt (Sigma, St. Louis, MO), was added to [^{73}As]arsenous acid to produce the desired iAs^{III} concentration. Methylarsonic (As^V) acid was obtained from Ventron (Danvers, MA), and dimethylarsinic (As^V) acid was obtained from Strem (Newburyport, MA). Methyloxoarsine (As^{III}) and iododimethylarsine (As^{III}) were generously provided by Professor William R. Cullen.

Cloning of rcyt19 and Purification of Rat Recombinant cyt19. The cloning of rat cyt19 and the expression and purification of recombinant cyt19 have been previously described (*18*). For present studies, recombinant protein was expressed in 300 mL cultures incubated at 22 °C overnight. Following washing of the charged Pro-bond Ni-NTA resin column with 50 mL of 50 mM Na phosphate buffer, pH 7.4, containing 50 mM NaCl and 10 mM imidazole, rrcyt19 was eluted from the column with 50 mM Na phosphate buffer, pH 7.4, containing 50 mM NaCl and 500 mM imidazole. Purified recombinant protein was dialyzed against 50 mM Na phosphate buffer, pH 7.4, containing 50 mM NaCl or 100 mM tris, pH 7.4, containing 50 mM NaCl and was stored at −20 °C before use. Protein concentrations were determined with a BCA kit (Sigma) with bovine serum albumin as a standard.

Methylation of Inorganic As^{III}. The rrcyt19-catalyzed conversion of iAs^{III} to methylated arsenicals was typically monitored by measuring conversion of [^{73}As]-labeled arsenite to methylated products. Reaction mixtures were usually prepared in a 100 mM tris and a 100 mM phosphate, pH 7.4, buffer. DTT, GSH, baker's yeast GR, DHLA, LA, *Escherichia coli* Trx, NADPH, and ATG were obtained from Sigma. TCEP was obtained from Pierce (Rockport, IL). TR was either purified from livers of adult male Fischer 344 rats by a previously described procedure (*19*) or obtained as recombinant rat TR (American Diagnostica, Greenwich, CT). Recombinant *E. coli* Grx1 was a gift from Professor Barry P. Rosen. After incubation at 37 °C, all arsenicals in reaction mixtures were oxidized by treatment with H_2O_2 (final concentration of 10%) for 2 h at room temperature and were separated by TLC in an acetone:water:acetic acid (3:1:1) solvent system (*20*). In some experiments, a portion of a cytosolic fraction prepared from guinea pig liver was added to reaction mixtures. This fraction was prepared by centrifugation at 100 000*g* for 30 min at 4 °C of a 20% (w/v) homogenate of the liver of an adult (∼350 g) male Hartley guinea pig (Hilltop Labs, Scottsdale, PA) in 50 mM phosphate, 250 mM glucose, and 10 mM GSH (pH 7.4). Guinea pig liver cytosol was stored at −80 °C until used. Reaction mixtures containing guinea pig liver cytosol were treated with CuCl to liberate protein-bound arsenicals and then oxidized by treatment with H_2O_2 (final concentration of 10%) for 2 h at room temperature before TLC in an 2-propanol:water:acetic acid (10:2.5:1) or an acetone:water:acetic acid (3:1:1) solvent system (*21*). Radiolabeled arsenicals separated by TLC were quantified using a model FLA-2000 fluorescence image analyzer (Fujifilm, Stamford, CT) and Image Gauge software (version 3.0).

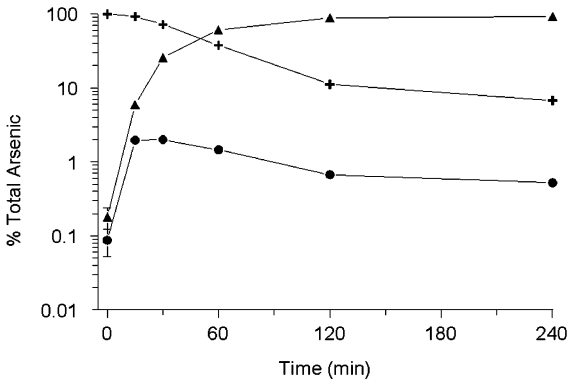

Figure 1. Time course for rrcyt19-catalyzed conversion of arsenite into methylated arsenicals. Reaction mixtures containing 5 μg of rrcyt19, 1 μM [^{73}As]iAs^{III}, 1 mM AdoMet in 100 mM tris/100 mM Na phosphate buffer, pH 7.4, with 1 mM DTT (50 μL final volume) were incubated at 37 °C for up to 2 h. Percentages of total As accounted for by inorganic As (+), methyl As (●), and dimethyl As (▲) shown. Note log scale on ordinate (*y*) axis.

Determination of the Oxidation State of As in Arsenicals. pH selective generation of arsines was used to determine the oxidation state of As in inorganic and methylated arsenicals. This method exploits a pH-dependent difference in the generation of arsines from arsenicals containing As^V or As^{III}. Reduction of arsenicals at pH 6 generates arsines from arsenicals containing As^{III}; reduction at pH 1 generates arsine from arsenicals containing either As^V or As^{III}. Hence, analyses at each pH permit determination of the quantities of all arsenical species containing As^V or As^{III}. Conditions, standards for calibration, and instrumentation for selective generation of arsines and detection by atomic absorption spectrometry have been described (*22*). To minimize oxidation of arsenicals before analysis, an aliquot of each reaction mixture was taken after incubation at 37 °C and immediately submitted for analysis at pH 6. Another aliquot of each reaction mixture was concurrently snap frozen in liquid nitrogen and held at −20°. These samples were quickly thawed in 1 M HCL and submitted for analysis at pH 1.

Results

Characterization of Recombinant Rat cyt19 Activity. In rrcyt19-catalyzed reactions containing 5 μg of

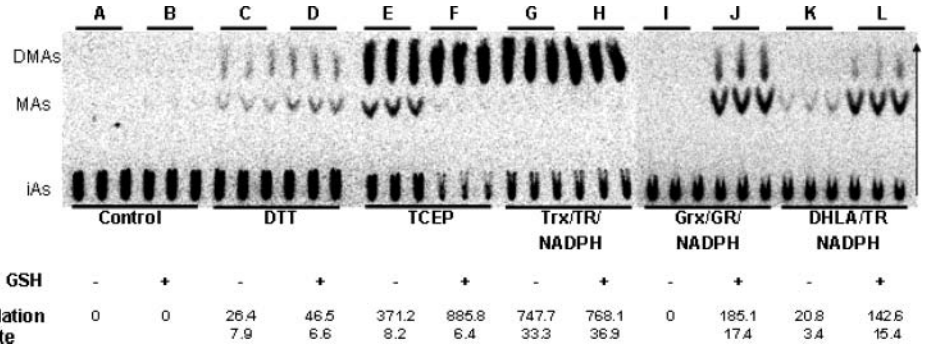

Figure 2. Effects of reductants on rrcyt19-catalyzed conversion of arsenite to methylated arsenicals. Reaction mixtures that contained 5 μg of rrcyt19, 1 mM AdoMet, and 1 μM [^{73}As]iAs^{III} in 100 mM tris/100 mM Na phosphate buffer, pH 7.4, with or without reductant were incubated at 37 °C for 20 min. Group A, no reductants; B, 5 mM GSH; C, 1 mM DTT; D, 1 mM DTT and 5 mM GSH; E, 1 mM TCEP; F, 1 mM TCEP and 5 mM GSH; G, 10 μM *E. coli* Trx, 3 μM rat liver TR, and 300 μM NADPH; H, 10 μM *E. coli* Trx, 3 μM rat liver TR, 300 μM NADPH, and 5 mM GSH; I, 1 μM *E. coli* Grx1, 15 nM yeast GR, 300 μM NADPH; J, 1 μM *E. coli* Grx1, 15 nM yeast GR, 300 μM NADPH, and 5 mM GSH; K, 100 μM DHLA, 3 μM rat liver TR, and 300 μM NADPH; L, 100 μM DHLA, 3 μM rat liver TR, 300 μM NADPH, and 5 mM GSH. Methylation rate calculated as nmols of arsenite methylated per μg of protein per 20 min. Mean (upper) and standard deviation (lower) shown for three assays. Arrow indicates direction of migration in TLC.

rrcyt19, 1 μM [^{73}As]iAs^{III}, 1 mM AdoMet in 100 mM tris/100 mM Na phosphate buffer, pH 7.4, with 1 mM DTT, conversion of iAs^{III} to DMAs was rapid and nearly quantitative; MAs never accounted for more than 2% of the total As in reaction mixtures (Figure 1). AdoMet (0.05−1 mM) was used as the methyl group donor in reaction mixtures containing 1 mM DTT or a TR/Trx/NADPH-coupled reaction system (data not shown). An oxyanion, phosphate, stimulated rrcyt19 activity. Addition of 100 mM phosphate to reaction mixtures increased the yield of DMAs in the presence of either 1 mM DTT or the TR/Trx/NADPH-coupled system but had little effect on MAs production (data not shown). Hence, reaction mixtures typically contained 1 mM AdoMet and 100 mM phosphate.

Reductants and Catalysis by rrcyt19. We examined the effects of two chemical reductants, 1 mM DTT or 1 mM TCEP, on rrcyt19-catalyzed methylation of iAs^{III} (Figure 2). Without a reductant, the enzyme was inactive; in the presence of either reductant, MAs and DMAs were formed. In short-term (20 min) assays, addition of 5 mM GSH alone did not result in formation of methylated arsenicals; however, in the presence of DTT or TCEP, GSH increased the yield of methylated products from 2- to 5-fold.

Three coupled reaction systems consisting of an endogenous reductant, an enzyme that catalyzes reduction of this reductant, and NADPH were examined as sources of reducing equivalents to support rrcyt19's function. We also evaluated the effect of 5 mM GSH on rrcyt19 activity in the presence of these coupled systems (Figure 2). Among endogenous reductants, the Trx/TR/NADPH-coupled system most strongly stimulated rrcyt19's activity. After 20 min of incubation, almost all iAs^{III} was converted to DMAs in Trx/TR/NADPH-containing reaction mixtures. For this reductant, GSH had little effect on reaction yield. The action of the Grx/GR/NADPH-coupled system was absolutely dependent on the presence of 5 mM GSH. Little methylation of iAs^{III} was seen in reaction mixtures containing DHLA/TR/NADPH. However, addition of 5 mM GSH to these reaction mixtures stimulated MAs production.

We investigated the role of Trx in rrcyt19 catalysis by omitting components of the coupled system and by inhibiting regeneration of Trx with ATG. Omission of any component of the coupled system (Trx, TR, NADPH) essentially stopped conversion of iAs^{III} (Figure 3). Addition of 10 μM ATG to reaction mixtures containing the Trx/TR/NADPH-coupled system reduced iAs^{III} methylation by about 80% as compared to uninhibited reactions (data not shown). This suggests that the Trx/TR/NADPH-coupled system is required to reduce Trx needed for rrcyt19's function.

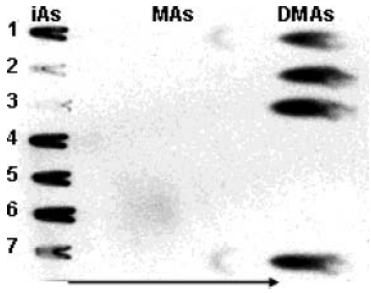

Figure 3. Effects of omission of components of the Trx/TR/NADPH-coupled system on rrcyt19-catalyzed conversion of arsenite. Reaction mixtures contained 5 μg of rrcyt19, 1 mM AdoMet, and 1 μM [^{73}As]iAs^{III} in 100 mM tris/100 mM Na phosphate buffer, pH 7.4, and were incubated at 37 °C. Lane 1, 1 mM DTT incubated for 1 h; lane 2, 1 mM DTT incubated for 16 h; lane 3, 1 mM DTT and the 10 μM Trx/3 μM TR/300 μM NADPH-coupled system incubated for 16 h; lane 4, 3 μM TR and 300 μM NADPH incubated for 16 h; lane 5, 10 μM Trx and 300 μM NADPH incubated for 16 h; lane 6, 3 μM TR and 10 μM Trx incubated for 16 h; lane 7, 10 μM Trx/3 μM TR/300 μM NADPH incubated for 0.25 h. Arrow indicates direction of migration in TLC.

The oxidation state of As in arsenicals in reaction mixtures was ascertained by pH selective generation of arsines. Figure 4 shows profiles for metabolites formed in reaction mixtures containing 1 mM TCEP or the Trx/TR/NADPH-coupled system. In the presence of 1 mM TCEP, methyl and dimethyl arsenicals containing As^{III} or As^{V} were present in reaction mixtures. With increasing time, DMAs^{III} became the predominant metabolite. In reaction mixtures containing the Trx/TR/NADPH-coupled system, the rate of methylation was higher and DMAs^{III} was always the predominant metabolite.

Catalysis by rrcyt19 was examined in a cellular environment that contained multiple physiological reductants. Here, guinea pig liver cytosol was added to reaction mixtures containing rrcyt19, AdoMet, and iAs^{III}. Guinea pigs are poor methylators of iAs (*23*, *24*), and cultured primary guinea pig hepatocytes do not methylate iAs (*24*); hence, liver is an ideal source of cytosol that contains physiological reductants without endogenous As

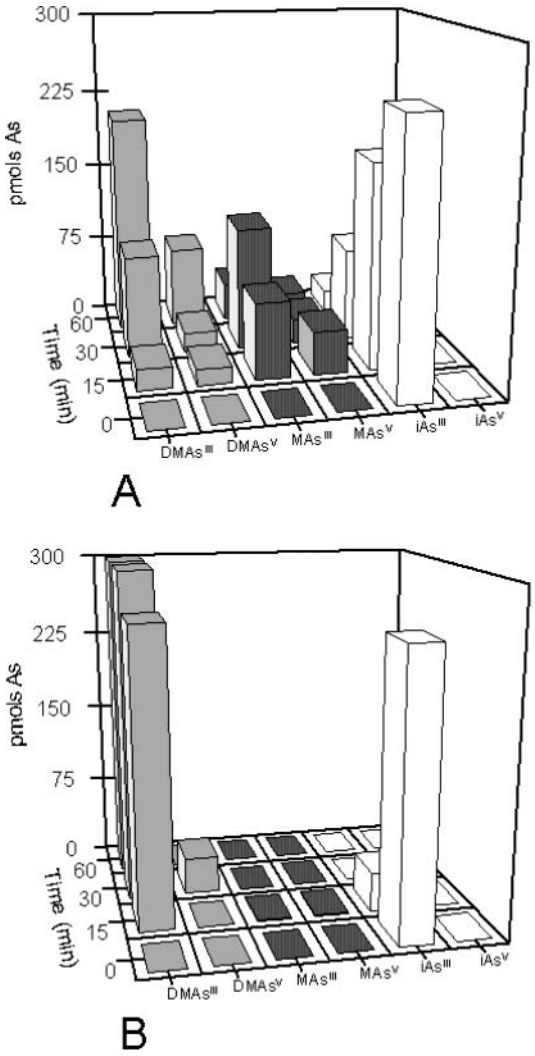

Figure 4. Distribution of arsenical metabolites in rrcyt19-catalyzed reactions with different reductants. Reaction mixtures (2 mL final volume) containing 1 μM iAs^{III}, 40 μg of rrcyt19, 1 mM AdoMet, 5 mM GSH, and (A) 1 mM TCEP or (B) 10 μM Trx/3 μM recombinant rat liver TR/300 μM NADPH were incubated at 37 °C for up to 60 min. Arsenicals speciated by oxidation state using pH selective hydride generation−atomic absorption spectrophotometry.

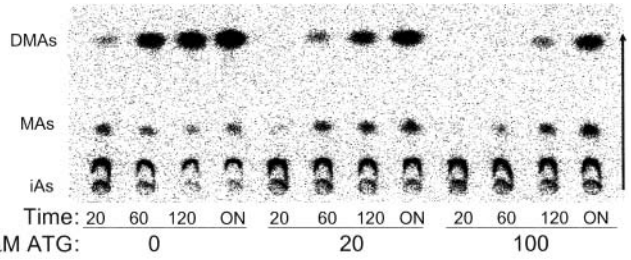

Figure 5. Effect of guinea pig liver cytosol on rrcyt19-catalyzed conversion of arsenite. ATG (0, 20, and 100 μM) added to reaction mixtures (100 μL final volume) containing 2 μg of rrcyt19, 1 mM AdoMet, 1 μM [^{73}As]iAs^{III} in 100 mM tris/100 mM Na phosphate buffer, pH 7.4, and 40% (v/v) guinea pig liver cytosol (4 mM GSH). Reaction mixtures were incubated for 20, 60, 120 min, or 18 h (overnight, ON) at 37 °C. Arrow indicates direction of migration in TLC.

methyltransferase activity. Methylation of iAs^{III} was minimal in reaction mixtures containing guinea pig liver cytosol. Less than 5% of iAs^{III} was methylated in reactions containing 40% (v/v) guinea pig cytosol (4 mM GSH), 1 μM radiolabeled iAs^{III}, and 1 mM AdoMet incubated at 37 °C for 24 h. In contrast, iAs^{III} was converted to MAs and DMAs in reactions mixtures containing rrcyt19 and guinea pig cytosol (Figure 5). Addition of ATG to these reaction mixtures slowed the rate of iAs^{III} methylation. To evaluate the possible role of endogenous reductants and reductases of guinea pig liver cytosol in the function of cyt19, reaction mixtures containing 2 μg of rrcyt19, 1 μM radiolabeled iAs^{III}, and 1 mM AdoMet and 40% (v/v) guinea pig cytosol that had been heated to 100 °C for 10 min (4 mM GSH) were incubated at 37 °C for up to 24 h. At 24 h, about 23% of iAs^{III} had been converted to MAs or DMAs in assays containing heat-treated guinea pig cytosol (data not shown). In contrast, about 52% of iAs^{III} had been converted to methylated arsenicals in assays containing untreated guinea pig cytosol.

Discussion

The rat *cyt19* gene encodes a 42 kDa protein that catalyzes the AdoMet-dependent production of methylated arsenicals from iAs^{III}. Rat cyt19 protein resembles the predicted products of orthologous *cyt19* genes of mouse and human. Multiple sequence alignment (*26*) of the cyt19 proteins for these species (GenBank accession numbers: rat, NP_543166; mouse, AAH13468; human, AK057833) identifies striking similarities, including 87% identity between rat and mouse cyt19 and 76% identity between rat and human cyt19. Each protein is cysteine rich, containing 12 (mouse), 13 (rat), or 14 (human) cysteine residues, contains sequence motifs (motif I, post I, and motifs II and III) common to nonnucleic acid methyltransferases (*27*), and contains a common UbiE methylase domain. Thus, rat cyt19 can be studied as a prototype for this family of AdoMet-dependent As^{III} methyltransferases. Notably, predicted cyt19 protein sequences do not resemble that of thioether-*S*-methyltransferase, an enzyme that converts dimethylated selenium to a trimethylated form (*28*, *29*) or those of members of a family of Zn-dependent methyltransferases that use thiols or selenols as substrates (*30*, *31*). Hence, cyt19 proteins may be a unique family of As^{III} methyltransferases.

In the presence of reductants, rrcyt19 catalyzes reactions that produce MAs and DMAs from iAs^{III}. These reactions produce DMAs as the major metabolite; MAs is always present as a minor metabolite. DMAs is the predominant metabolite formed in in vitro systems or in organisms exposed to iAs^{III} (*32*). Because preliminary studies found rrcyt19 sufficient to catalyze the conversion of iAs^{III} to methylated products and that its activity was absolutely dependent on the presence of reductants, we hypothesized that rrcyt19 catalyzes both steps in the reaction scheme—the oxidative methylation of As^{III} and the reduction of As^{V}-containing products to the trivalent oxidation state. We also hypothesized that rrcyt19, like other As^{V} reductases, uses endogenous reductants (Trx, Grx, and LA) to reduce As^{V}.

A Trx/TR/NADPH-coupled system provided reducing equivalents for the catalytic function of rrcyt19. Omission of any component of this coupled system or addition of ATG abolished rrcyt19's activity. Continuous generation of reduced Trx is also required by ArsC, an As^{V} reductase from *S. aureus* (*33*, *34*). Dependence of the Grx/GSH/GR/NADPH-coupled system upon GSH is consistent with roles for GR and GSH in reduction of oxidized Grx (*35*).

Catalysis by Acr2p, an As^V reductase from *S. cerevisiae*, requires both Grx and GSH (*36*). The DHLA/TR/NAPDH-coupled system supported rrcyt19 catalysis. DHLA is a reductant for PNP in its function as an As^V reductase (*10*). Mammalian TR reduces LA (*37*) permitting regeneration of DHLA. The basis for the enhancement by GSH of the effectiveness of the DHLA/TR/NADPH-coupled system is unknown. Because lipoamide reduces GSSG (*38*), the cycling of reducing equivalents to cyt19 may be increased in the presence of DHLA and GSH. As compared with other As^V reductases, rrcyt19 was relatively indiscriminant in use of endogenous reductants. For example, ArsC cannot use GSH or Grx to support iAs^V reduction (*33*) and Acr2p cannot use Trx as a reductant (*36*). A partially characterized human liver iAs^V reductase used DTT but did not use GSH, Trx, or DHLA (*7*). It should be noted that the activities of potential reductants Trx, Grx, and LA were not tested under equivalent experimental conditions in the studies reported here. For example, different concentrations of reductant and reducing enzyme were used in evaluations of the potency of Trx and Grx. The tested concentrations approximated those used in other studies of the role of reductants in the catalytic function of well-characterized As^V reductases (*34*, *36*). Further studies are needed to provide better quantitative estimates of the relative potencies of these reductants in support of the catalytic function of cyt19.

We also examined the effects of various reductants on the oxidation state of As in the products of rrcyt19-catalyzed reactions. These reaction mixtures contained 5 mM GSH to maximize product yield. The presence of the Trx/TR/NADPH-coupled system clearly favored the formation of $DMAs^{III}$. In the presence of 1 mM TCEP, a more complex pattern of metabolites was observed; however, $DMAs^{III}$ was the predominant product of this reaction. Notably, although each intermediate of the postulated metabolic scheme (MAs^{III}, MAs^V, $DMAs^{III}$, and $DMAs^V$) was found in reaction mixtures, neither iAs^V nor MAs^V was reduced or methylated in reaction mixtures containing TCEP or the Trx/TR/NADPH coupled system. The lack of activity suggests that steric or thermodynamic factors hinder interactions of these putative substrates with cyt19.

We examined rrcyt19 activity in reaction mixtures that contained guinea pig liver cytosol, a tissue fraction with little As methyltransferase activity. Because guinea pig liver cytosol fraction used in these studies contained 10 mM GSH, its addition to reaction mixtures also added GSH. Although addition of ATG to reaction mixtures containing rrcyt19 and guinea pig liver cytosol markedly slowed the rate of iAs^{III} methylation, other reductants besides the Trx/TR/NADPH-coupled system apparently provided reducing equivalents for rrcyt19 catalysis. These endogenous reductants could include Grx or LA, which are typically present in cells at low or submicromolar concentrations (*39*, *40*). We also found that use of heat-treated guinea pig cytosol (100 °C for 10 min) markedly reduced the rate of iAs^{III} methylation by rrcyt19 (data not shown). Hence, uncharacterized heat labile constituents of cytosol support the catalytic function of cyt19. It is possible that some of the activity of rrcyt19 found in reaction mixtures containing heat-treated guinea pig cytosol is dependent on the presence of 4 mM GSH. Further studies are needed to quantify the function of GSH as a relatively weak reductant for cyt19. Coupling rrcyt19's low discrimination among reductants and the redundancy of sources of reducing equivalents in cells ensures its activity when demand for and availability of each of several endogenous reductants fluctuates. A potential locus for interaction between the cyt19 and the Trx/TR/NADPH-coupled system involves the effect of methylated arsenicals on TR. MAs^{III} potently inhibited the activity of purified mouse liver TR (*19*), and in cultured rat hepatocytes, the extent of TR inhibition strongly correlated with intracellular MAs concentration (*41*). Thus, in cells, cyt19-catalyzed production of MAs^{III} could inhibit TR activity, altering regeneration of reduced Trx and thereby reducing cyt19's activity.

Phosphate stimulation of rrcyt19 activity is consistent with As^V reductase function. Phosphate and other oxyanions stimulate the activity of *S. aureus* ArsC, a Trx-dependent iAs^V reductase (*34*). For ArsC, oxyanions bind to residues in a catalytic P-loop sequence motif (CX5R) common to iAs^V reductases from *S. aureus* and *S. cerevisiae* and to low molecular weight phosphotyrosine phosphatases (*42*, *43*). In rat cyt19, a CX7R sequence (^{250}CRFVSATFR^{258}) may be functionally homologous to the iAs^V-binding P-loop of ArsC (*44*). The two phenyalanine residues in cyt19's putative P-loop may interact hydrophobically with methyl groups of MAs^V or $DMAs^V$. The C-terminal half of cyt19, which is dissimilar in sequence to other methyltransferases, may function catalytically in reduction of As^V-containing intermediates. An analogous C-terminal region in *Thermotoga maritima* isoaspartyl methyltransferase also lacks sequences commonly associated with methyltransferase activity but is needed for activity and structural stabilization (*45*). Notably, the CX7R sequence and four conserved aspartate residues are common to rat, mouse, and human cyt19 in the protein's C-terminal half. Mutation of analogous aspartate residues in ArsC decreases As^V reductase activity (*44*). Mutations in rrcyt19's putative P-loop and in C-terminal aspartate residues should yield insights into the structural basis for arsenic reduction by this protein.

Acknowledgment. We thank Dr. Michael F. Hughes, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection, for helpful discussions. Professor William R. Cullen, Department of Chemistry, University of British Columbia, generously provided arsenicals. Professor Barry P. Rosen, Department of Biochemistry, Wayne State University School of Medicine, generously provided *E. coli* Grx1. Professor Steven G. Clarke, Department of Chemistry and Biochemistry, University of California at Los Angeles, provided valuable advice on protein motif assignments. S.B.W. is a postdoctoral fellow in the Curriculum in Toxicology, University of North Carolina at Chapel Hill and is supported by Training Grant T901915 of the U.S. Environmental Protection Agency—University of North Carolina Toxicology Research Program. V.D. is a recipient of a Fulbright Commission Fellowship administered by the Ministry of Education, Culture, and Sport, Spain. M.S. is supported by NIH Grant ES09941 and a Clinical Nutrition Research Center Grant DK 56350. Disclaimer: This manuscript has been reviewed in accordance with the policy of the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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TX0342161