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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 V_{max}; 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 monomeric 37-kDa band on SDS-polyacrylamide gel electrophoresis (PAGE) and two distinct species when analyzed using nondenaturing 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 ars*C*) 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[1]-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 $MgCl_2$ to a final concentration of 5 mM. Protein determination was carried out using the Bradford assay (13).

Nondenaturing Polyacrylamide Gel Electrophoresis and Western Blotting of Cellular Lysates of hASNA-I-transfected Human Cells—A cell population of adenovirus (AD5) E1A-transformed human embryonal 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 nondenaturing 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 13 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-ethylcabodiimide hydrochloride; MOPS, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin.

borate buffer, pH 7.2 (adjusted with HCl), and 5 mM Tris, 39 mM glycine, pH 8.9, as the running buffer. For Western blotting, electrophoretically separated proteins were blotted onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA) using the Bio-Rad transblot cell. Transfers were incubated with a 1:5,000 dilution of the rabbit polyclonal GST-hASNA-I antiserum (10), and signals were visualized using a peroxidase-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech) and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Chemical Cross-linking of the hASNA-I Protein—Cross-linking with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) (EEDQ) (Sigma) and 1-(3-dimethylaminopropyl)-3-ethylcabodiimide hydrochloride (EDAC) (Aldrich) was performed basically as described previously (15). Purified GST-hASNA-I fusion protein (5 μg) and control proteins, purified bovine serum albumin and GST protein produced from the pGEX-3X empty vector (5 μg), were incubated for 30 min at 37 °C in 40 μl of 50 mM MOPS, pH 7.5, and 20% glycerol, in the presence of EEDQ and for 60 min at 30 °C in the presence of EDAC. A preincubation in the presence of 1 mM sodium arsenite for 15 min at 37 °C was carried out for the study of the effect of arsenite on chemical cross-linking with EEDQ. The reactions were terminated by the addition of 2 × SDS sample buffer, and the products were separated by SDS-polyacrylamide gel electrophoresis (16). Visualization was carried out by staining with Coomassie Blue.

RESULTS

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 preparation, 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 antimonyl 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 K_m 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 presence and absence of arsenite, respectively. A V_{max} of 16.6 nmol/min/mg was found for the basal ATPase activity, whereas the V_{max} 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 V_{max} 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
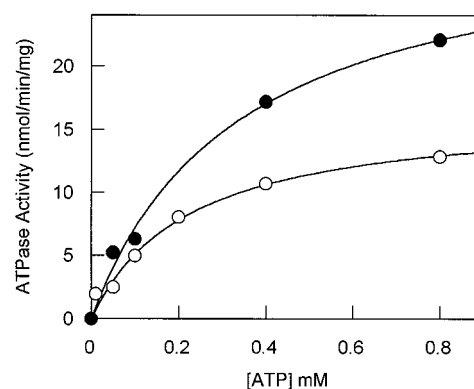
	Sodium Arsenite	
	0 μM	100 μM
V_{max} (nmol/min/mg ± standard error)[a]	17 ± 1	31 ± 3
K_m^{ATP} (mM ± standard error)[a]	0.22 ± 0.04	0.33 ± 0.07

(a) The kinetic parameters were determined using the computer software GraFit (21).

FIG. 1. **Kinetic parameters of the GST-hASNA-I protein.** The activity of purified GST-hASNA-I was measured over a range of ATP concentrations (0.1–0.8 mM) in the absence (*open circles*) or presence of 100 μM sodium arsenite (*closed circles*). A summary of the V_{max} and K_m^{ATP} in the presence and absence of arsenite, determined using the GraFit software (21), is shown under the plot.

nondenaturing 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 condition 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.

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 is shown in Fig. 3*A*. 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 EDAC concentrations ranging from 0.25 to 1 mM is shown in Fig. 3*C*. In addition to the monomeric

FIG. 2. Western blotting of cellular lysates separated using nondenaturing polyacrylamide gel electrophoresis. Lysates containing 50 µg of protein from *hASNA-I* cDNA transfected human embryo kidney 293 cells (*lane 1*), and from empty vector transfected 293 cells (*lane 2*), were separated on a 12% polyacryamide gel under nondenaturing conditions. Proteins were transferred to a polyvinylidene difluoride membrane and incubated with a rabbit hASNA-I antiserum. Detection was carried out using a peroxidase-conjugated donkey anti-rabbit antibody and developed using enhanced chemiluminescence.

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. 3*B*, *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 sulfhydryl-specific 1,4-di-[3′-(2′-pyridyldithio)propionamido]butane, the homobifunctional *N*-hydroxysuccimide 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 nondenaturing PAGE, showed two distinct bands representing two species of the hASNA-I protein. This finding was confirmed by the appear-
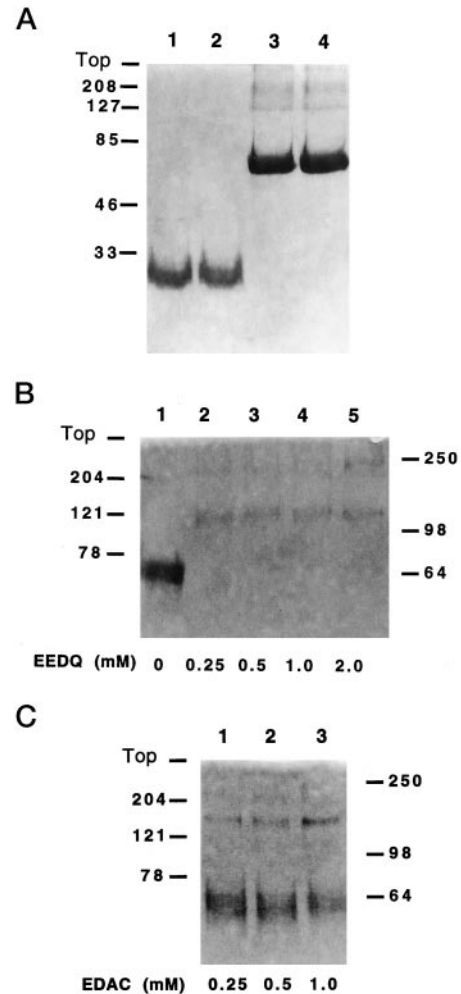

FIG. 3. Chemical cross-linking of the GST-hASNA-I fusion protein. *A*, purified control GST protein produced by the empty pGEX-3X vector and BSA (5 µg) treated with 2 mM EEDQ for 30 min at 37 °C. *Lane 1*, untreated GST; *lane 2*, EEDQ-treated GST protein; *lane 3*, untreated BSA; *lane 4*, EEDQ-treated BSA. *B*, purified GST-hASNA-I fusion protein (5 µg) treated for 30 min at 37 °C with EEDQ. *Lane 1*, untreated GST-hASNA-I; *lanes 2–5*, EEDQ-treated GST-hASNA-I with 0.25, 0.5, 1.0, and 2.0 mM, respectively. *C*, *lanes 1–3*, EDAC-treated GST-hASNA-I fusion protein for 60 min at 30 °C with 0.25, 0.5, and 1 mM, respectively. Proteins were visualized by Coomassie Blue staining. The *numbers* on the *right* and *left sides* depict the sizes of the molecular weight markers SeeBlue (Novex) and Kaleidoscope (Bio-Rad), respectively.

ance 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.

REFERENCES

1. Carlin, A., Shi, W., Dey, S., and Rosen, B. P. (1995) *J. Bacteriol.* **177,** 981–986
2. Diorio, C., Cai, J., Marmor, J., Shinder, R., and DuBow, M. S. (1995) *J. Bacteriol.* **177,** 2050–2056
3. Hedges, R. W., and Baumberg, S. (1973) *J. Bacteriol.* **115,** 459–460
4. Novick, R. P., and Roth, C. (1968) *J. Bacteriol.* **95,** 1335–1342
5. Rosen, B. P., Hsu, C. M., Karkaria, C. E., Kaur, P., Owolabi, J. B., and Tisa, L. S. (1990) *Biochim. Biophys. Acta* **1018,** 203–205
6. Gladysheva, T. B., Oden, K. L., and Rosen, B. P. (1994) *Biochemistry* **33,** 7288–7293
7. Ji, G., and Silver, S. (1992) *J. Bacteriol.* **174,** 3684–3694
8. Rosenstein, R., Peschel, A., Wieland, B., and Gotz, F. (1992) *J. Bacteriol.* **174,** 3676–3683
9. Wang, Z., Dey, S., Rosen, B. P., and Rossman, T. G. (1996) *Toxicol. Appl. Pharmacol.* **137,** 112–119
10. Kurdi-Haidar, B., Aebi, S., Heath, D., Enns, R. E., Naredi, P., Hom, D. K., and Howell, S. B. (1996) *Genomics* **36,** 486–491
11. Smith, D. B., and Corcoran, L. M. (1992) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Stehl, K., eds) Vol. 2, pp. 16.7.1–16.7.8, John Wiley & Sons, Inc., New York
12. Vogel, G., and Steinhart, R. (1976) *Biochemistry* **15,** 208–216
13. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
14. Nakatsuji, T., and Miwa, S. (1981) *Anal. Biochem.* **112,** 52–54
15. Hsu, C. M., Kaur, P., Karkaria, C. E., Steiner, R. F., and Rosen, B. P. (1991) *J. Biol. Chem.* **266,** 2327–2332
16. Laemmli, U. K. (1970) *Nature* **227,** 680–685
17. O'Leary, K. A., and Tracy, J. W. (1988) *Arch. Biochem. Biophys.* **264,** 1–12
18. Koonin, E. V. (1993) *J. Mol. Biol.* **229,** 1165–1174
19. Hsu, C. M., and Rosen, B. P. (1989) *J. Biol. Chem.* **264,** 17349–17354
20. Dupuis, A., Lunardi, J., Issartel, J. P., and Vignais, P. V. (1985) *Biochemistry* **24,** 734–739
21. Leatherbarrow, R. J. (1992) *GraFit*, Version 3.0, Erithacus Software, Staines, UK