Metalloregulatory Properties of the ArsD Repressor*

(Received for publication, January 10, 1997, and in revised form, April 7, 1997)

Yanxiang Chen and Barry P. Rosen‡
From the Department of Biochemistry and Molecular Biology, Wayne State University, School of Medicine, Detroit, Michigan 48201

The plasmid-encoded arsenical resistance (ars) operon of plasmid R773 produces resistance to trivalent and pentavalent salts of the metalloids arsenic and antimony in cells of Escherichia coli. The first two genes in the operon, arsC and arsR, were previously shown to encode trans-acting repressor proteins. ArsR controls the basal level of expression of the operon, while ArsD controls maximal expression. Thus, action of the two repressors form a homeostatic regulatory circuit that maintains the level of ars expression within a narrow range. In this study, we demonstrate that ArsD binds to the same site on the ars promoter element as ArsR but with 2 orders of magnitude lower affinity. The results of gel shift assays demonstrate that ArsD is released from the ars DNA promoter by phenylarsine oxide, sodium arsenite, and potassium antimonyl tartrate (in order of effectiveness), the same inducers to which ArsR responds. Using the quenching of intrinsic tryptophan fluorescence to measure the affinity of the repressor for inducers, apparent 

\[ K_d \]

values for Sb(III) and As(III) of 2 and 60 \( \mu \)M, respectively, were obtained. These results demonstrate that the ars-R-arsD pair provide a sensitive mechanism for sensing a wide range of environmental heavy metals.

The arsenical resistance (ars) operon of resistance plasmids R773 and R46 encodes an oxyanion extrusion pump that produces resistance to arsenite and antimonite (1). The operon has five genes, ArsR, -D, -A, -B, and -C (2–5). ArsR and ArsD are both trans-acting repressor proteins that homeostatically regulate the levels of ars transcript (4, 6). Although both are 13-kDa homodimers, they share no sequence similarity. ArsR is an ArsRI/ArsBII-responsive repressor with high affinity for its operator site that controls the basal level of expression of the operon (7). Binding of arsenite or antimonite produces dissociation of ArsR from the operator site, producing transcription. As the levels of transcript rise, synthesis of the integral membrane ArsB protein becomes toxic, limiting growth. ArsD is a second regulator that controls the upper level of expression of the operon, preventing overexpression of ArsB (4). Together, ArsR and ArsD form a regulatory circuit that controls the basal and maximal levels of expression of the ars operon. Dual regulatory proteins in a single operon are rare; in metalloregulatory systems only the MerR/MerD pair has been reported (8).

Preparation of plasmid DNA was performed by DNA Manipulation—

**MATERIALS AND METHODS**

Escherichia coli Strains, Plasmids, and Media—The bacterial strains and plasmids used in this study are described in Table I. E. coli cells were grown in Luria-Bertani medium at 37 °C. Ampicillin (100 \( \mu \)g/ml), kanamycin (80 \( \mu \)g/ml), tetracycline (15 \( \mu \)g/ml) or chloramphenicol (20 \( \mu \)g/ml) were added as required. For protein expression, 0.5 mm isopropyl-\( \beta \)-D-thiogalactopyranoside (IPTG) or 20 \( \mu \)M sodium arsenite was used as inducer, except where otherwise noted.

**DNA Manipulation**—Preparation of plasmid DNA was performed by using a Wizard DNA purification kit (Promega). Endo- and exonuclease digestions, DNA fragments separations and isolations, ligations, transformations, and Klenow fragment fill-in were performed according to standard procedures (9) unless otherwise noted.

Expression and Purification of ArsD—ArsD was purified from culture of strain BL21(DE3) bearing plasmid pT7-5-D (4). Cells were grown at 37 °C overnight with aeration in 0.2 liters of LB medium containing 0.1 mg/ml ampicillin. The cultures were diluted into 2 liters of prewarmed Luria-Bertani medium containing 0.1 mg/ml ampicillin. At an \( A_{600} \) of 0.6–0.8, production of ArsD was induced by the addition of 0.5 mm isopropyl-\( \beta \)-D-thiogalactopyranoside for 4 h. Induced cells were harvested by centrifugation and washed once with buffer A (10 mm Tris-HCl, pH 7.5, 1 mm EDTA, and 2 mm dithiothreitol). The pellets were suspended in 5 ml of buffer A/\( \gamma \)g of wet cells and disrupted by a single passage through a French pressure cell at 20,000 p.s.i. Unbroken cells and membranes were removed by centrifugation at 150,000 \( \times g \) for 1 h. The supernatant solution containing ArsD was loaded onto a 2.5-cm diameter column filled to 20 cm with Q-Sepharose (Pharmacia Biotech Inc.) pre-equilibrated with buffer A. The column was eluted with 512 ml of a linear gradient of 0–0.3 M NaCl in the same buffer. Fractions of 4 ml were collected and analyzed by SDS-PAGE (10). Fractions containing ArsD were pooled and concentrated. The protein

\* This work was supported by United States Public Health Service Grant AI19793. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence and requests for reprints should be addressed: Dept. of Biochemistry and Molecular Biology, Wayne State University, School of Medicine, 540 E. Canfield Ave., Detroit, MI 48201. Tel.: 313-577-1512; Fax: 313-577-2765; E-mail: brosen@med.wayne.edu.

1 The abbreviations used are: PAO, phenylarsine oxide; IPTG, isopropyl-\( \beta \)-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; ONPG, \( O \)-nitrophenyl-\( \beta \)-D-galactopyranoside.
Regulation of the *ars* Operon

**TABLE I**

| Strain/plasmid | Genotype/description | Reference |
|----------------|----------------------|-----------|
| JM109 recA supE44 endA1 hsdR17 gyrA96 relA1 thi Dlac-proAB F' [traD36 proAB lacIq lacZAM15] | | 9 |
| BL21(DE3) hsdS gal (λcits857 ind1 Sam7 nin5 lacUV5-T7genel) | | 9 |
| HMS174(DE3) HMS174(F', recA, rif*) lysojen with integration of T7 RNA polymerase gene under control of lacUV5 promoter | | 18 |
| Plasmid | | |
| pBS663 blaM' gene fusion vector | | 19 |
| pACYC184 Cloning vector (Cm’ and Tcr) | | 20 |
| pT7–5-D arsD gene cloned into pT7–5 under control of T7 promoter | | 4 |
| pArsD arsD gene under control of T7 promoter from pT7–5-D cloned into pACYC184 | | 4 |
| pBGD23 arsD::lacZ fusion under control of ars promoter | | 13 |
| pBGDAR1 arsR gene of pBGD23 was deleted | | 13 |

**FIG. 1. Regulation of an arsD::lacZ reporter gene by arsR and/or arsD.** Cells of *E. coli* strain HMS174(DE3) bearing the indicated plasmids were induced with the indicated concentrations of sodium arsenite and/or IPTG. A and B, no inducer (lane 1); 10 μM sodium arsenite (lane 2); 10 μM sodium arsenite + 10 μM IPTG (lane 3); 10 μM arsenite + 20 μM IPTG (lane 4); 10 μM arsenite + 50 μM IPTG (lane 5); and 10 μM sodium arsenite + 100 μM IPTG (lane 6). C and D, no inducer (lane 1); 10 μM sodium arsenite (lane 2); 10 μM sodium arsenite + 10 μM IPTG (lane 3); 20 μM sodium arsenite + 10 μM IPTG (lane 4); 50 μM sodium arsenite + 10 μM IPTG (lane 5); 100 μM sodium arsenite + 10 μM IPTG (lane 6); and 200 μM sodium arsenite + 10 μM IPTG (lane 7). E and F, no inducer (lane 1); 5 μM IPTG (lane 2); 10 μM IPTG (lane 3); 20 μM IPTG (lane 4); and 50 μM IPTG (lane 5). G and H, 10 μM IPTG (lane 1); 10 μM IPTG + 10 μM sodium arsenite (lane 2); 10 μM IPTG + 20 μM sodium arsenite (lane 3); 10 μM IPTG + 50 μM sodium arsenite (lane 4); 10 μM IPTG + 100 μM sodium arsenite (lane 5); and 200 μM sodium arsenite (lane 6).

was applied to a 2-cm diameter column filled to 100 cm with Sepharclay S-200 (Pharmacia) and eluted with buffer A. ArsD-containing fractions were pooled, concentrated, and stored at 4 °C. The concentration of ArsD in purified preparations was determined using a modification of the method of Lowry et al. (11).

**Gel Mobility Shift and DNase I Footprinting Assays**—Gel mobility shift and DNase I footprinting assays were performed as described previously (7). A 160-base pair DNA fragment containing the arsR operator/promoter and partial arsR gene was generated by PCR. After digestion with restriction endonucleases, either end of this fragment was labeled with [α-32P]dATP using the Klenow fragment of DNA polymerase I and purified with Wizard cleanup system (Promega).

**β-Galactosidase Assays**—Overnight cultures of *E. coli* strain HMS174 (DE3) harboring pBGD23 or pBGDAR1 and pArsD plasmids were diluted 50-fold to 2 ml of fresh Luria-Bertani medium containing ampicillin and chloramphenicol. After a 2-h incubation at 37 °C, cells were induced with varying concentrations of sodium arsenite and IPTG and grown for another 2 h. One ml of cells was pelleted by centrifugation and suspended in 0.5 ml of a buffer consisting of 60 mM NaH2PO4, 40 mM Na2HPO4, 10 mM KCl, 1 mM MgSO4 and 50 mM β-mercaptoethanol, pH 8.0 (Z buffer) (12). The cells were permeabilized by adding 1 drop of 0.1% SDS and 2 drops of chloroform with mixing. The reaction mixture contained 0.02 ml of cells, 0.1 ml of 8 mg/ml O-nitrophenyl-β-D-galactopyranoside, and 0.88 ml of Z buffer. Enzyme activity was estimated from the release of nitrophenol at 420 nm and 37 °C and expressed in Miller units (12).

**Measurements of Tryptophan Fluorescence**—Intrinsic tryptophan fluorescence was measured at room temperature using an SLM 8000 spectrofluorometer. The excitation wavelength was 295 nm. The slit widths for excitation and emission were 4 nm. Reagents were added in 1–2-μl volumes with a microliter syringe through a light-protected port to the cuvette containing 2-ml samples, and the solutions were continuously stirred during the measurement. Dilution effects were negligible. Fluorescence intensity changes were recorded at 336 nm.
RESULTS

**ArsD Is an Arsenite-responsive Repressor**—To investigate the in vivo repression of ars expression by ArsD, two reporter gene constructs were used. In the first, pBGD23, the lacZ gene was fused in frame with the 23rd codon of arsD. From this plasmid, both ArsR and an ArsD-β-galactosidase reporter are synthesized. In the second, pBGDΔR1, base pairs 27–259 of the 351-base pair arsR gene were deleted (13). The arsD gene was present on a compatible plasmid, pARS. In this plasmid, arsD is under control of the T7 promoter, and in strains with the DE3 lysogen, the T7 RNA polymerase, which is under control of the lac promoter, can be induced with IPTG. In the absence of IPTG, expression of the reporter gene from plasmid pBGD23 was low due to the presence of ArsR (Fig. 1A). Addition of 10 μM sodium arsenite in the absence of IPTG induced reporter gene expression by dissociation of ArsR from the operator/promoter. Simultaneous induction of ArsD with IPTG decreased reporter gene expression, demonstrating repression by ArsD. In the absence of the arsD gene, there was no effect of IPTG on repression by ArsR (Fig. 1B). The higher level of reporter gene expression observed in this experiment compared with the presence of ArsD (Fig. 1A) is most likely due to the leakiness of the lac promoter so that some ArsD is produced even in the absence of IPTG.

Although it was previously reported that repression by ArsD was not affected by inducer, in that experiment, ArsD was expressed at levels that were too high to respond to arsenite (4). In this study, when the concentration of sodium arsenite was increased, repression by ArsD was relieved, indicating that ArsD responds to inducer (Fig. 1C). Derepression of ArsD was further confirmed using a mobility shift assay (Fig. 1D). The control experiments with vector plasmid in place of pARS show that the effects of IPTG and arsenite require ArsD (Fig. 1, F and H). The ArsD and ArsR act independently and do not require each other for repression. Expression of the reporter gene from plasmid pBGDΔR1, which lacks the arsR gene, was constitutive, not requiring arsenite (Fig. 1E). Addition of IPTG to induce ArsD from plasmid pARS produced repression, demonstrating that ArsD does not require ArsR for repression. In the absence of ArsR, repression by ArsD was relieved by high concentrations of sodium arsenite, demonstrating that ArsD alone responds to inducer (Fig. 1G). The control experiments with vector plasmid in place of pARS show that the effects of IPTG and arsenite require ArsD (Fig. 1, F and H).

**Purification of the ArsD Repressor**—ArsD was purified by a combination of anion exchange chromatography and size exclusion chromatography, as described under “Materials and Methods.” Approximately 35 mg of purified ArsD could be obtained from 2 liters of cell culture. From the intensity of Coomassie Blue staining of samples separated by SDS-PAGE, ArsD was
judged to be >95% homogeneous (Fig. 2, inset). A small amount of dimer was observed in the purified preparations, as judged by immunoblotting with anti-ArsD serum. Considering the number of cysteines in ArsD, it is possible that the dimer is held together by disulfide bonds despite denaturation in the presence of β-mercaptoethanol. The molecular mass of purified ArsD was determined by gel filtration chromatography using a Sephacryl S-200 column (Fig. 2). From the nucleotide sequence of the arsD gene, the predicted mass of ArsD is 13,218 Da (4). From its elution position, a mass of approximately 26 kDa was determined, consistent with an ArsD homodimer.

**ArsD Is a DNA Binding Protein**—Gel mobility shift assays were used to examine the DNA binding activity of ArsD. A 160-base pair 32P-labeled PCR fragment containing the R773 ars promoter was used as target DNA. ArsD was able to retard the migration of this DNA probe (Fig. 3, top). No retardation of the labeled probe was observed when a 50-fold excess of unla-beled target DNA was added. From the least squares fit, the $K_d$ of ArsD for the operator/promoter DNA was calculated to be 65 μM (Fig. 3, bottom). This compares with a $K_d$ of 0.33 μM for ArsR (13), 2 orders of magnitude less than ArsD.

The repressor could be dissociated from its promoter by addition of inducer (Fig. 4). Either arsenite or antimonite produced dissociation although at relatively high amounts. In gel shift experiments, ArsR similarly dissociated only at high concentrations of inducer (7). On the other hand, the organoarseni-cal PAO was 100-fold more effective than either of the inorganic oxyanions. ArsR similarly is induced most effectively with PAO (13, 14).

**DNase I Footprint Analysis of the Binding Site for ArsD in the ars Regulatory Region**—Using purified ArsD, the site of binding to the R773 ars regulatory region was analyzed by DNase I protection assays. Protected regions found on both the coding strand from nucleotides −61 to −37 (Fig. 5A) and the noncoding strand from nucleotides −64 to −40 (Fig. 5B). Protection was prevented by addition of arsenite (Fig. 5B). This is the same region protected by ArsR (7).

**Intrinsic Tryptophan Fluorescence of ArsD**—ArsD has two tryptophan residues, Trp35 and Trp97. The intrinsic fluorescence of ArsD tryptophans exhibited a considerable enhancement and blue shift of the maximum emission wavelength (Fig. 6, curve A) compared with free tryptophan (Fig. 6, curve C). The fluorescence of ArsD following denaturation with guanidine...
Arsenic resistance (ars) operons produce resistance to the metalloids As(III) and Sb(III) by encoding an active efflux system for their oxyanions (1). Resistance to As(V) is conferred by an additional protein, ArsC, that reduces pentavalent to trivalent arsenical, the substrate of the extrusion system. The resistance to high levels of the pump proteins is itself toxic (4, 15) so that there must be a balance between detoxification of the metalloid and expression of the pump genes. Thus, action of the two repressors forms a homeostatic regulatory circuit that maintains the level of ars expression within a narrow range, with ArsR controlling basal level of expression and ArsD controlling maximal expression (Fig. 8).

ArsD responds to the same range of inducers as ArsR, with higher affinity for Sb(III) than for As(III), and essentially no response to other heavy metals or metalloids. It is of interest to a basal level, with induction through sensing of environmental metalloid. The role of ArsD is related to prevention of toxicity resulting from production of the membrane protein ArsB.

In this study, ArsD was shown to bind to the same operator region of ars DNA as ArsR. Neither required the other for DNA binding. Physiologically, their binding would be temporally distinct. The affinity of ArsD for ars operator/promoter DNA was shown to be 2 orders of magnitude less than ArsR. Small amounts of both ArsR and ArsD would be synthesized at a basal level in the absence of inducer, but ArsR would preferentially bind to the operator site, repressing ars expression. In vivo repression by ArsR can be fully relieved with 10 μM sodium arsenite (13), while ArsD repression requires approximately 100 μM sodium arsenite for induction (Fig. 1). These results suggest that ArsR has higher affinity for inducer than ArsD. Therefore, a low level of environmental metalloid would cause dissociation of ArsR, resulting in transcription of the ars message and increasing amounts of ArsD. As the intracellular concentration of ArsD exceeded the Kd for ars DNA, it would fill the ars operator site. Since its affinity for inducer is less than that of ArsR, the relatively low level of inducer present in the cell would not prevent its binding. On the other hand, exposure to high levels of environmental metalloid would cause dissociation of ArsD, effecting further expression of the ars genes and increased synthesis of the Ars efflux pump. Synthesis of high levels of the pump proteins is itself toxic (4, 15) so that there must be a balance between detoxification of the metalloid and expression of the pump genes. Thus, action of the two repressors forms a homeostatic regulatory circuit that maintains the level of ars expression within a narrow range, with ArsR controlling basal level of expression and ArsD controlling maximal expression (Fig. 8).

FIG. 8. A model of the ArsR-ArsD metalloregulatory circuit. A regulatory circuit provides homeostasis in sensing a wide range of environmental arsenicals and antimonials. Step 1, in the absence of inducer, transcription is repressed by a basal level of ArsR synthesis. Step 2, in the presence of low to moderate concentrations of inducer, the ArsR-inducer complex dissociates from the DNA, resulting in transcription of the ars operon. Step 3, when the concentration of ArsD increases sufficiently to allow this low affinity DNA binding protein to bind to the operator site, transcription is again repressed. Step 4, in the presence of high concentrations of inducer, the ArsD-inducer complex dissociates from the DNA, resulting in a further increase in ars transcription.

DISCUSSION

Arsenical resistance (ars) operons produce resistance to the metalloids As(III) and Sb(III) by encoding an active efflux system for their oxyanions (1). Resistance to As(V) is conferred by an additional protein, ArsC, that reduces pentavalent to trivalent arsenical, the substrate of the extrusion system. The first two genes of the ars operon of E. coli plasmid R773, arsR and arsD, encode trans-acting regulatory proteins (4, 6). Although both are small proteins (117 amino acid residues for ArsR and 120 for ArsD), they exhibit no significant sequence similarity. While ArsR had been shown to bind to the ars operator/promoter (7), binding of ArsD could not be measured (4). The role of ArsR was shown to be repression of the operon was similar to free tryptophan (Fig. 6, curve B). These results indicate that one or both tryptophan residues are in less polar environment than free tryptophan. ArsD tryptophan fluorescence reported inducer binding. In the presence of inducer, the fluorescence was quenched. The magnitude of fluorescence quenching was dependent on inducer concentration (Fig. 7A). Quenching as a function of inducer concentration allowed the determination of apparent affinity constants of approximately 2 μM for Sb(III) and 60 μM for As(III) (Fig. 7B).
that the R773 ars operon encodes three proteins that are regulated by these soft metals; in addition to ArsR and ArsD, the ArsA ATPase is allosterically regulated specifically by binding of Sb(III) or As(III) (16, 17). These three proteins have no sequence similarity but have apparently evolved independent binding sites for the two soft metals. ArsR has been shown to bind to the R773 ars promoter at the sequence TCAT-NNNNNNNTTTG, which is just upstream of the −35 site (7). The results of the DNase I protection show that ArsD and ArsR bind to the same sequence. Thus ArsR and ArsD have also evolved binding sites for the same element on the DNA.

REFERENCES
1. Rosen, B. P. (1996) J. Biol. Inorg. Chem. 1, 273–277
2. Chen, C.-M., Misra, T. K., Silver, S., and Rosen, B. P. (1986) J. Biol. Chem. 261, 15030–15038
3. San Francisco, M. J. D., Hope, C. L., Owolabi, J. B., Tisa, L. S., and Rosen, B. P. (1990) Nucleic Acids Res. 18, 619–624
4. Wu, J. H., and Rosen, B. P. (1993) Mol. Microbiol. 8, 615–623