Biochemical Characterization of the Human Arsenite-stimulated ATPase (hASNA-I)*

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Arsenic is a potent toxin and carcinogen. In prokaryotes, arsenic detoxification is accomplished by chromosomal and plasmid-borne operon-encoded efflux systems. We have previously reported the cloning of hASNA-I, a human homologue of arsA encoding the ATPase component of the Escherichia coli arsenite transporter. Purified glutathione S-transferase (GST)-hASNA-I fusion protein was biochemically characterized, and its properties were compared with those of ArsA. The GST-hASNA-I exhibited a basal level of ATPase activity of 18.5 ± 8 nmol/min/mg in the absence of arsenite. Arsenite produced a 1.6 ± 0.1-fold stimulation of activity (p = 0.0044), which was related to an increase in Vmax; antimonite did not stimulate activity. Two lines of evidence suggest that an oligomer is the most likely native form of hASNA-I. First, lysates of human embryo kidney 293 cells overproducing recombinant hASNA-I produced a single monomorphic 37-kDa band on SDS-polyacrylamide gel electrophoresis (PAGE) and two distinct species when analyzed using non-denaturing PAGE. Second, chemical cross-linking of the 63-kDa GST-hASNA-I resulted in the formation of dimeric and tetrameric protein forms. The results indicate that hASNA-I is a distinct human arsenite-stimulated ATPase belonging to the same superfamily of ATPases represented by the E. coli ArsA protein.

Arsenic is a toxic metalloid whose reactive trivalent and pentavalent ions can influence a number of biochemical processes. In bacteria, arsenic detoxification is mediated by specific chromosomal (1, 2) as well as plasmid-transmissible operons (3, 4) encoding efflux systems that confer low and high level resistance to arsenic, respectively. The well characterized plasmid-borne ars operon of Escherichia coli is composed of two regulatory (arsR and arsD) and three structural (arsA, arsB, and arsC) genes. An oxyanion-dependent ATPase is encoded by the arsA gene and associates with the channel-forming transmembrane protein encoded for by arsB (5). ArsC is an arsenate reductase (6). In contrast to the plasmid-borne ars operon of E. coli, both its chromosomal ars operon and the plasmid-borne operon in Gram-positive bacteria lack arsD and arsA (7, 8).

In mammalian cells, although evidence for the presence of an ATP-dependent arsenite efflux system has been reported (9), none of its components have been molecularly isolated. As part of our efforts to identify genes involved in drug and arsenite resistance, we focused our efforts on the isolation of the human homologue of the bacterial ATP-binding ArsA protein, a putative component of an arsenite efflux pump in human cells. The ArsA protein is a member of a superfamily of ATP-binding proteins with a distinct nucleotide (NTP)-binding motif different from that of other ATPases, including the cation-translocating transporters. We have previously isolated the human hASNA-I cDNA utilizing homology to the distinct NTP-binding motif. It codes for a 37-kDa ATPase with a single ATP-binding cassette and is the first reported mammalian member of this superfamily of ATPases (10). In this report, we describe the biochemical characterization of hASNA-I expressed either as a fusion protein with glutathione S-transferase (GST-hASNA-I) in bacteria or as a native protein in hASNA-I-transfected human embryo kidney 293 cells. The results indicate that hASNA-I is biochemically a distinct arsenite-stimulated rather than an arsenite-dependent ATPase and that it shares some of the biochemical properties of the bacterial ArsA.

EXPERIMENTAL PROCEDURES

Purification of Recombinant GST-hASNA-I Fusion Protein Expressed in E. coli—The GST-hASNA-I fusion protein was produced in E. coli using the previously described prokaryotic recombinant plasmid pGEX-3X-hASNA-I (10). The recombinant GST-hASNA-I fusion protein was affinity-purified using a glutathione Sepharose 4B resin (Amersham Pharmacia Biotech) as described (11).

ATPase Assay—Freshly purified GST-hASNA-I protein maintained at 4 °C was used for the biochemical characterization throughout this work. The ATPase activity was measured spectrophotometrically at room temperature from a decrease in NADH concentration at 340 nm using a coupled assay (12). The reaction was carried out in an assay mixture containing 50 mM HEPES-HCl, pH 7.5, 30 mM KCl, 4 mM phosphoenolpyruvate, 0.4 mM NADH, 5 mM ATP, 21 mg/ml lactate dehydrogenase (Boehringer Mannheim), 42 mg/ml pyruvate kinase (Boehringer Mannheim). The GST-hASNA-I was preincubated at room temperature in the reaction mixture for 10 min before the reaction was started by the addition of MgCl2 to a final concentration of 5 mM. Protein determination was carried out using the Bradford assay (13).

Non-denaturing Polyacrylamide Gel Electrophoresis and Western Blotting of Cellular Lysates of hASNA-I-transfected Human Cells—A cell population of adenosvirus (AD5) E1A-transformed human embryo kidney 293 cells overproducing the hASNA-I protein was previously generated by Lipofecting a eukaryotic expression vector engineered to overexpress the hASNA-I cDNA. High levels of the recombinant hASNA-I were previously demonstrated (10). Cellular lysates from control empty vector-transfected and hASNA-I overproducing 293 cells were prepared, and 50 µg were analyzed using polyacrylamide gels under non-denaturing conditions. Running conditions were modified from (14) using a 12% resolving gel in 56 mM Tris, 31 mM borate buffer, pH 8.8 (adjusted with HCl), a 2.5% stacking gel in 31 mM Tris, 13 mM

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1 The abbreviations used are: GST, glutathione S-transferase; EEDQ, N-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; EDAC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; MOPS, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin.
boration of 1 mM dithiothreitol in the buffer had no effect on either basal ATPase activity (data not shown). Likewise, inclusion of tartrate to the reaction mixture had any impact on the measured ATPase activity varied slightly between different experiments. The difference in ATPase activity was statistically significant (p = 0.0044, two-sided paired t test). This 1.6 ± 0.1 (S.D.)-fold stimulation of activity was not dependent on preincubation of the enzyme with 100 μM sodium arsenite prior to initiation of the ATPase assay. Neither preincubation with nor concurrent addition of 100 μM potassium antimycin tartrate to the reaction mixture had any impact on the measured basal ATPase activity (data not shown). Likewise, inclusion of 1 mM dithiothreitol in the buffer had no effect on either basal or arsenite-stimulated activity.

**Effect of Arsenite on ATPase Activity**—The specific activity of GST-hASNA-I fusion protein in the presence and absence of 100 μM arsenite was determined using the recombinant GST-hASNA-I fusion protein. No ATPase activity was detected for the GST protein lacking the fused hASNA-I portion. In the absence of arsenite, a basal oxyanion-independent ATPase activity of 18.5 ± 8 (S.D.) nmol/min/mg was measured using four different GST-hASNA-I fusion protein preparations that increased to 28.6 ± 10 (S.D.) nmol/min/mg in the presence of a saturating concentration of 100 μM arsenite. Even though the measured ATPase activity varied slightly between different preparations, the activity was consistently higher in the presence of arsenite. The difference in ATPase activity was statistically significant (p = 0.0044, two-sided paired t test). This 1.6 ± 0.1 (S.D.)-fold stimulation of activity was not dependent on preincubation of the enzyme with 100 μM sodium arsenite prior to initiation of the ATPase assay. Neither preincubation with nor concurrent addition of 100 μM potassium antimycin tartrate to the reaction mixture had any impact on the measured basal ATPase activity (data not shown). Likewise, inclusion of 1 mM dithiothreitol in the buffer had no effect on either basal or arsenite-stimulated activity.

**Affinity of GST-hASNA-I Protein for ATP**—To examine the effect of arsenite on the affinity for ATP, the apparent Kₘ for ATP was determined at pH 7.5 in the absence and presence of the saturating concentration of 100 μM arsenite (Fig. 1) using a single GST-hASNA-I protein preparation. Similar values of 0.22 and 0.33 mM were obtained in the absence and presence of arsenite, respectively. A Vₘₐₓ of 16.6 nmol/min/mg was found for the basal ATPase activity, whereas the Vₘₐₓ of the arsenite-stimulated ATPase was 31.4 nmol/min/mg (Fig. 1). The arsenite-induced stimulation of the ATPase activity is thus due to a 1.9-fold increase in the Vₘₐₓ rather than to increased affinity of the GST-hASNA-I for ATP.

**Western Analysis of hASNA-I Overproduced in Human 293 Cells under Nondenaturing Conditions**—We previously showed that, in cellular lysates from human embryo kidney 293 cells engineered to overproduce hASNA-I, Western blot analysis demonstrated a single protein band of 37 kDa representing the monomeric form of the hASNA-I protein when the proteins were separated using SDS-polyacrylamide gel electrophoresis (10). To determine whether hASNA-I could form dimers or tetramers, Western blot analysis was carried out on cellular lysates from the same 293 cells that were separated using non-denaturing polyacrylamide gel electrophoresis. Fig. 2 shows that two distinct bands were observed in the lysate of the hASNA-I overproducing cells (lane 1), but no bands were detected in the control empty vector-transfected 293 cells (lane 2). Thus under the conditions used for this analysis only the overproduced protein was detected. Because migration of any one protein in polyacrylamide gels under nondenaturing conditions is a function of surface charge as well as size, this analysis was limited by the inability of a molecular weight marker to help establish whether the bands observed represented monomeric, dimeric, or even larger oligomeric species. However, the presence of two distinct bands in lane 1 is consistent with the interpretation that hASNA-I exists in more than one form.

**Chemical cross-linking of GST-hASNA-I Protein**—Chemical cross-linking was performed to further assess whether hASNA-I forms dimers or oligomers. The effect of treatment with 2 mM of the zero-length cross-linker EEDQ for 30 min on the control proteins: bovine serum albumin (BSA) and the GST portion of the fusion protein produced from the pGEX-3X empty vector was shown in Fig. 3A. The latter vector encodes the *Schistosoma mansoni* GST, which unlike all other studied GST proteins, has previously been demonstrated to be a catalytically active monomer (17). No change in band intensity or shift in size was observed for either BSA (lanes 1 and 2) or the 26-kDa *S. mansoni* GST (lanes 3 and 4). In contrast, treatment of the GST-hASNA-I for 30 min with EEDQ concentrations ranging from 0.25 to 2 mM resulted in disappearance of the 63-kDa GST-hASNA-I band (Fig. 3, lane 1) and appearance of two higher molecular species (lanes 2–5). The estimated sizes of the two latter species were consistent with a dimer at 126 kDa and a tetramer at 252 kDa. These results indicated that the cross-linking of the GST-hASNA-I resulted from self-interaction of the hASNA-I portion of the fusion protein resulting in the appearance of a dimers and tetramers. Treatment of the GST-hASNA-I for 60 min with EEDQ concentrations ranging from 0.25 to 1 mM is shown in Fig. 3C. In addition to the monomeric
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GST-hASNA-I, a higher molecular weight species (lanes 1–3) of molecular weight consistent with a dimer was obtained. A slight difference in the apparent molecular weight of the dimeric form was obtained with the two cross-linkers (Fig. 3B, lanes 2–5, and C, lanes 1–3), consistent with more binding of the EDAC to the GST-hASNA-I. Similar cross-linked GST-hASNA-I dimeric and/or tertrameric species were obtained with a number chemical cross-linkers, including the homobifunctional sulphydryl-specific 1,4-di-[3’-(2’-pyridylidithio)propionamido]butane, the homobifunctional N-hydroxy succinimide ester, disuccinimidyl glutarate, and the aryl halide containing 1,5-difluoro-2,4-dinitrobenzene (data not shown). No difference in the cross-linked products was observed when the GST-hASNA-I was preincubated in the presence of sodium arsenite even at concentrations up to 1 mM EEDQ (data not shown).

DISCUSSION

We have previously reported the cloning of the hASNA-I cDNA (10), encoding the first eukaryotic member of the superfamily of ATPases represented by the bacterial ArsA (18). In the absence of a simple method to purify the hASNA-I protein itself, we used a GST-hASNA-I fusion protein. A number of features distinguish this human ATPase from its bacterial counterpart. An oxyanion-independent basal ATPase activity of 18.5 nmol/min/mg was measured for the hASNA-I, that is 3-fold lower than that of the basal ATPase activity of the E. coli ArsA. Unlike the bacterial enzyme whose activity is induced 4-fold by arsenite and 32-fold by antimonite (19), the basal activity of hASNA-I was stimulated only 1.6-fold in the presence of arsenite and was not affected by the presence of antimonite.

The E. coli ArsA is a 63-kDa protein with two ATP-binding cassettes, functions as a homodimer that can be chemically cross-linked (15, 20), and its functional form contains four ATP-binding domains. DNA sequence information indicated that hASNA-I contained a single ATP-binding cassette and a predicted size of 37 kDa, half the size of the bacterial ArsA. The monomeric size of the human hASNA-I was confirmed by Western blotting using SDS-PAGE and an anti-hASNA-I antibody (10). In contrast, Western analysis of lysates from hASNA-I overproducing cells, carried out under non-denaturing PAGE, showed two distinct bands representing two species of the hASNA-I protein. This finding was confirmed by the appearance of dimers and tetramers of chemically cross-linked purified GST-hASNA-I fusion protein. These findings are consistent with the hypothesis that, similar to the E. coli ArsA, the active form of hASNA-I is likely to contain four ATP-binding domains assembled as a tetramer.

It is noteworthy that, although the hASNA-I cDNA was isolated using homology to the bacterial arsA and the protein it encodes is functionally an ATPase, the hASNA-I is a biochemically distinct enzyme. The activities of both prokaryotic and the eukaryotic enzymes are stimulated in the presence of arsenite; however, the underlying mechanism by which this takes place appears to be different. The activation and dimerization of the E. coli ArsA are dependent on the presence of oxyanions, with antimonite rather than arsenite being the major effector (15, 19). On the other hand, although the activity of the hASNA-I is mildly affected by the presence of arsenite and antimonite had no effect. Likewise, the oligomeric state of the GST-hASNA-I was not influenced by the presence of arsenite.

The presence of the distinct NTP-binding motif in the
hASNA-I and the bacterial ArsA places both within the same superfamily of ATPases. In *E. coli* the ArsA operates in concert with the ArsB, the transmembrane channel, to efflux arsenite. Our current knowledge does not allow us to establish whether the hASNA-I and the *E. coli* ArsA are orthologs, with an evolutionary conserved function, or whether they are functionally distinct paralogs. Isolation of the human ArsB homolog, the putative transmembrane channel is necessary before the role of hASNA-I as a component of a human efflux pump for arsenite detoxification can be tested. It remains possible that both the hASNA-I and the *E. coli* ArsA are paralogs having descended from an ancestral arsenite-responsive ATPase and may play different roles in cellular metabolism.

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