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Evidence for Cooperativity between the Four Binding Sites of Dimeric ArsD, an As(III)-responsive Transcriptional Regulator*

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ArsD is a trans-acting repressor of the arsRDABC operon that confers resistance to arsenicals and antimonials in Escherichia coli. It possesses two-pairs of vicinal cysteine residues, Cys^{122}-Cys^{133} and Cys^{112}-Cys^{113}, that potentially form separate binding sites for the metalloids that trigger dissociation of ArsD from the operon. However, as a homodimer it has four vicinal cysteine pairs. Titration of the steady-state fluorescence of ArsD with metalloids revealed positive cooperativity, with a Hill coefficient of 2, between these sites. Disruption of the Cys^{112}-Cys^{113} site by mutagenesis of arsD, but not the Cys^{122}-Cys^{133} site, largely abolished this cooperativity, indicative of interactions between adjacent Cys^{112}-Cys^{113} sites within the dimer. The kinetics of metalloid binding were determined by stopped flow spectroscopy; the rate increased in a sigmoidal manner, with a Hill coefficient of 4, indicating that the pre-steady-state measurements reported cooperativity between all four sites of the dimer rather than just the intermolecular interactions reported by the steady-state measurements. The kinetics of Sb(III) displacement by As(III) revealed that the metalloid-binding sites behave differentially, with the rapid exchange of As(III) for Sb(III) at one site retarding the release of Sb(III) from the other sites. We propose a model involving the sequential binding and release of metalloids by the four binding sites of dimeric ArsD, with only one site releasing free metalloids.

All cells possess regulatory mechanisms to tightly control the concentration of soft metals, including both essential metals, such as zinc and copper, and toxic metalloids, such as arsenite and antimony (1). A common homeostatic mechanism is the use of efflux systems, such as transport ATPases, to control the level of these soft metals in the cell. Failure of these regulatory systems can lead to genetic disorders, such as Menkes' disease and Wilson's disease in humans, caused by mutations in two Cu(I) pumps (2–6). Consequently, understanding the mechanism controlling the expression of these efflux pumps and the basis of their selectivity for toxic soft metals is of paramount importance. A common feature of the protein components of these regulatory systems is the use of pairs of cysteine residues to chelate the soft metals, with multiple copies of these soft metal-chelating sites occurring within each protein. However, there have been no transient kinetic studies, with the potential to define cooperative interactions, of metal or metalloid binding to such sites; consequently, the binding mechanism has not been elucidated, and our knowledge of the role played by the individual binding sites is still rudimentary.

High level resistance to arsenite and antimonite in Escherichia coli is conferred by the arsRDABC operon of plasmid R773 (7). The arsA and arsR genes encode the ATPase and membrane translocase subunits of an arsenical pump (8), whereas the arsC gene encodes an arsenate reductase that is required to catalyze the reduction of arsenate to arsenite prior to extrusion (9–13). The products of the arsD and arsR genes, which encode trans-acting repressors that bind to the ars operon, control the expression of the operon (14, 15). ArsR is a 117-residue protein that functions as a homodimer, repressing expression of the operon to a basal level in the absence of arsenite or antimonite, but in the presence of these metalloids it dissociates from the DNA, and transcription ensues. ArsD is also a homodimer of two 120-residue subunits that binds to the same operator site as ArsR but with an affinity that is 2 orders of magnitude less than that of ArsR. Consequently, ArsD only binds to the ars operon when produced in high concentrations, such as after prolonged stimulation of transcription of the arsD gene following induction by arsenite or antimonite. It has been proposed that ArsD controls the maximal level of expression of the operon, preventing the build-up of ArsB, which at high levels appears toxic to the cell (15). Although ArsD has an affinity for arsenite that is an order of magnitude lower than ArsR, at sufficiently high levels of arsenite, ArsD dissociates, and the operon is again transcribed. In both ArsR and ArsD vicinal pairs of cysteine residues have been shown to be involved in coordinating the metalloid (16, 17). The homodimeric ArsR has only a single metalloid binding site/monomer, utilizing the Cys^{122}-Cys^{133} pair to coordinate metalloids, whereas ArsD appears to have two, or four one-half metalloid-binding sites/monomer, utilizing the Cys^{112}-Cys^{113} and Cys^{112}-Cys^{113} pairs to coordinate metalloids. Substitution of either of these cysteine pairs in ArsD abolished metalloid inducibility but did not affect repression, indicating that these cysteine residues form metalloid-binding sites (17). The aim of the present study was to determine whether these sites are independent or whether they interact and to elucidate the mechanism of metalloid binding to these sites.

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purified as described previously (17), quickly frozen, and stored in small aliquots at \(-80^\circ\text{C}\). The concentrations of purified ArsD and its derivatives were determined using a Bio-Rad protein assay kit. An ArsD derivative in which the Cys\(^{119}\) and Cys\(^{120}\) residues were deleted and in which a His\(_6\) tag was added to the C terminus was used as the basis for the construction of further derivatives in which the other cysteine residues were systematically changed to alanines.

**Fluorescence Measurements**—Fluorescence measurements were made in a Jasco FP750 fluorimeter at 20 °C. Tryphtophan fluorescence was excited at 295 nm, and the emission wavelength was scanned between 300 and 400 nm. For titrations, Sb(III) was added as potassium antimonal tartrate from 1, 10, or 100 mM stock solutions to 2 ml of 1 \(\mu\text{M}\) ArsD protein (in 50 mM MOPS-KOH, \(pH\) 7.5, 0.25 mM EDTA), so that the original sample was not diluted by more than 10%. The protein fluorescence and the Sb(III) concentration were corrected for the dilution effect.

Time-resolved fluorescence measurements were also carried out in an Applied Photophysics (London, UK) SX.18MV stopped flow instrument at 20 °C. For measurements of the change in tryptophan fluorescence, the samples were excited with light at 295 nm and selected with a monochromator, and the emission was monitored at wavelengths above 335 nm using a cut-off filter. Routinely, equal volumes of the reactants were mixed together in the stopped flow instrument, using two syringes of equal volume. The concentration of ArsD was 1 \(\mu\text{M}\), unless otherwise noted, in 50 mM MOPS-KOH, \(pH\) 7.5, 0.25 mM EDTA.

All of the concentrations are for the mixing chamber unless stated otherwise, so that the concentrations in the syringe were twice those quoted for the mixing chamber. To set up the stopped flow instrument, 1 \(\mu\text{M}\) ArsD protein was mixed with buffer, and the photomultiplier tube voltage was increased until a 4 V signal was achieved. Fluorescence changes were recorded as an increase or decrease in this 4 V signal (i.e., an increase in the signal from 4 to 4.1 V would correspond to a 2.5% increase in fluorescence and a decrease from 4 to 3.9 V would correspond to a 2.5% quench in fluorescence), which was backed off to zero, and changes above and below this zero base line were recorded.

**Data Analysis**—Stopped flow traces were analyzed by fitting to single (\(s = A \times \text{exp}^{-k_i t}\)) or multiple exponential functions (\(s = A_1 \times \text{exp}^{-k_1 t} + A_2 \times \text{exp}^{-k_2 t} + A_3 \times \text{exp}^{-k_3 t}\), for a triple exponential function) using the nonlinear regression software with the Applied Photophysics stopped flow program. Concentration dependence data were analyzed by nonlinear regression fitting to sigmoidal or hyperbolic functions, as required, using Sigmaplot 4.0. Drug titration curves were fitted to a sigmoidal function by nonlinear regression using the program Sigma Plot from Jandel Scientific.

\[
\Delta F = \Delta F_{\text{max}} \left[\frac{[\text{Sb(III)}]}{K_{i_{\text{H}}}} + [\text{Sb(III)}]\right]^{n} \tag{1}
\]

where \(\Delta F\) is the quench in protein fluorescence, \(\Delta F_{\text{max}}\) is the maximum fluorescence quench, \(K_{i_{\text{H}}}\) is the half-saturation constant, and \(n\) is the Hill coefficient.

**RESULTS**

The Steady-state Kinetics of Sb(III) and As(III) Binding to ArsD—The binding of Sb(III) and As(III) to ArsD induces a quench in the protein fluorescence (17) that was used to titrate the ArsD metalloid-binding site. ArsD contains only two tryptophan residues, Trp\(^{35}\) and Trp\(^{97}\), of which only the latter participates to metalloid responsiveness. In a previous study we established that although the vicinal cysteine pair Cys\(^{119}\)-Cys\(^{120}\) bound metalloids, it did not have an apparent effect on repression or metalloid responsiveness in vivo or in vitro (17). Accordingly, to avoid complications in the interpretation of the data for the binding of Sb(III) to ArsD and to provide the base-line data for our study, we produced and characterized a C-terminal His-tagged Cys\(^{119}\)-Cys\(^{120}\) deletion derivative of ArsD, which, for simplicity, we refer to as ArsD throughout the manuscript and into which further amino acid substitutions were introduced. The titration curves for the binding of Sb(III) and As(III) to 1 \(\mu\text{M}\) ArsD (i.e., the Cys\(^{119}\)-Cys\(^{120}\) deletion derivative) indicated that binding is a cooperative process (Fig. 1) and was characterized by Hill coefficients of 2.1 \pm 0.2 and 2.1 \pm 0.1 and maximal quenches in the protein fluorescence of 45 \% and 20 \% for Sb(III) and As(III), respectively. However, the titration curve for Sb(III) was to the left of that for As(III), with \(K_{i_{\text{H}}}\) values of 1.7 \pm 0.6 and 11.2 \pm 4.5 \(\mu\text{M}\), respectively, indicating that ArsD has higher affinity for Sb(III) than As(III). Comparatively, the His-tagged wild type protein (termed wild type ArsD), which possessed the Cys\(^{119}\) and Cys\(^{120}\) residues, behaved in an identical manner (Table I).

**Evidence for Cooperativity between Adjacent Protoners of Dimeric ArsD**—To investigate the molecular basis of the cooperative interactions between the metalloid-responsive binding sites of dimeric ArsD, Cys\(^{12}\), Cys\(^{13}\), Cys\(^{112}\), and Cys\(^{113}\) were systematically substituted by alanine residues to disrupt each potential binding site. Our previous studies suggested that the vicinal cysteine pairs Cys\(^{12}\)-Cys\(^{13}\) and Cys\(^{112}\)-Cys\(^{113}\) form separate metalloid-binding sites. As shown in Table I, disruption of the Cys\(^{12}\)-Cys\(^{13}\) site by replacing either Cys\(^{12}\), Cys\(^{13}\), or both did not affect the cooperativity in the binding of Sb(III). In contrast, disruption of the Cys\(^{112}\)-Cys\(^{113}\) site caused a major reduction in the binding cooperativity. This behavior indicates that the observed cooperativity probably does not arise from interactions between the Cys\(^{12}\)-Cys\(^{13}\) and Cys\(^{112}\)-Cys\(^{113}\) sites but more likely arises from interactions between Cys\(^{12}\)-Cys\(^{113}\) sites of the dimer. Indeed, not only does disruption of the Cys\(^{112}\)-Cys\(^{113}\) site reduce cooperativity but also causes a 75–80% reduction in the fluorescence response of the protein to the binding of Sb(III), suggesting that the changes in ArsD fluorescence are primarily reporting events at the Cys\(^{112}\)-Cys\(^{113}\) site. Consequently, the titration curves for ArsD might only reflect filling of the two Cys\(^{112}\)-Cys\(^{113}\) sites in dimeric ArsD, and cooperative interactions with the Cys\(^{12}\) and Cys\(^{13}\) sites might go unobserved. It is important to note that although disruption of the Cys\(^{112}\)-Cys\(^{113}\) sites dramatically reduces the cooperative effects, these are not totally abolished. For example, the Hill coefficient for C112A/C113A ArsD was 1.1. These data suggest...
Cooperative Binding of Metalloids to ArsD

**TABLE I**

| ArsD derivatives | Fluorescence quench | Hill coefficient |
|------------------|---------------------|------------------|
|                  | %                   |                  |
| Native           |                     |                  |
| Wild type ArsD (Sb)  | 52 ± 5              | 2.0 ± 0.2        |
| ArsD (Cys^{119}-Cys^{120} deletion) (Sb) | 55 ± 5 | 2.2 ± 0.2 |
| ArsD (Cys^{119}-Cys^{120} deletion) (As) | 26 ± 4 | 2.1 ± 0.2 |
| Single substitutions  |                  |                  |
| C12A             | 52 ± 5              | 2.0 ± 0.2        |
| C13A             | 57 ± 3              | 2.2 ± 0.2        |
| C112A            | 14 ± 3              | 1.3 ± 0.2        |
| C113A            | 17 ± 3              | 1.2 ± 0.2        |
| Double substitutions   |                  |                  |
| C12A/C13A        | 52 ± 4              | 2.1 ± 0.2        |
| C112A/C113A      | 11 ± 2              | 1.1 ± 0.1        |
| Triple and quadruple substitutions | 13 ± 3 | 417 ± 24 μM |
| C12A/C13A/C112A/C113A | No response |                  |

**a** Wild-type ArsD bearing a C-terminal His₆ tag.

**b** ArsD Cys-Ala derivatives were constructed in the ArsD (Cys^{119}-Cys^{120} deletion) derivative.

**c** The data was adequately fitted to a hyperbolic function.

A Hill coefficient of 4.0 ± 0.7, 45 ± 2 μM, 246 ± 14 s⁻¹, and 29 ± 9 s⁻¹, respectively (Fig. 3, top panel), whereas for phase 2, these constants had values of 3.7 ± 0.6, 44 ± 2 μM, 24 ± 1 s⁻¹, and 4.8 ± 0.8 s⁻¹, respectively (Fig. 3, bottom panel). The fact that the rates of both phase 1 and 2 are dependent upon the Sb(III) concentration suggests that ArsD has two nonidentical binding sites for Sb(III).

Interestingly, for concentrations of Sb(III) below 10 μM, there was a deviation away from the best fit curves in Fig. 3 for phases 1 and 2, with the rates of these phases dropping off.
more rapidly than predicted. Accordingly, we reinvestigated this part of the titration curve, obtaining more data points between 0 and 50 μM Sb(III). As shown in Fig. 4 (top panel), over this concentration range the rate of phase 1 increased in an apparently sigmoidal manner with the Sb(III) concentration and was best fitted to Equation 2 with values for the Hill coefficient, $K_0$, $k_{\text{max}}$, and $k_{\text{min}}$ of $2.2 \pm 0.4$, $9.8 \pm 1$ μM, $67 \pm 7$ s$^{-1}$, and $9.3 \pm 2.0$, s$^{-1}$, respectively. Consistent with the data in Fig. 3, the rate increased more rapidly for concentrations above 40 μM. Phase 2 behaved in a parallel manner, indicating a Hill coefficient of $1.7 \pm 0.3$ (Fig. 4, middle panel). It is notable that this sigmoidal behavior occurs over the same concentration range as the steady-state titration curve for ArsD (Fig. 1), and, although the corresponding amplitude data were more variable than the steady-state measurements, the concentration dependence of the amplitude data (e.g., total percentage of fluorescence change) was best fitted to a sigmoidal function with a Hill coefficient of $2.0 \pm 0.5$ and a Δ$F_{\text{max}}$ of $39 \pm 1$% (Fig. 4, bottom panel).

It is tempting to speculate that phases 1 and 2 correspond to the filling of the two metalloid-binding sites (e.g., Cys$^{12}$-Cys$^{13}$ and Cys$^{12}$-Cys$^{113}$, respectively) within the monomer. However, as noted above, the concentration dependence of each phase is characterized by two sigmoidal dependences, at low (0–20 μM) and high (20–150 μM) concentrations of Sb(III). Moreover, for each phase, the increase in rate over the higher concentration range was characterized by a Hill coefficient of about 4, indicating that all four sites of dimeric ArsD are involved. On the other hand, the steady-state titration curve indicated that ArsD approaches saturation with Sb(III) at 20 μM, suggesting that the high affinity (detected by steady-state measurements) and the low affinity (detected by pre-steady-state measurements) ArsD-Sb(III) complexes are kinetically coupled; the latter is the initial ArsD-Sb(III) complex, and the former is the final ArsD-Sb(III) complex; these complexes are connected by a conformational change from one to the other. As
illustrated by Fig. 5, the titration curve for formation of the initial ArsD-Sb(III) complex, defined by the Sb(III) concentration dependence of the rate of binding of Sb(III) (e.g., either phase 1 or 2), is displaced to the right of the titration curve for the final ArsD-Sb(III) complex, defined by the Sb(III) concentration dependence of the steady-state fluorescence of ArsD, with half-saturation points for the initial and final complexes of about 44 and 1.7 μM, respectively. The simplest explanation for this behavior is that following the binding of Sb(III) to ArsD, the protein undergoes a conformational change that tightens the hold on Sb(III), probably in adopting a conformation that causes ArsD to dissociate from the arsRDABC promoter. Indeed, the equilibrium between these conformational states should be defined by k_{max} and k_{min} (e.g., forward and backward rate constants) determined for the binding of Sb(III) by the following relationship: 

\[ K_{\text{final}} = K_{\text{initial}}(1 + k_{\text{max}}/k_{\text{min}}) \]

where \( K_{\text{initial}} \) and \( K_{\text{final}} \) are the half-saturation constants for the initial and final ArsD-Sb(III) complexes, respectively. Applying this relationship, with the values determined for phase 1 for k_{max}, k_{min}, and K_{initial} of 246 s^{-1}, 29 s^{-1}, and 44 μM, respectively, gave a calculated value for K_{final} of 4.6 μM. Although this value is slightly higher than the expected value of 1.7 μM, this difference could easily be accounted for by an additional conformational change that further tightens the binding. Indeed, we note that phase 2 is characterized by k_{max} and k_{min} values of 23.6 s^{-1} and 4.8 s^{-1}, respectively, indicative of another conformational change that would yield a K_{final} of 0.8 μM. There is still a remaining question as to what process is being measured at low Sb(III) concentrations (i.e., below 10 μM). Two possibilities are that 1) the measurements detect filling of the first two sites, which is necessary to “activate” ArsD so that it can undergo the conformational change to the final state or 2) a slow conformational change subsequent to formation of the initial ArsD-Sb(III) complex is being measured. The former scenario is more likely because this occurs with a Hill coefficient of 2, not 4, as would be expected for the latter scenario. Furthermore, the steady-state studies indicate that the fluorescence quench is largely attributable to the binding of metalloids to the Cys^{112}-Cys^{113} site, and the measurements detect the filling of these sites within the dimer preferentially.

The Kinetics of the Formation of the ArsD-As(III) Complex—
The stopped flow traces for the binding of As(III) to ArsD were biphasic, but only the rate of the fast phase, which accounted for about 90% of the signal amplitude, was dependent upon the As(III) concentration (data not shown). The rate of this fast phase increased in a sigmoidal manner with the As(III) concentration, and a fit of the data to Equation 2 indicated values for the Hill coefficient, \( K_{\text{Hill}} \), k_{max}, and k_{min} of 3.6 ± 0.8, 7 ± 1 μM, 8.7 ± 0.6 s^{-1}, and 0.8 ± 0.4 s^{-1}, respectively (Fig. 6). However, as noted for the binding of Sb(III) (Figs. 2 and 3), the first few data points deviated from the best fit sigmoidal curve. Thus, although a second concentration-dependent phase for As(III) was not observed, possibly because this is obscured by the lower signal amplitude, As(III) behaves in a manner similar to that of Sb(III). These data confirm the cooperative binding of metalloids by all four binding sites of dimeric ArsD but indicate that the conformational change from the initial to the final ArsD-As(III) complex occurs at a rate that is more than an order of magnitude slower than for Sb(III). Interestingly, there is a discrepancy between the calculated overall K_{Hill} (i.e., \( K_{\text{final}} = K_{\text{initial}}(1 + k_{\text{max}}/k_{\text{min}}) = 660 μM(1 + 8.7 s^{-1}/0.8 s^{-1}) = 54 μM) \) and that obtained from the steady-state titration curve (e.g. \( K_{\text{Hill}} = 11.2 μM, \) Fig. 1); this is indicative of a further conformational change (i.e., the binding of As(III) to ArsD is at least a three-step process) that is rate-limiting.

**Dissociation of Sb(III) from the ArsD-Sb(III) Complex**—Dilution of the ArsD-Sb(III) complex with DTT to sequester the Sb(III) caused dissociation of the complex, as indicated by the increase in fluorescence of ArsD as the Sb(III) dissociated (Fig. 7), but no increase in protein fluorescence was observed when unliganded ArsD was mixed with DTT (data not shown). As expected, the ArsD-Sb(III) complex could not be dissociated by dilution with buffer in the stopped flow, because this only produced a drop in the Sb(III) concentration from 20 to 10 μM, which is still above the \( K_{\text{Hill}} \); however, when 2 μM ArsD/5 μM Sb(III) was diluted 1:1 with buffer to drop the Sb(III) concentration below the K_{Hill}, the Sb(III) dissociated very slowly over several hours (data not shown). The stopped flow traces shown in Fig. 7 for the mixing of 1 μM ArsD/20 μM Sb(III) with 0.5 mM DTT (trace A) and 5 mM DTT (trace B) were best fitted to biphasic exponential equation with rate constants of 20.0 ± 0.2 s^{-1} (86% of total signal amplitude) and 0.55 ± 0.02 s^{-1} (14% of total signal amplitude) for trace A and 166 ± 3 s^{-1} (90% of total signal amplitude) and 5.2 ± 0.2 s^{-1} (10% of total signal ampli-
FIG. 7. DTT induced dissociation of the ArsD-Sb(III) complex. Two semi-logarithmic plots of stopped flow traces generated by mixing 1 μM ArsD/20 μM Sb(III) with 0.5 mM DTT (trace A) and 5.0 mM DTT (trace B). Changes in the ArsD fluorescence were recorded with excitation = 295 nm and emission > 335 nm. The smooth curve through the traces are the best fits to a double-exponential function, with rate constants of 20.0 ± 0.2 s⁻¹ (86% of total signal amplitude) and 0.55 ± 0.02 s⁻¹ (14% of total signal amplitude) for trace A and 166 ± 3 s⁻¹ (90% of total signal amplitude) and 5.2 ± 0.2 s⁻¹ (10% of total signal amplitude) for trace B. Both 0.5 and 5 mM DTT caused a 31–32% increase in fluorescence of ArsD-Sb(III).

FIG. 8. The concentration dependence of the DTT-induced dissociation of the ArsD-Sb(III) complex. A series of stopped flow records were generated by mixing 1 μM ArsD/20 μM Sb(III) with DTT at the indicated concentrations in a stopped flow device. The rates of the fast and slow increase in the fluorescence of ArsD are plotted as a function of the DTT concentration. Two data sets are shown that were generated with ArsD (○, △) and C12A/C13A ArsD (■, ●). The fast phase for ArsD and C12A/C13A ArsD increased in a linear manner, indicating second order rate constants of 36 ± 5 and 3 ± 1 s⁻¹, respectively, whereas that for the slow phase had little concentration dependence, suggestive of a conformational change in ArsD subsequent to Sb(III) dissociation (Fig. 8). Over the studied concentration range, of 0.1–5.0 mM DTT, the total signal amplitude was constant, indicating that all of the Sb(III), which could be sequestered by DTT, was displaced from the ArsD-Sb(III) complex. However, we note that the signal amplitude is slightly less than expected from the Sb(III)-induced quench in the ArsD fluorescence (e.g. 39% in stopped flow experiments), so that we cannot exclude the possibility that some Sb(III) remains bound to ArsD in the presence of DTT. The fact that the rate of DTT-induced dissociation of the ArsD-Sb(III) complex increases linearly with the DTT concentration is consistent with the dissociation process being rate limited by the collision or bimolecular association of DTT with the Sb(III)-ArsD complex. As discussed above, dissociation of Sb(III) from ArsD is too slow to be rate-limiting for this process, supporting our conclusion that Sb(III) is transferred directly from ArsD to bound DTT.

FIG. 9. As(III)-induced dissociation of the ArsD-Sb(III) complex. Five semi-logarithmic plots of stopped flow traces generated by mixing 1 μM ArsD/20 μM Sb(III) with buffer (trace A), 1 mM As(III) (trace B), 30 mM As(III) (trace C), 40 mM As(III) (trace D), and 1 μM ArsD mixed with buffer (trace E). Trace B was adequately defined by a single-exponential function, indicating a rate of 0.0091 ± 0.0001 s⁻¹, whereas trace C was more complex being best fitted to a triple exponential function that indicated rates of 14.5 ± 0.5, 1.50 ± 0.03, and 0.070 ± 0.002 s⁻¹. Because of the decrease in fluorescence that occurred after the first second of the reaction, only the fast phase of trace D could be analyzed, which indicated a rate of 23.1 ± 0.3 s⁻¹ when fitted to a single-exponential function.

FIG. 10. The concentration dependence of the As(III)-induced dissociation of the ArsD-Sb(III) complex. A series of stopped flow records were generated by mixing 1 μM ArsD/20 μM Sb(III) with As(III), at the indicated concentrations, in a stopped flow device. The rates of the fast (top panel) and intermediate (bottom panel) increase in the fluorescence of ArsD are plotted as functions of the As(III) concentration. The rate of the fast phase increased in a linear manner, with a second-order rate constant of 0.52 ± 0.04 mM⁻¹ s⁻¹; although the maximal rate could not be defined, the rate of the intermediate phase clearly increased in a sigmoidal manner.
 ArsD produces about a 56% quench in the protein fluorescence, whereas As(III) causes only a 26% quench, allowing the displacement of Sb(III) by As(III) to be monitored as an increase in protein fluorescence (17). A couple of illustrative stopped flow traces are shown in Fig. 9 for the mixing of 1 μM ArsD/20 μM Sb(III) with 1 mM (trace A) and 30 mM (trace C) As(III). Although the total signal amplitude, measured as the difference between the initial and final fluorescence of ArsD in each trace, only increased from 27 to 31%, there was clearly a dramatic increase in the rate of Sb(III) displacement upon increasing the As(III) concentration from 1 to 30 mM. The trace for 1 mM As(III) was adequately defined by a single exponential function, indicating a rate of 0.0091 ± 0.0001 s⁻¹ (intermediate phase or phase 2), whereas the trace for 30 mM As(III) was more complex, being best fitted to a triple exponential function with rate constants of 266 ± 5 s⁻¹ (intermediate phase 1, 43% of total signal amplitude), 0.03 ± 0.005 s⁻¹ (intermediate phase 2, 51.9% of total signal amplitude), and 0.588 ± 0.005 s⁻¹ (57% of total signal amplitude). The rates of the fast (1) and intermediate (2) phases increase in a sigmoidal manner, whereas that for the slow phase was apparently independent of the Sb(III) concentration. The curve through the data points for the fast phase is the best fit to a sigmoidal equation (Equation 2), indicating values for the Hill coefficients, \( k_{\text{Hill}} \), and \( K_{\text{Hill}} \), of 2.0 ± 0.3, 38 ± 2 μM, 225 ± 15 s⁻¹, and 59 ± 8 s⁻¹, respectively. The corresponding data for ArsD (©) are shown for comparison, illustrating the higher degree of cooperativity for ArsD in comparison with C12A/C13A ArsD.

Sb(III) by different concentrations of As(III), the As(III) concentration dependence of the rate of formation of the C12A/C13A ArsD-Sb(III) complex was examined. Both phase 1 and 3 were only well defined at the higher As(III) concentrations used, where these phases represented a reasonably substantial proportion of the total signal amplitude. Thus, for As(III) concentrations above 6 mM, the data were fitted to a single exponential function, but below this concentration, the data were fitted to a single-exponential function. As shown in Fig. 10, the rate of phase 1 (Fig. 10, top panel) increased in an apparently linear manner with the As(III) concentration, whereas phases 2 (Fig. 10, bottom panel) and 3 (data not shown) increased in a sigmoidal manner. For As(III) concentrations above 30 mM, the amplitudes of phases 2 and 3 rapidly declined, and eventually these phases were replaced by a slow decrease in fluorescence (Fig. 9, trace B), making it impossible to obtain data at sufficiently high concentrations to define the maximal rates of phases 2 or 3. However, under such conditions the fast phase was still apparent (Fig. 9, trace D), and the rate of this phase was determined for concentrations as high as 200 mM As(III) (\( k = 132 \) s⁻¹); no
deviation from a linear dependence was observed (data not shown). This behavior leads us to attribute this fast phase to the binding of As(III) to ArsD, which is rate-limited by the association of As(III) with ArsD, rather than by Sb(III) dissociation from ArsD. Furthermore, because the association of As(III) with the dissociating ArsD-Sb(III) complex was not rate-limited by the slow conformational change that rate limits As(III) association with unliganded ArsD (Fig. 6; $k_{\text{max}} = 8.7$ s$^{-1}$), this suggests that dissociating Sb(III) leaves ArsD in a similar conformational state to that of the final ArsD-As(III) complex. In other words, there is rapid exchange of metalloids on ArsD. Interestingly, because the fast phase in the As(III)-induced dissociation of the ArsD-Sb(III) complex only accounts for about a quarter of the total signal amplitude, it is tempting to suggest that this phase corresponds to only one site. This would explain the disappearance of the intermediate and slow increase in fluorescence, which are replaced by a decrease in fluorescence for As(III) concentrations above 30 mM; the binding of As(III) to this fast exchange site induces a quench in the ArsD fluorescence that eventually overrides the increase in fluorescence because of Sb(III) release from the other sites. However, nearly saturating concentrations of As(III) only quench the fluorescence of ArsD by about 25%, whereas the maximum difference in the Sb(III) and As(III) quenches is about 30%. How then can As(III) binding to only one site override the increase in fluorescence because of Sb(III) release from the other sites? An alternative explanation might be that As(III) binding to the fast exchange site retards the release of Sb(III) from the other sites. To test this hypothesis 1 $\mu$M ArsD/20 $\mu$M Sb(III) was mixed with 5 and 50 mM As(III) in the fluorimeter; as expected, 5 mM As(III) induced a slow increase in fluorescence of about 35%, whereas 50 mM As(III) induced a rapid increase in fluorescence but of only about 10%, thus suggesting that 50 mM As(III) blocked the slow release of Sb(III) from ArsD (Fig. 11), which is consistent with the other sites only very slowly exchanging metalloids.

In common with the kinetics of As(III) binding to unliganded ArsD, the rate of phase 2 increased in a sigmoidal manner (Fig. 10, bottom panel). Moreover, this phase occurred at a rate similar to that for the binding of As(III) to unliganded ArsD (Fig. 6). However, although the rate of phase 2 was still increasing at concentrations above 30 mM As(III), the rate of binding of As(III) to unliganded ArsD had reached a saturable level with 30 mM As(III). This difference could easily be explained by the Sb(III) competing with the As(III) for binding to the remaining sites. Indeed, assuming simple competition, the $K_{\text{app}}$ for As(III) would increase from about 7 to 89 mM, i.e., the apparent $K_{\text{app}} = K_{\text{d,As(III)log}}(1 + [\text{Sb(III)}]/K_{\text{d, Sb(III)}})$, with a $K_{\text{d, Sb(III)}}$ of 1.7 $\mu$M and for As(III) of 7 mM, in the presence of 20 $\mu$M Sb(III). Under these conditions, 30 mM As(III) would only give 25% saturation of the ArsD, and the rate of binding of As(III) should be about 2.2 s$^{-1}$, which is reasonably consistent with the measured value of 1.3 s$^{-1}$ (Fig. 10, bottom panel). We propose that, following the rapid binding of As(III) to the first unoccupied site, binding of As(III) to the remaining sites of ArsD is rate-limited by the same conformational change that rate limits As(III) association with unliganded ArsD.

Characterization of the Kinetics of the Binding of Sb(III) to C12A/C13A ArsD—To elucidate the origins of the cooperativity in metalloid binding to ArsD, the kinetics of the binding of Sb(III) to an ArsD substitution in which one of the binding sites had been disrupted by mutation of $arsD$ was examined. For this analysis C12A/C13A ArsD was used because steady-state studies revealed that this protein was optically responsive to the binding of metalloids, whereas only small changes in the fluorescence of C112A/C113A ArsD were apparent, severely hindering detailed stopped flow fluorescence studies of this protein. The stopped flow traces for the binding of 120 $\mu$M Sb(III) to ArsD and C12A/C13A ArsD are compared in Fig. 12. In the case of C12A/C13A ArsD, there was no intermediate binding phase (e.g., phase 2) as found for ArsD, and the data were adequately fitted to a biphasic exponential function with rate constants of 266 ± 5 s$^{-1}$ (43% of total signal amplitude) and 0.588 ± 0.006 s$^{-1}$ (57% of total signal amplitude). In contrast to ArsD, only the fast phase of the binding of Sb(III) to C12A/C13A ArsD was characterized by a single concentration dependent phase, equivalent to phase 1 of the wild type, whereas phase 2 was clearly missing. These data suggest that phase 2 is attributable to metalloid binding to the Cys$^{12}$-Cys$^{13}$ site. The apparent rate of binding of Sb(III) to C12A/C13A ArsD increased in a sigmoidal manner but with less sigmoidicity than for ArsD (Fig. 13). As for the wild type, the first few data points deviated systematically from the best fit curve, consistent with our hypothesis that the fluorescence quench at these low concentrations is attributable to the initial filling of two of the sites, which must be the Cys$^{112}$-Cys$^{113}$ sites. As might be expected, a fit of the data points above 20 $\mu$M indicated a Hill coefficient of 2.0 ± 0.3, presumably because the Cys$^{12}$-Cys$^{13}$ sites are unavailable for metalloid binding.

The kinetics of the displacement of Sb(III) from C12A/C13A ArsD by DTT were identical to those for wild type ArsD (Fig. 8). In many respects the kinetics of Sb(III) displacement from C12A/C13A ArsD by As(III) also resembled those of the wild type but with two significant differences. First, the appearance of the decrease in protein fluorescence that overrides the As(III)-induced increase in fluorescence occurs at much lower concentrations of As(III) (~10 mM as compared with 30 mM for the wild type). Consequently, none of the stopped flow traces for the increase in fluorescence induced by As(III) could be resolved into both fast and slow phases. Clearly though, for As(III) concentrations above 10 mM, there is a fast phase, because the rate of increase in protein fluorescence induced by As(III) is fast and increases linearly above this critical concentration (data not shown). Second, the concentration dependence curve for the slow phase is displaced to the left of that for the wild type (Fig. 14). The displacement of the data for C12A/C13A ArsD relative to that for ArsD might be attributed to the
Sb(III) competing less effectively with As(III) for binding to C12A/C13A ArsD than ArsD. However, 30 mM As(III) was insufficient to cause near saturation of C12A/C13A ArsD, whereas 10 mM As(III) was sufficient to cause near saturation of unliganded ArsD, suggesting that Sb(III) is still competing with As(III) for binding to C12A/C13A ArsD. A more plausible explanation for the displacement of the data for C12A/C13A ArsD relative to that for ArsD is to attribute this effect to a reduced degree of cooperativity in the binding of As(III) to C12A/C13A ArsD relative to that for ArsD.

DISCUSSION

Although the mechanisms underlying the binding of trans-acting repressors to their target operator have been studied in detail, often identifying cooperativity in the binding process, there have been few detailed studies of the mechanisms of binding of inducers to these repressors that trigger the conformational changes that cause dissociation from the operon. To date, the most detailed studies have been performed on the tetrameric LacI and dimeric TetR repressors, inserting tryptophan residues at defined positions within tryptophan-free derivatives and monitoring the exposure of these residues as the protein undergoes conformational changes induced by the binding of the inducer (18–21). These studies have been useful for elucidating the protein dynamics and in conjunction with structural data derived from x-ray crystallographic studies used to model the conformational changes that induce dissociation from the operon (22, 23). However, the conformational changes induced by the binding of the inducers have not been time-resolved, nor has any cooperativity between the inducer-binding sites been explored in those studies. Here we describe the kinetics of binding of metalloids to ArsD and cooperativity between the metalloid-binding sites of ArsD, a trans-acting repressor of the arsRDABC operon that confers resistance to arsenicals and antimonials in E. coli, were examined.

In a previous study, in vivo assays of repression and induction and in vitro studies of DNA binding revealed that both the Cys119-Cys120 and Cys112-Cys113 pairs but not the Cys119-Cys120 pair were required for responses to metalloid inducers (17). According to experiments designed to test whether these sites act independently in triggering the conformational changes in ArsD that induce it to dissociate from the operon, titration of the protein fluorescence of ArsD (in which Cys119 and Cys120 had been deleted) revealed cooperativity in the binding process, which occurred with a Hill coefficient of 2. However, the binding of metalloids to a C12A/C13A substitution, constructed by further mutation of the C119/C120 ArsD deletion, was also cooperative, with a Hill coefficient of 2, indicating that cooperativity exists between the Cys112-Cys113...
sites of adjacent ArsD monomers within the functional dimer. Consistent with this finding, C112A/C113A ArsD displayed little cooperativity, C12A/C13A/C112A ArsD was noncooperative, and C12A/C13A/C112A/C113A ArsD was unresponsive. On the other hand, the Cys12-Cys13 pair is required for inducibility, suggesting that there are interactions between the two metalloid-binding sites within the ArsD monomer. The maximal fluorescence quench of C112A/C113A ArsD but not C12A/C13A ArsD was substantially reduced, indicating that the fluorescence changes are largely reporting on effects at the Cys112-Cys113 sites. Indeed, our previous studies indicated that, of the two tryptophans present in ArsD, Trp35 and Trp97, only Trp97, which is presumably close to the Cys112-Cys113 site, reports metalloid binding to ArsD (17).

Stopped flow studies revealed that the binding of metalloids is a rapid process that is rate-limited by a change in the conformation of the ArsD-metalloid complex. Formation of this conformational state, which presumably has a lower affinity for the operon, is 25-fold faster for Sb(III) than As(III), consistent with Sb(III) being a better inducer. The rate of this conformational change increases as a function of Sb(III) concentration with a Hill coefficient of 4, indicating that all four binding sites of the dimer interact in a positive cooperative manner. Binding of Sb(III) to C12A/C13A ArsD was also rate-limited by a conformational change in the C12A/C13A Ars-Sb(III) complex that occurred at a similar rate to that for ArsD. However, the binding process was characterized by a Hill coefficient of 2, consistent with the deletion of the Cys12-Cys13 sites within the dimer. Furthermore, although two concentration-dependent phases for the binding of Sb(III) to ArsD were apparent, only one concentration-dependent phase was apparent for C12A/C13A ArsD, suggesting that the second slow phase for ArsD is attributable to the binding of Sb(III) to the Cys12-Cys13 sites. Binding of Sb(III) to these sites induces a 10-fold slower conformational change in the ArsD-Sb(III) complex, which is presumably necessary for induction.

Interestingly, the binding sites behave differentially, with the replacement of Sb(III) by As(III) at one site rate-limited by the bimolecular association of As(III) with ArsD rather than by the subsequent conformational change in the ArsD-As(III) complex that rate limits the binding process at the other sites, but this behavior contrasts with that of DTT, which apparently induces rapid dissociation of bound Sb(III) from all the sites. Indeed, binding of As(III) to this so-called fast exchange site occludes the Sb(III) from being replaced by As(III) at the other sites. Furthermore, the kinetics of the binding of Sb(III) indicates that at least one site must near saturation before the subsequent conformational changes in the ArsD-Sb(III) complex are triggered by binding of Sb(III) to the other sites. In these respects, this site can bind Sb(III) independently of the other sites and appears to play a regulatory role over the other sites. This site must be one of the Cys12-Cys13 sites within the dimer because similar kinetic behavior is noted for C12A/C13A ArsD, which lacks the Cys12-Cys13 sites. How can we account for this behavior? One possibility is that the binding sites fill and empty sequentially, as indicated by their positive cooperativity, because of metalloid exchange between the sites. We propose that As(III) is able to exchange with Sb(III) at the fast exchange site but that this blocks release from the other sites because the Sb(III) in these sites only dissociate by exchange with the fast exchange site, but this would be very slow because the other sites will have a higher affinity for metalloids because of the positive cooperativity (Fig. 15A). In other words, there is sequential exchange of the metalloids, with only the fast exchange site liberating free metalloid (Fig. 15B). Furthermore, to account for the cooperativity between the subunits of the ArsD dimer, which occurs with a Hill coefficient of 4, we propose that the binding sites are formed by and between the equivalent cysteine residues in each subunit (i.e. the Cys113 residues in each subunit form a binding site). Indeed, the fact that a C112A/C113A/C12A substitution of ArsD, which only possesses one cysteine residue, can still bind metalloids implies that each monomer contributes a cysteine residue to each metalloid-binding site. In contrast, metalloid exchange can occur between each site and bound DTT, facilitating the rapid dissociation of metalloids from ArsD. Such exchange of metalloids between thiol-containing binding sites has been noted previously for both organic molecules (24) and proteins (25). Indeed, ArsD may be useful as a model in understanding how metal exchange occurs between proteins, such as between copper chaperones and their target proteins (26–28).

Apparently at odds with the simple role proposed for ArsD, that of primarily acting as an unliganded repressor that prevents the expression of ArsB at levels that become toxic for the cell, the present investigation has revealed a complex pattern of positive cooperativity between the metalloid-binding sites. One might have expected that negative cooperativity between these sites, with only the fully complexed protein being released from the operon, would have been more consistent with such a role. However, consider the isothermal curve for the formation of the initial complex that triggers the conformational change that presumably leads to induction (Fig. 4). The first site binds Sb(III) with a $K_{1/2}$ of about 10 μM, but full activation requires all the sites to be occupied (which is, in effect, negative cooperativity), and full activation occurs with a $K_{1/2}$ of about 44 μM. Furthermore, the isothermal curve for the C12A/C13A ArsD derivative, which only has two cooperating sites, is displaced to the left of the curve for ArsD (Fig. 13). Accordingly, although there is positive cooperativity in the binding of Sb(III) to ArsD, these interactions also cause the titration curve for formation of the initial complex to be displaced to the right, decreasing the overall affinity of ArsD for Sb(III) and increasing the concentration of Sb(III) needed for induction. These features of ArsD would be useful in allowing it to control the upper limit of expression of the operon. However, considering the tightness and capacity for binding metalloids by ArsD, it might play an additional role in buffering the free concentration of toxic metalloids within the cytoplasm and could conceivably act as a chaperone to deliver these metalloids to the pump.

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