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Imparting As(III) Responsiveness to the Choline Response Transcriptional Regulator BetI

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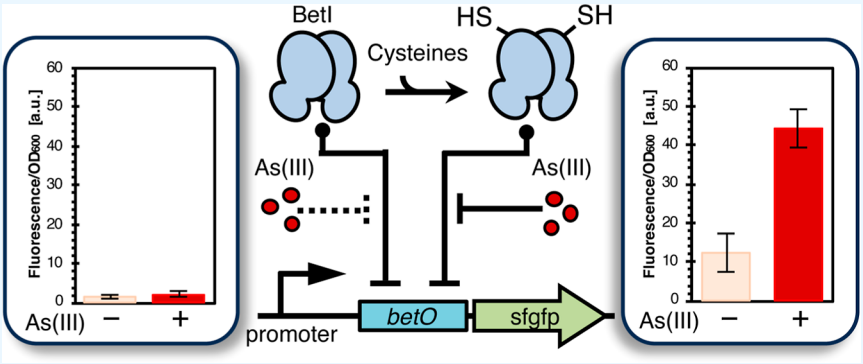

ABSTRACT: The development of a low-cost and user-friendly sensor using microorganisms to monitor the presence of As(III) on earth has garnered significant attention. In conventional research on microbial As(III) sensors, the focus has been on transcription factor ArsR, which plays a role in As(III) metabolism. However, we recently discovered that LuxR, a quorum-sensing control factor in *Vibrio fischeri* that contains multiple cysteine residues, acted as an As(III) sensor despite having no role in As(III) metabolism. This finding suggested that any protein could be an As(III) sensor if cysteine residues were incorporated. In this study, we aimed to confer As(III) responsiveness to BetI, a transcriptional repressor of the TetR family involved in osmotic regulation of the choline response, unrelated to As(III) metabolism. Based on the BetI structure constructed using molecular dynamics calculations, we generated a series of mutants in which each of the three amino acids not critical for function was substituted with cysteine. Subsequent examination of their response to As(III) revealed that the cysteine-substituted mutant, incorporating all three substitutions, demonstrated As(III) responsiveness. This was evidenced by the fluorescence intensity of the downstream reporter superfolder green fluorescent protein expression regulated by the operator region. Intriguingly, the BetI cysteine mutant maintained its binding responsiveness to the natural ligand choline. We successfully engineered an OR logic gate capable of responding to two orthogonal ligands using a single protein.

INTRODUCTION

Arsenite [As(III)] naturally occurs throughout the earth. It is toxic, and its toxicity varies depending on its chemical form, with inorganic As(III) being particularly hazardous. The World Health Organization has established a safe limit of 10 ppb for As(III) concentration, highlighting the crucial importance of monitoring and detecting As(III) in the environment.[1] Detection of As(III) in the environment usually involves the use of expensive analytical equipment such as atomic absorption spectroscopy, gas chromatography−mass spectrometry, and induced coupled plasma-mass spectrometry.[2−4] Although these instruments offer high sensitivity, their substantial cost and the need for specialized expertise in both operation and analysis circumscribe their accessibility to the general public.

The main focus of As(III) detection has been on using the arsenic resistance (*ars*) gene system in bacteria, which is associated with As(III) metabolism. When As(III) binds to the transcriptional regulator of the *ars* operon, known as ArsR, it releases the binding between ArsR and the DNA operator region in P_{ars},[5−7] which results in the lifting of transcriptional repression. As an As(III) sensor, this system detects the expression of reporter genes such as β-galactosidase or green fluorescent protein (GFP) downstream of P_{ars}.[8−13] As(III) is known to have high affinity for the thiol group of cysteine,[13,14] and ArsR has an As(III)-binding domain that contains three cysteines, C32, C34, and C37.[15−17] ArsR undergoes significant changes in structure when As(III) binds to these cysteines,

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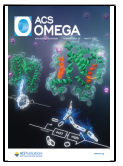

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leading to its dissociation from P_{ars} and the subsequent lifting of transcriptional repression.[7] Because this system is well-established, it is widely used in whole-cell sensors.[18] Recently, we reported that LuxR, the quorum-sensing regulator in *Vibrio fischeri*,[19−21] which possesses multiple cysteine residues, can function as an As(III) sensor despite having no functional relationship with As(III) metabolism. LuxR contains nine cysteine residues and is precipitated when the As(III) concentration exceeds 10 ppm, resulting in inhibition of its transcriptional activation function.[22] This finding implies that by leveraging the binding of As(III) with cysteine residues to induce structural changes or aggregation of proteins, any protein can be engineered to act as an As(III)-responsive sensor.

The aim of this study was to engineer an As(III)-responsive protein from scratch by introducing cysteine residues into BetI, a choline-responsive transcriptional repressor that lacks cysteine residues (Figure 1). In *Escherichia coli* (*E. coli*), the

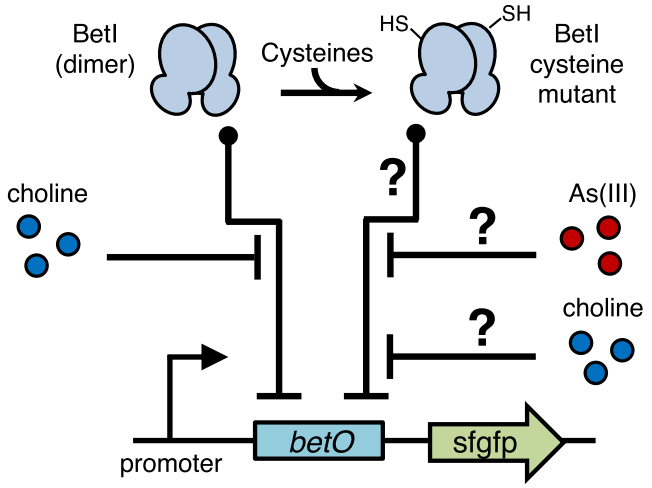

Figure 1. Schematic illustration of imparting As(III) responsiveness to the choline response protein BetI by introducing cysteine residues. Choline-responsive transcriptional repressor BetI regulates the expression of downstream reporter superfolder GFP (sfGFP) by binding and detachment to the *betO* regulatory sequence. A cysteine (SH) can be introduced at a position designed for BetI to respond to As(III).

bet operon is known to respond to three external stresses, choline, oxygen, and osmotic pressure, with its expression regulated by BetI.[23,24] BetI belongs to the TetR family.[25] In the absence of choline, BetI binds to the DNA operator sequence *betO*, thereby suppressing transcription of the downstream *bet* operon. No other ligands or chemical species that BetI responds to have been reported. In this study, we have engineered BetI so that it responds to As(III), while preserving its cognate function as choline-responding transcription factors. As a result, we successfully constructed a BetI/*betO* system that behaves as an As(III)/choline OR logic gate.

RESULTS AND DISCUSSION

Response to As(III) in Cysteine-Free Protein BetI and Its Cysteine Substitution. In a previous study,[22] we discovered that proteins containing cysteine residues can respond to As(III). This responsiveness to As(III) was not limited to proteins involved in As(III) metabolism, such as the Ars family.[22] Building upon this finding, we hypothesized that proteins that lack natural cysteine residues could be engineered to develop an As(III)-dependent functional switch system by introducing cysteine residues by artificial design. In this study, our goal was to create such a system using BetI, a choline-responsive transcriptional repressor in *E. coli* that does not contain cysteine residues. To confer As(III) responsiveness, we artificially introduced cysteine residues into BetI. Previously, we performed evolutionary engineering to enhance the responsiveness of BetI to choline because the wild-type BetI exhibited very low response to choline; the fluorescence intensity of the reporter sfGFP was too low to detect choline binding.[26] Introduction of the I22T mutation by directed evolution significantly improved the responsiveness of BetI to choline, resulting in $BetI_{I22T}$, a highly sensitive transcriptional regulator for choline induction. In this study, we used $BetI_{I22T}$ as the parent protein and confirmed that it did not respond to As(III) and did not lose its function when As(III) was added by examining its responsiveness to As(III) and choline. *E. coli* strain MG1655 transformed with the BetI expression plasmid pAC-P_{j23116}-$betI_{I22T}$ and the reporter plasmid pET23d-$P_{T5(betO)}$-sfgfp[27] was cultured in Luria−Bertani (LB) liquid medium supplemented with 10 ppm As(III) or 100 mM choline chloride. The fluorescence intensity of sfGFP was monitored to assess the As(III) and choline responsiveness of $BetI_{I22T}$, confirming that it is nonresponsive to As(III) (Figure 2a, left panel).

To confer As(III) responsiveness to $BetI_{I22T}$, a three-dimensional structure of $BetI_{I22T}$ was used for identifying suitable sites for cysteine substitution. Given the absence of an experimentally determined structure, we conducted MD simulations using a homology-modeled structure of $BetI_{I22T}$. The average trajectories and secondary structure analysis showed that the structural model for the $BetI_{I22T}$ homodimer folds into nine α helices and their symmetric counterparts (Figure 2a, right panel), as seen in other members of the TetR family.[25] Helices $\alpha1\alpha3$ and their symmetric counterparts include the helix-turn-helix motif ($\alpha2$-turn-$\alpha3$), which is believed to be the DNA-binding domains. The average Cα−Cα distance of the threonine residues at position 43 (T43) in the helix-turn-helix motifs was 34.6 Å, which is similar to the pitch of canonical B-form DNA, which is approximately 34 Å.[28] Helices $\alpha4\alpha9$ and their symmetric counterparts can form a regulatory core domain that includes cavities for putative choline-binding sites (Figure 2a, right panel).

To determine the cysteine substitution sites for $BetI_{I22T}$, we focused on three amino acid residues, L79, Q81, and S102. The MD analysis indicated that L79 and Q81 are located at a reverse turn that connects helices $\alpha4$ and $\alpha5$, and S102 is located at a turn between helices $\alpha5$ and $\alpha6$ (Figure 2a, right panel). Being placed in loops, these residues were expected to be mutation-tolerant, as was supported by FoldX calculation (Figure S1). Additionally, these three residues are not involved in either putative DNA-binding or choline-binding domains. Further MD analysis on $BetI_{3cys}$, wherein L79, Q81, and S102 amino acids in each $BetI_{I22T}$ monomer were replaced with cysteine, did not produce a large structural deformation (Figure 2b, right panel); the average Cα−Cα distance of T43 in the helix-turn-helix motifs was 35.0 Å, which is similar to that of $BetI_{I22T}$. The observed fluorescence intensity when assessing the choline responsiveness of $BetI_{3cys}$ also indicated that $BetI_{3cys}$ retains its functional structure (Figure 2b, left panel). These results strongly suggest that L79, Q81, and S102 are good sites for replacement with cysteine residues.

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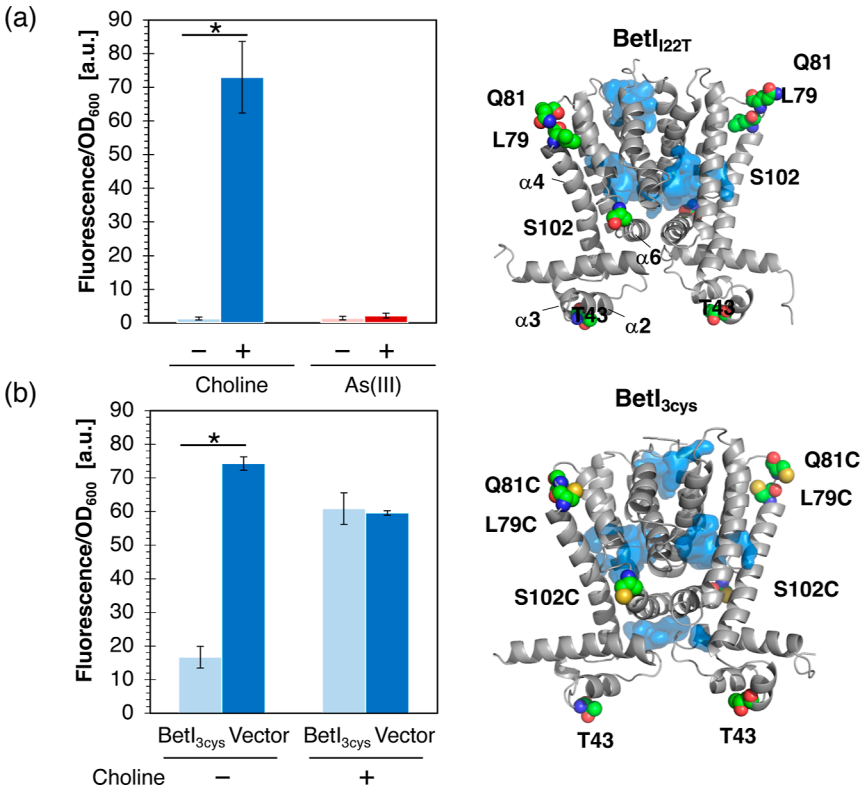

Figure 2. Response to As(III) in cysteine-free protein $BetI_{I22T}$and response to choline in cysteine-substituted protein $BetI_{3cys}$. (a) Responsiveness of $BetI_{I22T}$ to choline and As(III) (left), and representative molecular dynamics (MD) snapshot of $BetI_{I22T}$ (right). Left: Choline concentrations are 0 (−) and 100 mM (+) and As(III) concentrations are 0 (−) and 10 ppm (+). Each bar is the average of three independent biological replicates; error bars indicate standard deviations. Asterisk (*) indicates a significant difference ($p < 0.05$) compared with the absence of choline (unpaired, two-tailed Student's *t*-test). Right: three amino acid residues (L79, Q81, and S102), the cysteine-substituted mutants, and T43 are shown as balls; internal cavities are shown in blue. (b) Transcriptional repression of $BetI_{3cys}$ in the absence of choline and its responsiveness to choline compared to a strain without sensor plasmid denoted as the vector (left), and representative MD snapshot of $BetI_{3cys}$ (right). Left: Choline concentrations are 0 (−) and 100 mM (+). Asterisk (*) indicates a significant difference ($p < 0.05$) compared to the vector (unpaired, two-tailed Student's *t*-test).

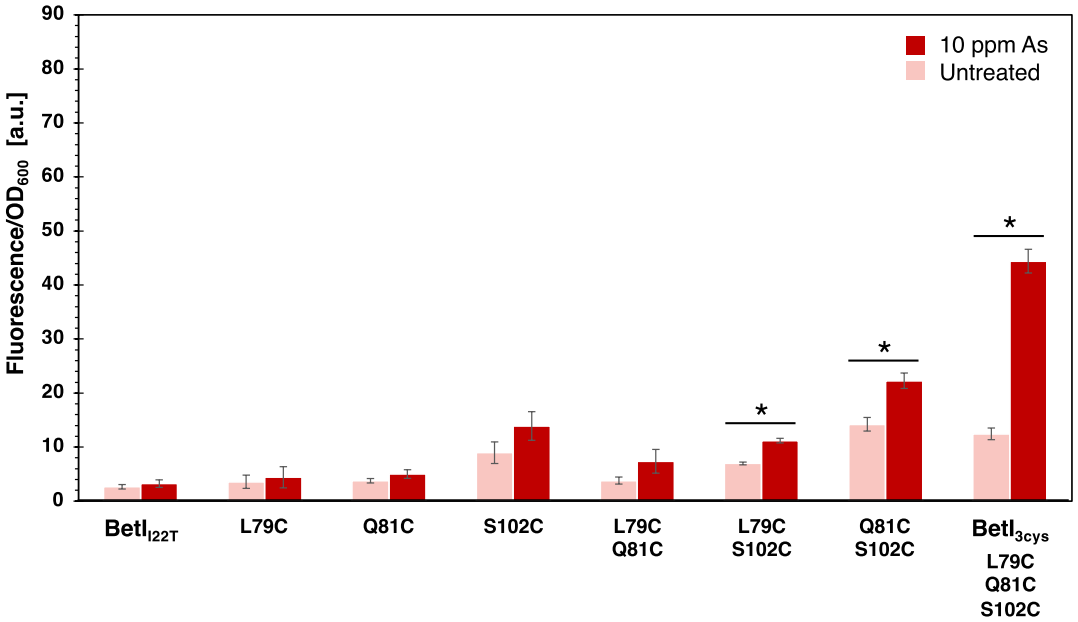

Figure 3. Responsiveness of the BetI cysteine mutants to As(III) with sfGFP as the reporter. Fluorescence of the sfGFP was scored in the presence (red) and absence (pink) of 10 ppm of As(III). Each bar represents the mean value derived from three independent biological replicates. Error bars indicate standard deviations. Asterisks (*) denote statistically significant differences ($p < 0.05$) in comparison to the control group without As(III), as determined by an unpaired, two-tailed Student's *t*-test.

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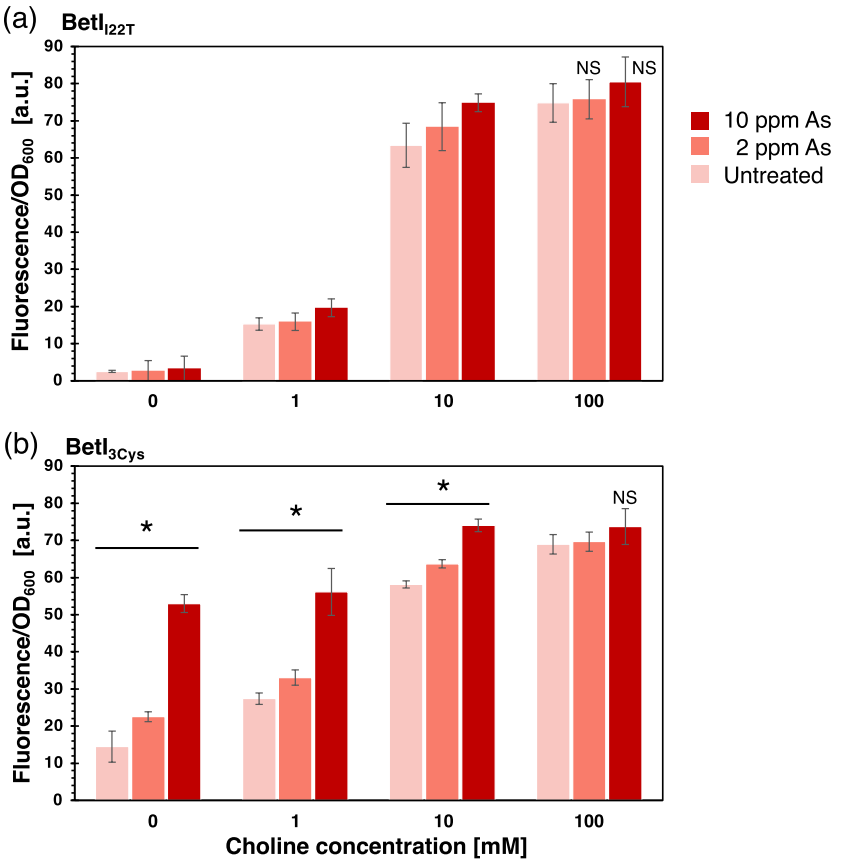

Figure 4. Two-input OR logic gate of BetI$_{3cys}$. (a) Fluorescence output characteristics of BetI$_{I22T}$ in response to varying As(III) and choline concentrations. Each bar is the mean value derived from three independent biological replicates; error bars indicate the standard deviations. NS indicates not significant ($p > 0.05$) compared with the same As(III) concentration in 10 mM choline concentration (unpaired, two-tailed Student's *t*-test). (b) Fluorescence output characteristics of BetI$_{3cys}$ in response to varying As(III) and choline concentrations. Each bar is the mean value derived from three independent biological replicates; error bars indicate standard deviations. Asterisk (*) indicates a significant difference of each As(III) concentration ($p < 0.05$) compared with the absence of As(III) (unpaired, two-tailed Student's *t*-test). NS indicates not significant ($p > 0.05$) compared with the same As(III) concentration in 10 mM choline concentration.

Imparting As(III) Responsiveness via Cysteine Substitution. To test whether the introduction of cysteine substitutions at L79, Q81, and S102 conferred As(III) responsiveness to BetI$_{I22T}$, a cysteine-free protein, we generated seven mutants. Three of the mutants had one cysteine substitution each, and the other four mutants had combinations of any two or all three cysteine substitutions. The response of each mutant to As(III) was examined. *E. coli* strain MG1655 was transformed with plasmids that expressed each of the mutants along with the reporter pET23d-P$_{T5(betO)}$-sfGFP. The resulting colonies were grown in LB liquid medium supplemented with 10 ppm of As(III) or 100 mM choline chloride, and the response to each ligand was evaluated. We found that there was no statistically significant difference in the fluorescence intensity of sfGFP per optical density with or without As(III) in all single cysteine substitution mutants (Figure 3).

Next, we examined the response of the double cysteine substitution mutants to As(III) by combining L79, Q81, and S102 to create double cysteine substitutions (Figure 3). We found that both the double L79C S102C and the double Q81C S102C substitutions increased the As(III) response in the presence of As(III) compared with the absence of As(III). These double mutants displayed a statistically significant difference in fluorescence intensity in the presence or absence of As(III), despite the observation of leakage expressions of sfGFP in the absence of As(III) ($p < 0.05$) (Figure 3).

BetI$_{3cys}$ with all three cysteine substitutions showed a 3.6-fold increase in the ratio of the fluorescence intensity of sfGFP without As(III) to that with As(III) (on/off ratio), indicating a clear response to As(III). The obtained results suggest that this is not an effect of As(III)-induced oxidative stress, for the following reasons. In a separate investigation, we scrutinized the As(III)-induced proteome of *E. coli* under As(III) concentrations of 10 ppm. Our analysis revealed that the expression levels of factors associated with oxidative stress, including the SOD system (sodA and sodB), catalase (katG and katE), and chaperones (hslO), remained unchanged. Furthermore, an assessment of *E. coli* growth, conducted by varying As(III) concentrations from 1 to 62.5 ppm, demonstrated no significant alterations at 10 ppm (in preparation). These findings collectively lead us to posit that no discernible effect of oxidative stress manifests at an As(III) concentration of 10 ppm. Taken together, we suggest that BetI$_{3cys}$ exhibited a heightened responsiveness to As(III) due to the irreversible covalent binding of cysteine to As(III).[17]

Two-Input OR Logic Gate of BetI$_{3cys}$. To investigate the output characteristics of BetI$_{3cys}$ with respect to As(III) and its natural ligand choline, we measured the fluorescence output of BetI$_{3cys}$ at various concentrations of As(III) and choline. The

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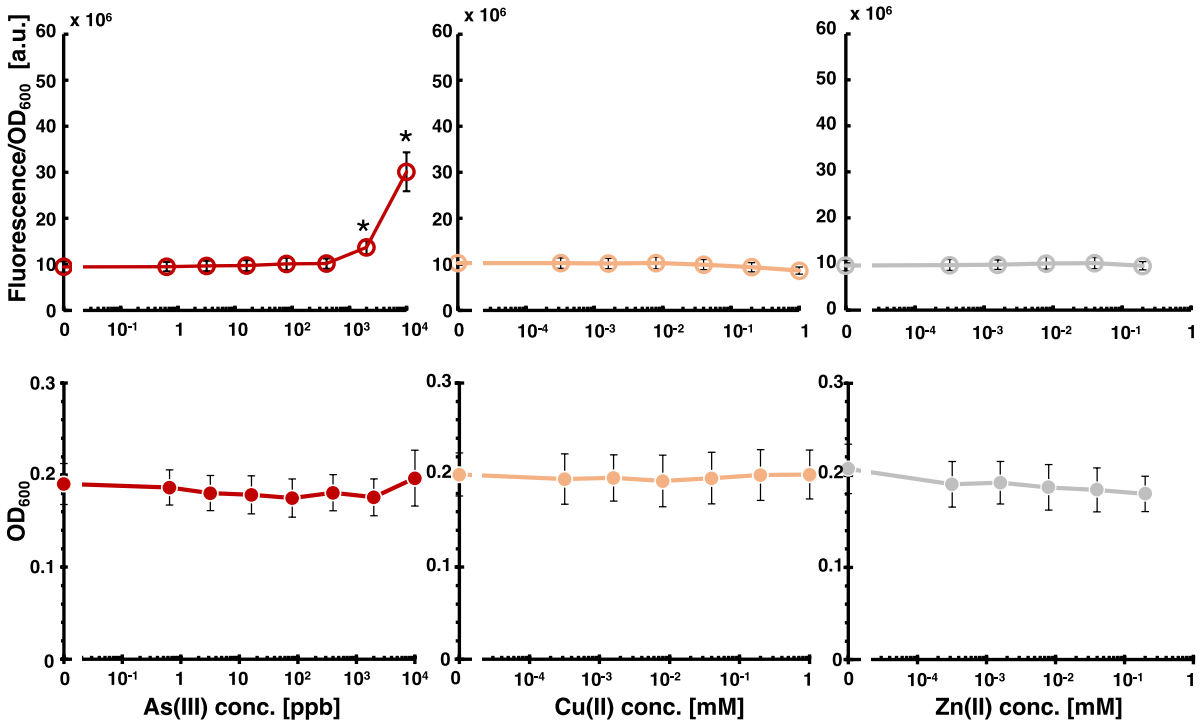

Figure 5. As(III) specificity of $BetI_{3cys}$. Fluorescence output characteristics of $BetI_{3cys}$ in response to varying As(III), Cu(II), and Zn(II) concentrations. Each plot is the average derived from three independent biological replicates; error bars indicate standard deviations. Asterisk (*) indicates a significant difference of each metal ion concentration ($p < 0.05$) compared with the absence of metal ion (unpaired, two-tailed Student's *t*-test).

MG1655 strain expressing $BetI_{I22T}$ without cysteine displayed a high output only when choline was introduced (Figures 4a and S4). On the other hand, in the presence of 2 and 10 ppm As(III) conditions, a significant elevation in fluorescence output of $BetI_{3cys}$ was noted under the choline-free condition compared to the condition without As(III) (Figures 4b and S4). In particular, under the conditions of 10 ppm As(III), a 4-fold increase in fluorescence output was observed compared to the fluorescence output in the absence of As(III) condition. The basal expression based on $BetI_{3cys}$ without As(III) was marginally higher than that of $BetI_{I22T}$. This slight increase is likely attributed to the substitution of cysteines at three specific sites. This structural distortion does not appear to significantly compromise functionality, as the observed effect on expression is subtle.

Next, we investigated the response of $BetI_{3cys}$ in the presence of both As(III) and choline ligands. On increasing the choline concentration to 0, 1, and 10 mM, we observed a significant enhancement in fluorescence output on the addition of As(III), along with an additive response to choline. This was evident when comparing the conditions with no As(III) and 2 ppm of As(III) at each choline concentration. The 10 ppm of As(III) condition also exhibited an additive response to both ligands, similar to the response observed with 2 ppm of As(III). However, under the 100 mM choline conditions, the fluorescence values were nearly identical across all As(III) concentrations, with no significant difference in fluorescence output between the various As(III) concentrations. Furthermore, the fluorescence output for 10 ppm As(III) at each choline concentration displayed a notable increase due to choline, in addition to the As(III) response, when comparing the 1 and 10 mM choline concentrations. Nevertheless, the fluorescence value reached a plateau at the 10 mM choline concentration and did not increase further at the 100 mM choline concentration (Figure 4b).

These results indicate that $BetI_{3cys}$ maintains its ability to bind choline (Figures 4b and S3). The results of the examination of the choline response for all cysteine substitutions indicate that each substitution exhibited choline responsiveness equivalent to that of $BetI_{I22T}$. These findings suggest that the cysteine substitutions at these locations do not impede the binding affinity for choline or the conformational changes triggered by choline binding (Figure S3). The lack of an additive As(III) response when 100 mM choline was added may indicate that the amount of choline within the cells was relatively higher than the amount of As(III), potentially masking the As(III) response of BetI through the choline response. This implies that $BetI_{3cys}$ can function as an OR-type logic gate, responding to two orthogonal ligands as input factors with a single protein as the output.

Testing of $BetI_{3cys}$ Responsiveness to Other Metal Ions. In order to use $BetI_{3cys}$ as an As(III) sensor, specificity for As(III) is required. Therefore, to investigate the response of $BetI_{3cys}$ to other metals, the fluorescence output was measured by using Cu(II) and Zn(II). In the concentration range that did not affect the survival of *E. coli*, $BetI_{3cys}$ showed a clear response only to As(III) but not to both Cu(II) and Zn(II) metals (Figure 5). For Cu(II), a decrease in viability was observed at concentrations higher than 1 mM, while for Zn(II), viability was maintained down to 0.2 mM. These results indicate that $BetI_{3cys}$ is a sensor that specifically responds to As(III) in living cells.

Implications of As(III) Binding on the $BetI_{3cys}$ Structure: Insights from MD Simulations. To understand the effect of As(III) binding to the $BetI_{3cys}$ structure, we performed MD simulations on its As(III)-bound form. The

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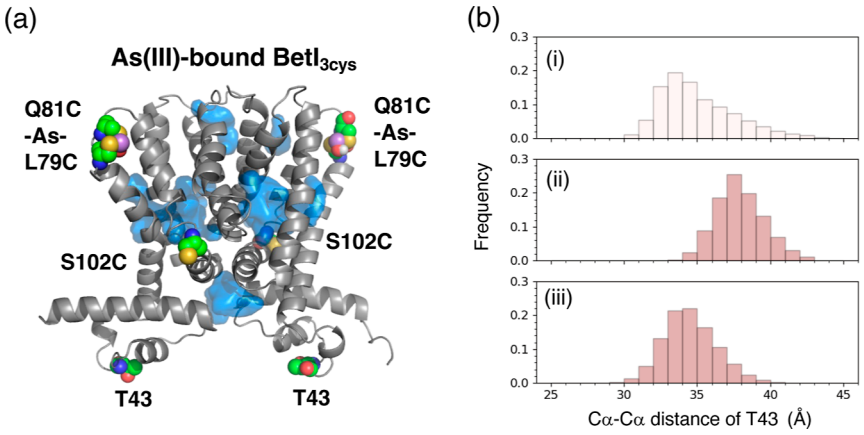

Figure 6. MD observation of As(III) binding's influence on BetI proteins. (a) Representative MD snapshot of As(III)-bound $BetI_{3cys}$ in which As(III) binds to L79C and Q81C on both monomers of the $BetI_{3cys}$ dimer. As(III) atoms are represented by purple spheres, while residues L79C, Q81C, S102C, and T43 are shown as balls. Internal cavities are shown in blue. (b) Cα−Cα distance of T43 in the helix-turn-helix motifs for $BetI_{3cys}$; (i) no As(III) bound to $BetI_{3cys}$, (ii) As(III) bound to L79C and Q81C on both monomers of the $BetI_{3cys}$ dimer, and (iii) As(III) bound to L79C and Q81C of only one monomer in the $BetI_{3cys}$ dimer.

model of the As(III)-bound form was constructed by coordinating As(III) to the L79C and Q81C on both monomers of the $BetI_{3cys}$ dimer. In this model, As(III) was assumed to form covalent bonds with L79C and Q81C, forming L79C-S−AsOH−S-Q81C (Figure 6a); this configuration is informed by the experimental evidence suggesting that $As(OH)_3$ has the most stable structure in solution at neutral pH.[29] Such a configuration could be feasible owing to the potential of the cysteines of L79C and Q81C to act as dithiol sites. Generally, dithiol sites, composed of two cysteines separated by a few amino acid residues, can easily be occupied by trivalent arsenicals, exhibiting a K_d value ranging from 1 to 20 μM.[30] This range is much lower than the present As(III) concentration of 10 ppm (about 130 μM). Furthermore, the L79C and Q81C residues constitute a CXC amino acid sequence (C and X represent cysteine and any amino acid residue, respectively), which is analogous to the sequence of the As(III)-binding domain of ArsR.[17] As a point of comparison, we constructed an alternative model of the As(III)-bound form by binding As(III) to L79C and Q81C of only one monomer in the $BetI_{3cys}$ dimer.

Figure 6b shows the Cα−Cα distance of T43 in the helix-turn-helix motifs for the As(III)-unbound and -bound states of $BetI_{3cys}$. When As(III) was bound to L79C and Q81C on both monomers of the dimer, the median Cα−Cα distance of T43 increased by approximately 3 Å compared with the distance in the absence of As(III) (Figure 6bi,ii). Such elongation was not observed when As(III) was bound to L79C and Q81C of only one monomer (Figure 6biii). The increase in the median Cα−Cα distance of the T43 residues could be triggered by two structural changes. First, the binding of As(III) to L79C and Q81C resulted in a shift of helices α4 and α5 (Figure S2a). Secondary structure analysis showed that the occurrence of the α helical structure decreased at the C-terminal part of helix α4 (residues 74−78) and N-terminal part of helix α5 (residues 84 and 85) (Figure S2b); a significant decrease in the frequency of the bend structure was also observed at residues 79 and 83 (Figure S2b). Second, the displacement in helices α4 and α5 caused a shift of helices α3 and α6 in a similar direction as that observed for helices α4 and α5 (Figure S2a), with an increase in the α helical structure at the N-terminal region of helix α4 (residues 53 and 54) and helix α6 (residues 103−105) (Figure S2b). These structural features are similar to the conformational changes seen in members of the TetR family, where a pendulum-like motion of helix α4 resulted in an increase in the separation of the DNA-binding domains.[25] Thus, we hypothesize that the binding of As(III) to $BetI_{3cys}$ turns off the DNA-binding ability through an on/off switch mechanism similar to that of TetR. While this notion is speculative at this stage, it offers a compelling conceptual framework for the As(III)-mediated functional modulation of proteins.

CONCLUSIONS

In this study, we succeeded in imparting As(III) responsiveness to $BetI_{I22T}$, a cysteine-free protein that does not exhibit a response to As(III), by introducing cysteines at sites that do not affect its function. This investigation demonstrates that not only conventional ArsR but also various proteins can serve as whole-cell sensors for As(III). Furthermore, the $BetI_{3cys}$ mutant obtained in this study retained responsiveness to its native ligand, choline. In essence, we believe that this study has established the groundwork for a new paradigm in sensor engineering, wherein the function of a single protein molecule as a logic circuit is embedded within that same protein molecule. While the As(III) sensor developed in this study may not exhibit high sensitivity, our research proposes a groundbreaking approach by utilizing proteins other than those traditionally associated with As(III) sensors. This study suggests new possibilities for constructing As(III) sensors, diverging from the well-known proteins in this context.

METHODS

Bacterial Strain, Media, and Chemicals. *E. coli* strain MG1655 was used throughout this work, and *E. coli* XL10-Gold (Kan) (Stratagene, La Jolla, CA) was used for cloning. *E. coli* strain MG1655 was incubated in LB medium or on LB-agar plates [2.0% (w/v) LB, 1.5% (w/v) agar; Nacalai Tesque, Kyoto, Japan] at 37 °C. Then, 30 μg/mL chloramphenicol (*Cm*; Nacalai Tesque) was used to maintain the pAC-based vector, and 100 μg/mL ampicillin (Amp; Sigma-Aldrich, St. Louis, MO) was used to maintain the pET-based vector. Stock solutions (1 M) of choline chloride (Sigma-Aldrich, St. Louis, MO), copper sulfate, and zinc sulfate (Nacalai Tesque) were prepared by dissolving appropriate amounts of the compound

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in LB medium and filter-sterilized through a 0.2 μm cellulose acetate filter (MN Sterilizer CA, Macherey-Nagel GmbH & Co. KG, Düren, Germany) and stored at 4 °C. The As(III) used in this study was As(III) standard solution: As 1000 mg/L, composition: As_2O_3 and NaOH in water with HCl, pH 5.0 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Plasmid Construction and Cysteine Insertion. All of the plasmids used in this study are listed in Table S1. Primers were designed to introduce a TGC sequence at the site of cysteine insertion (Table S2), and site-specific mutagenesis was performed on pAC-Pj_{23116}-$betI_{I22T}$ using a KOD One PCR Master Mix and Gibson assembly. The obtained plasmids were transformed into *E. coli* XL10-Gold, and three randomly selected colonies were checked for the insertion by sequencing. The confirmed colonies were cultured in LB liquid medium, and the plasmids were isolated.

Analysis of BetI Cysteine Mutants to As(III) with sfGFP as the Reporter. The reporter plasmid pET23d-$P_{T5(betO)}$-sfgfp and a plasmid expressing BetI were co-transformed into *E. coli* strain MG1655 by electroporation. The vector pAC [Void; BetI (−)], which does not encode BetI, was used as a control. Transformed colonies were grown on solid LB (Amp/*Cm*) medium, and three colonies were randomly selected and inoculated into 96-well deep well plates containing 500 μL of LB (Amp/*Cm*) liquid medium, followed by incubation at 37 °C for 10 h. Subsequently, each culture was inoculated with 1/100 volume into 100 μL of LB (Amp/*Cm*) liquid medium containing varying concentrations of As(III) (0−10 ppm), choline chloride (0−100 mM), or both, within the range of concentrations previously shown to induce a response in *E. coli*. The cultures were then incubated at 37 °C for 12 h. Cells were re-suspended in physiological saline before measuring the cell density and fluorescence intensity. Cell density (OD600) was measured on a SpectraMax (Molecular Devices, San Jose, CA, USA), and fluorescence intensity (excitation: 485 nm, emission: 510 nm) was measured using Fluoroskan Ascent (Thermo Scientific, Waltham, MA, USA) for Figures 2−4 and FilterMax F5 (Molecular Devices, Sunnyvale, CA, USA) for Figure 5.

MD Simulations. All MD simulations conducted for each system in this study were performed using GROMACS 2019 software (Abraham MJ, http://www.gromacs.org.). We used the LINCS method for bond constraints[31] and the particle-mesh Ewald method with a cutoff of 10 Å for Coulomb interactions.[32] The following treatments were performed to equilibrate each system: (a) Energy minimization was performed for 50 000 steps; (b) A 100 ps MD equilibration was performed with the V-rescale thermostat at 300 K under a constant-NVT ensemble;[33] and (c) a 600 ps MD equilibration was performed with isotropic Berendsen exponential relaxation pressure coupling[34] at 300 K under 1 atm. Finally, a 100 ns MD simulation using a 2 fs time step was performed with an isotropic Parrinello−Rahman barostat[35] under a constant-NPT (300 K and 1 atm) ensemble as production runs and the last 80 ns were used for the analyses. To obtain statistically reliable trajectories, we performed 10 runs with different initial velocities for each system.

The structural model of $BetI_{I22T}$ was constructed by homology modeling based on the crystal structure of the transcriptional regulator from *Pseudomonas aeruginosa* PAO1 (PDB: 3E7Q). The protonation states of the protein were examined using the H++ server, with conditions set as pH 7 and ion concentration 0.15 M.[36] The protein was then solvated in a rectangular box of TIP3P water molecules under periodic boundary conditions. Neutralization was accomplished by adding Na^+ and Cl^- ions and ensuring an ion concentration of 0.15 M. The initial geometry of $BetI_{3cys}$ was constructed from a $BetI_{I22T}$ structure that was thermally equilibrated by 10,100 ns MD simulations. After replacing L79, Q81, and S102 with cysteine residues in AmberTools 22 (D.A. Case, 2023, https://ambermd.org/AmberMD.php) and equilibrating the system, a 10,100 ns production run at 300 K and 1 atm was performed. A thermally equilibrated structure of $BetI_{3cys}$ was used to construct the structural model of the As(III)-bound form. A calculation model of the As(III)-bound form was constructed by binding As(III) to L79C and Q81C on both monomers of a $BetI_{3cys}$ dimer. For comparative purposes, an alternative model was also constructed by binding As(III) to L79C and Q81C of only one monomer. The force field for the As(III)-bound residues was built using antechamber and MCPB.py in AmberTools 22;[37] the Amber ff14SB force field was used for standard residues. All of the parameters of the As(III) complexes were derived from quantum mechanical calculations by density functional theory (B3LYP/6-31G*) using Gaussian 16 software.[38] The force constants were calculated from the method proposed by Seminario,[39] and the restrained electrostatic potential fitting scheme was used for the partial charges.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09604.

Plasmids and primers used in this study, stability changes of BetI during cysteine substitution by FoldX analysis, stability changes ($\Delta\Delta G$ [kcal/mol]) upon site-specific cysteine substitution of all amino acid residues of BetI, comparison of $BetI_{3cys}$ without and with As(III), responsiveness of the BetI cysteine mutants to choline with sfGFP as the reporter, and fluorographs of cell suspensions of $BetI_{3cys}$ and $BetI_{I22T}$ in response to 10 ppm As(III) and 100 mM choline (PDF)

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ACS Omega 2024, 9, 16035−16043

Author Contributions

All authors designed the research. R.Y. performed all experiments with assistance from D.U., K.K., and S.K.-N. K.K. performed MD simulations. R.Y., T.Y., and K.K. analyzed the data. R.Y., D.U., K.K., and S.K.-N. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Amp, ampicillin; *ars*, arsenic resistance; As(III), arsenite; PDB, protein data bank; *Cm*, chloramphenicol; GFP, green fluorescent protein; LINCS, linear constraint solver for molecular simulations; MD, molecular dynamics; ppm, parts per million; sfGFP, superfolder green fluorescent protein

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