Arsenate reductase encoded by the chromosomal \textit{arsC} gene in \textit{Bacillus subtilis} catalyzes the intracellular reduction of arsenate to arsenite, which is then extruded from cells through an efficient and specific transport system. Herein, we present the solution structures and backbone dynamics of both the reduced and oxidized forms of arsenate reductase from \textit{B. subtilis}. The overall structures of both forms are similar to those of bovine low molecular weight protein-tyrosine phosphatase and arsenate reductase from \textit{Staphylococcus aureus}. However, several features of the tertiary structure and mobility are notably different between the reduced and oxidized forms of \textit{B. subtilis} arsenate reductase, particularly in the P-loop region and the segment Cys82–Cys89. The backbone dynamics results demonstrated that the reduced form of arsenate reductase undergoes millisecond conformational changes in the functional P-loop and Cys82–Cys89, which may facilitate the formation of covalent intermediates and subsequent reduction of arsenate. In the oxidized form, Cys82–Cys89 shows motional flexibility on both picosecond-to-nanosecond and possibly millisecond time scales, which may facilitate the reduction of the oxidized enzyme by thiolredoxin to regenerate the active enzyme. Overall, the internal dynamics and static structures of the enzyme provide insights into the molecular mechanism of arsenate reduction, especially the reversible conformational switch and changes in internal motions associated with the catalytic reaction.

Arsenate (As(V)) enters the cell through the phosphate transport system because of its chemical similarity to phosphate (1). Arsenic compounds are ubiquitous and abundant in nature. They are toxic to nearly all kinds of life forms and cause skin, kidney, lung, and bladder cancers in mammals (2, 3). As a consequence, many organisms have developed unique systems to confer arsenic resistance (4, 5). In bacteria, arsenic resistance is conferred by the \textit{ars} operon, which contains at least three genes: \textit{arsR}, \textit{arsB}, and \textit{arsC} (6). The \textit{arsR} protein is the arsenite (As(III))-responsive repressor of transcription; the \textit{arsB} protein is a membrane-associated arsenite efflux pump; and the \textit{arsC} protein functions as a cytoplasmic arsenate reductase that converts arsenate to arsenite (7–9). The higher toxicity associated with arsenite requires its immediate efflux by the \textit{arsB} protein, which is essential for the organism’s survival (10).

The \textit{arsC} gene product, arsenate reductase (\textit{ArsC}), is the key enzyme in the arsenate reduction process. Several families of \textit{arsC} have been identified and characterized (10–14). Among these, \textit{Staphylococcus aureus} \textit{ArsC} has been extensively studied (8, 15–21). These studies revealed that three redox active cysteine residues (Cys10, Cys82, and Cys89) are critical for arsenate reduction. A disulfide bridge (Cys82–Cys89) is formed after a single catalytic reaction cycle, converting the enzyme into the inactive form. \textit{ArsC} is subsequently regenerated by thioreredoxin, which converts the enzyme into the reduced form (21). The \textit{arsC} gene of the Gram-positive bacteria \textit{Bacillus subtilis} is located in the SKIN element in the chromosome and shares 64% identity with \textit{S. aureus} \textit{ArsC} (7, 22). The conserved Cys10, known as the P-loop, is proposed to be the arsenate-binding site (23). Four cysteine residues (Cys10, Cys15, Cys82, and Cys89) are conserved between the two \textit{ArsC} proteins.

The crystal structure of the reduced form of \textit{B. subtilis} \textit{ArsC} has been reported and contains four molecules (chains A–D) in an asymmetric unit (22). The segment Cys82–Val96 is missing in two chains (chains C and D) of the crystal structure, indicating a mixture of different conformations. To investigate the mechanism of arsenate reduction of \textit{B. subtilis} \textit{ArsC}, especially the conformational changes associated with the catalytic reaction, we have determined the solution structures of both the reduced and oxidized forms of \textit{B. subtilis} \textit{ArsC} by high resolution NMR spectroscopy. Furthermore, the changes in internal dynamics associated with arsenate reduction were determined by NMR relaxation measurements. A comparison of the solution structures and backbone dynamics between the oxidized and reduced forms of \textit{B. subtilis} \textit{ArsC} provided further insights into the molecular mechanism of arsenate reduction.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—A detailed description of the protein purification and purification of reduced \textit{B. subtilis} \textit{ArsC} was reported previously (24). Briefly, the \textit{B. subtilis} \textit{arsC} gene was cloned into the pET28a expression vector and expressed in \textit{Escherichia coli} strain BL21 pLysS(DE3). The culture was grown in LB medium; centrifuged; and resuspended in M9 minimal medium with antibiotics and 15NH4Cl in the presence and absence of [13C]glucose for preparation of 13C/15N-labeled and 15N-labeled samples, respectively (25). \textit{B. subtilis} \textit{ArsC} was purified by iron exchange chromatography (Mono Q) and gel filtration (Superdex 75) using an AKTA FPLC system (Amersham Biosciences). The purity was determined to be >95% as judged by SDS-PAGE.

NMR samples were prepared with 2 mM \textit{B. subtilis} \textit{ArsC} in buffer containing 20 mM Tris-HCl (pH 6.85), 40 mM KCl, and 20 mM Na2SO4 in 95% H2O and 5% D2O and were argon-flushed. The reduced form of...
Solution Structures and Backbone Dynamics of ArsC

ArsC was obtained by adding excess dithiothreitol (DTT; 25 mM). DTT titration experiments were performed to probe the DTT concentration dependence of ArsC conformations, which were monitored using a series of two-dimensional $^{15}$N-edited heteronuclear single quantum coherence (HSQC) spectra. The optimized molar ratio of DTT to enzyme is 5 or greater to obtain the pure reduced form of ArsC. For the preparation of the oxidized form, ArsC was first reduced by excess DTT, followed by dialysis to remove DTT, and then incubated with excess arsenate. The oxidation process of ArsC by arsenate was monitored using a series of two-dimensional $^{15}$N-edited HSQC spectra. ArsC purified without DTT showed an HSQC spectrum identical to that of the enzyme incubated with arsenate, demonstrating that the enzyme purified in the absence of DTT was in the oxidized form, which was used as the oxidized sample for the NMR experiments.

**NMR Spectroscopy**—The NMR experiments were carried out at 25 °C on Bruker Avance 500- and 800-MHz spectrometers equipped with four RF channels and triple resonance probes with pulsed field gradients. The chemical shifts were referenced to internal 2,2-dimethyl-2-silapentane-5-sulfonic acid. A set of two-dimensional $^{15}$N- and $^{13}$C-edited HSQC spectra and three-dimensional HNCACB, HNCO, HNCCACB, HBHA(CO)NH, CBCA(CO)NH, and (H)CC(CO)NH total correlation spectroscopy and (H)CCH COSY spectra were collected to obtain the backbone and side chain assignments (26–30). The three-dimensional $^{1}$H$^{−}$NQ NOESY-HSQC spectra (mixing times of 50 and 100 ms) were collected to confirm the backbone assignments and in combination with the three-dimensional $^{15}$C-edited NOE-HSQC spectra (mixing times of 50 and 100 ms) to obtain distance restraints for structure calculations (31). The three-dimensional HNHA experiment was performed to obtain dihedral angle restraints (32). The side chain assignments were confirmed with the three-dimensional $^{15}$N-edited total correlation spectroscopy-HSQC spectrum (mixing time of 80 ms). All NMR data were processed using the program NMRPipe (33) and analyzed using the program NMRView (34). A series of hydrogen-deuterium exchange experiments were performed to obtain hydrogen bond information.

**Structure Calculations**—The structures of the reduced and oxidized forms of ArsC were calculated using the program CYANA and refined by AMBER (35, 36). Distance restraints were derived from inter-proton NOEs. Dihedral angles (φ and ψ) were derived from backbone chemical shifts using TALOS (37), and φ angles were confirmed by the HHNA experiment (32). In addition, the $\chi_1$ values were obtained based on three-dimensional $^{15}$N NOESY-HSQC and $^{13}$C NOESY-HSQC spectra. Hydrogen bond restraints were derived from the hydrogen-deuterium exchange experiments and intermediate-range NOEs in conjunction with secondary structural information. Two-hundred structures were calculated by CYANA, and the 100 lowest energy structures were used as initial structures and refined using AMBER. Finally, the 20 lowest energy structures were selected to represent the reduced and oxidized forms of ArsC, respectively. The final structures were analyzed using the program packages MOLMOL and PROCHECK_NMR (38, 39).

**Spin Relaxation Measurements**—The longitudinal relaxation rates ($R_1$), transverse relaxation rates ($R_2$), and steady-state heteronuclear $^1$H$^{−}$NQ NOE values of the reduced and oxidized forms of ArsC were determined (40). The experiments were performed on a Bruker Avance 800-MHz NMR spectrometer at 25 °C. Spectral widths of 11,160.7 Hz for $^1$H and 2432.8 Hz for $^{15}$N were used. For the $R_1$ and $R_2$ measurements, 512 ($^1$H) and 128 ($^{15}$N) complex data points were collected with 32 transients/increment and a recycle delay of 2.5 s. The delays used for the $R_1$ experiments were 10 (×2), 100, 300, 500, 800, 1000, 1200, 1600, 2000, 2500, and 2800 ms, and those used for the $R_2$ experiments were 8 (×2), 32, 56, 80, 104, 128, 152, 176, 200, and 240 ms. The relaxation rate constants were obtained by fitting the peak intensities to a single exponential function using the nonlinear least-squares method as described (41). The $^1$H$^{−}$NQ NOE experiments were performed in the presence and absence of a 3-s proton presaturation period prior to the $^{15}$N excitation pulse and using recycle delays of 2 and 5 s, respectively (42). Forty-eight transients were collected for each experiment.

**RESULTS**

**Characteristics of the Reduced and Oxidized Forms of B. subtillis ArsC**—ArsC is a reductase that uses cysteine residues as electron donors. ArsC has four cysteine residues, and their redox states in both the reduced and oxidized forms of the enzyme were evaluated. Incubation of reduced ArsC with 5,5′-dithiobis(2-nitrobenzoic acid) released ~4.0 mol of dianionic 5-thio-2-nitrobenzoic acid/mol of ArsC, indicating that all four cysteine residues (Cys$^{10}$, Cys$^{15}$, Cys$^{82}$, and Cys$^{89}$) were in the reduced state. In addition, the Cβ chemical shifts of these cysteine residues were ~30 ppm, supporting the absence of disulfide bonds (43). The reduced state of all four cysteine residues was further confirmed by the NOE contacts from the three-dimensional NOESY-HSQC spectra (44).

Incubation of oxidized ArsC with 5,5′-dithiobis(2-nitrobenzoic acid) demonstrated that two of the four cysteine residues were in the oxidized state. We examined the Cβ chemical shifts of the cysteine residues. Cys$^{82}$ and Cys$^{89}$ showed Cβ chemical shifts of ~40 ppm, suggesting the formation of a disulfide bridge (Cys$^{82}$-Cys$^{89}$). However, Cys$^{10}$ and Cys$^{15}$ showed Cβ chemical shifts of ~30 ppm, characteristic of free cysteine residues. Formation of a Cys$^{82}$-Cys$^{89}$ (but not Cys$^{10}$-Cys$^{15}$) disulfide bridge was further confirmed by the NOE contacts from the three-dimensional NOE-HSQC spectra (44).

Next, we examined the enzyme activity of ArsC in either the reduced or oxidized form. As expected, the reduced form was able to reduce arsenate to arsenite, whereas the oxidized form was catalytically inactive.

**Reversible Conformational Transition Coupled with Arsenate Reducti**—The conformational differences between the reduced and oxidized forms of ArsC were examined using two-dimensional $^{15}$N-edited HSQC spectra. First, an HSQC spectrum of reduced ArsC without DTT was collected. The enzyme was then incubated with excess arsenate, and a series of HSQC spectra were acquired until a spectrum with a set of unique peaks corresponding to a single conformation (the oxidized form) was obtained. Fig. 1A shows an overlay of the HSQC spectra of the reduced and oxidized forms of ArsC, and Fig. 1B shows the composite $^1$H and $^{15}$N chemical shift changes versus residue number. Significant changes in many resonance peaks between the two spectra indicate considerable conformational changes upon arsenate reduction coupled with enzyme oxidation. The largest chemical shift changes between the reduced and oxidized forms of ArsC were observed in the Cys$^{82}$-Val$^{92}$ segment, suggesting significant structural changes in this region. This observation was further confirmed by the solution structures of both forms (see below). After addition of excess DTT, the spectrum of the oxidized form became identical to that of the
Reduced form. The reversible conformational transitions monitored by two-dimensional NMR spectroscopy along with the catalytic reaction provide insights into the mechanism of arsenate reduction. A similar result regarding the conformational switch in S. aureus ArsC was reported previously (18).

Solution Structures of the Reduced and Oxidized Forms of B. subtilis ArsC—To fully characterize the conformational changes associated with arsenate reduction, the NMR solution structures of the reduced and oxidized forms of ArsC were determined. The structures were calculated using inter-proton NOE-derived distance restraints in combination with the dihedral angle and hydrogen bond information. The superimpositions of 20 representative structures, together with the ribbon diagrams of the mean structures for each form, are shown in Fig. 2.

The structural statistics for both the reduced and oxidized forms are summarized in TABLE ONE. For the reduced form of ArsC, there is only one distance restraint violation >0.3 Å and two dihedral angle violations >5°. PROCHECK NMR analysis determined that 88.5% of the residues are within the most favored regions of the Ramachandran plot, 11.5% of the residues are in the allowed regions, and 0.1% of the residues are in the disallowed regions. For residues 3–139, the overall backbone root mean square deviation (r.m.s.d.) from the mean structure is 0.38 ± 0.05 Å, and that of the regular secondary structural elements is 0.26 ± 0.05 Å. For the oxidized form of ArsC, there is no distance restraint violation >0.3 Å and no angle violation >5°. As determined by PROCHECK NMR analysis, 85.5% of the residues are within the most favored regions of the Ramachandran plot, 15.4% of the residues are in the allowed regions, and 0.1% of the residues are in the disallowed regions. For residues 3–139, the overall backbone root mean square deviation (r.m.s.d.) from the mean structure is 0.31 ± 0.06 Å, and that of the regular secondary structural elements is 0.18 ± 0.03 Å.

The overall folds of the reduced and oxidized forms of ArsC are very similar, containing a single α/β-domain and showing a typical protein-tyrosine phosphatase I-like fold (22). The reduced form of ArsC consists of seven helices (residues 16–27 (α1), 46–54 (α2), 60–62 (3_10 helix 1), 69–72 (α3), 83–88 (3_10 helix 2), 106–108 (3_10 helix 3), and 113–137 (α4)) and a four-stranded β-sheet (residues 4–10 (β1), 32–38 (β2), 77–80 (β3), and 97–100 (β4)), which are packed into a single α/β-domain containing a central four-stranded parallel open twisted β-sheet flanked by four α-helices (Fig. 2, A and B). For the region from Gly83 to Lys88 in the reduced form of ArsC, the amide peaks of Gly83, Asp84, and Ala85 are missing in the NMR spectra, whereas the side chain atoms are assigned, except that of Asp84. The 3_10 helical structure in this region was determined using a total of 15 NOE-derived distance restraints. The oxidized form retains most of the tertiary structure of the reduced form (Fig. 2, C and D), whereas significant local structural differences are observed. The Cys82–Cys89 segment forms a short 3_10 helix in the reduced form, which is looped out in the oxidized form. As mentioned above, a Cys82–Cys89 disulfide bridge is formed, whereas residues Cys10 and Cys15 are in the reduced state.

Structural Comparison—Although the overall structures of the reduced and oxidized forms of ArsC are similar, significant differences are found. The backbone r.m.s.d. between the two forms is 2.48 Å, but is 3.29 Å for the Cys82–Val96 segment. This difference is consistent with the results from DTT titration experiments mentioned above, in which the same segment showed the largest chemical shift changes. After excluding this segment, the backbone r.m.s.d. decreases to 1.12 Å. An overlay of the Cα trace of the mean structures (energy-minimized using AMBER) is shown in Fig. 3A. In the oxidized form, Ala85 and Ala86 move 11.4 and 12.2 Å away from their positions in the reduced form, respectively. Cys89 shifts a distance of 8.5 Å toward Cys82 and forms a disulfide bridge (Cys82–Cys89) in the oxidized form. Therefore, the Cys82–Cys89 segment has a significant conformational change upon the formation of the Cys82–Cys89 disulfide bridge. This region has a helical structure in the reduced form and a coil conformation in the oxidized form, indicating that a helix-to-coil transition is coupled with the oxidation of ArsC.

The P-loop region consisting of Leu9–Ser17 is important for both substrate binding and catalysis. The structures of this region are similar in both forms, and the backbone r.m.s.d. between them is 0.56 Å. However, local structural differences exist (Fig. 3B). The H–N bond of Ser14 in the P-loop has different orientations between the two forms. Moreover, the side chain of Arg108 is in the vicinity of the Cys82–Cys89 segment and is spatially close to Ala85 and Ala86 in the oxidized enzyme, resulting in shrinking of the substrate entry to the P-loop.

The crystal structure of reduced ArsC has been reported (Protein Data Bank code 1JL3) and contains four chains (chains A–D) in an asymmetric unit (22). Residues 3–139 can be seen in chains A and B and are in good agreement with the solution structure of reduced ArsC. Fig. 3C shows the superimposed ribbon diagrams of the solution and crystal...
Solution Structures and Backbone Dynamics of ArsC

FIGURE 2. Solution structures of the reduced and oxidized forms of B. subtilis ArsC. A, superimposition of 20 representative structures of reduced ArsC; B, ribbon diagram representation of the secondary structural elements of reduced ArsC; C, superimposition of 20 representative structures of oxidized ArsC; D, ribbon diagram representation of the secondary structural elements of oxidized ArsC. A ball representation of thiols of redox functional cysteine residues (Cys10, Cys15, Cys82, and Cys89) is shown in yellow. All four cysteine residues (Cys10, Cys15, Cys82, and Cys89) are reduced in reduced ArsC, whereas a Cys82–Cys89 disulfide bridge is formed and the other two cysteine residues (Cys10 and Cys15) are free in oxidized ArsC. The figure was generated using MOLMOL (38).

| TABLE ONE |
| Structural statistics of the reduced and oxidized forms of B. subtilis ArsC |

|                              | Reduced   | Oxidized  |
|------------------------------|-----------|-----------|
| Distance restraints          |           |           |
| Intraresidue unambiguous NOEs| 745       | 709       |
| Sequential unambiguous NOEs  | 594       | 580       |
| Medium-range unambiguous NOEs| 565       | 513       |
| Long-range unambiguous NOEs  | 733       | 747       |
| Total unambiguous NOEs       | 2637      | 2549      |
| Total ambiguous NOEs         | 898       | 939       |
| Dihedral angles (φ and ψ)   | 68        | 185       |
| Hydrogen bonds               | 41        | 35        |
| S–S bond                     | 0         | 1         |
| r.m.s.d. from mean structure |           |           |
| Secondary structural backbone atoms | 0.25 ± 0.04 | 0.18 ± 0.03 |
| Secondary structural heavy atoms | 0.70 ± 0.06 | 0.66 ± 0.08 |
| All backbone atoms           | 0.38 ± 0.05 | 0.31 ± 0.06 |
| All heavy atoms              | 0.77 ± 0.06 | 0.73 ± 0.08 |
| CYANA target function value  | 2.34 ± 0.26 | 1.54 ± 0.11 |
| Mean AMBER energy (kcal/mol) | −7940.12 ± 11.17 | −7510.93 ± 10.91 |
| NOE distance restraint violation energy | 18.07 ± 1.73 | 19.58 ± 1.92 |
| Torsion angle restraint violation energy | 1.68 ± 0.24 | 0.59 ± 0.09 |
| Restraint violations         |           |           |
| Distance (/>0.3 Å)            | 1         | 0         |
| Dihedral angle (/>5°)         | 2         | 0         |
| Hydrogen bonds                | 0         | 0         |
| Ramachandran statistics      |           |           |
| Residues in most favored regions (%) | 88.5       | 85.5       |
| Residues in additional allowed regions (%) | 10.8       | 12.8       |
| Residues in generously allowed regions (%) | 0.7        | 1.6        |
| Residues in disallowed regions (%) | 0.1        | 0.1        |

(chain A) structures. The backbone r.m.s.d. between them is 0.96 Å. However, for chains C and D, the Cys82–Val96 segment is missing in the crystal structure, indicating a mixture of different conformations. Based on the nature of this enzyme, one possible cause of the mixed conformations is the reductant (DTT) concentration. To confirm this, we performed DTT titration experiments and monitored the ArsC conformation using two-dimensional 15N-edited HSQC spectra. Extra resonance peaks were observed in the HSQC spectra when the molar concentration of DTT was less than five times that of the enzyme. After increasing the concentration of DTT, the HSQC spectra showed a unique set of peaks corresponding to the pure reduced form of ArsC. Notably, the concentration of DDT used in the crystal preparation was approximately five times that of the enzyme (14, 22). In addition, the region from Cys82 to Val96 is well defined in the NMR structure of oxidized ArsC. Thus, we conclude that the crystal structure is most likely a representation of a mixture of the reduced and intermediate forms of the enzyme. A structural comparison shows that the backbone r.m.s.d. between chain C of the crystal and solution structures of the reduced form is 0.84 Å and that between chain C and the oxidized form is 0.95 Å when residues in the Cys82–Val96 segment are excluded. The backbone r.m.s.d. between chains A and C in the crystal structure is 0.55 Å after excluding this region. These results indicate that the transition between the two forms involves only local structural changes.

Despite the low sequence identity (18%), the structure of B. subtilis ArsC is surprisingly similar to that of bovine low molecular weight protein-tyrosine phosphatase (23). Fig. 3D shows an overlay of the Cα trace of reduced B. subtilis ArsC with that of bovine low molecular weight protein-tyrosine phosphatase (Protein Data Bank code 1PHR). The r.m.s.d. is 1.27 Å for 110 pairs of Cα atoms. Previous studies indicated that S. aureus ArsC is also structurally similar to bovine low molecular weight protein-tyrosine phosphatase (17). This suggests that the evolutionary origin of B. subtilis ArsC is similar to that of S. aureus ArsC, but different from that of E. coli or Saccharomyces cerevisiae arsenate reductase (5, 11, 17).

Structural Comparison between the B. subtilis and S. aureus ArsC Proteins—B. subtilis ArsC shares 64% sequence identity with S. aureus ArsC. The overlays of the Cα trace of B. subtilis ArsC with that of S. aureus ArsC (Protein Data Bank codes 1LJL and 1JFV, respectively) in both the reduced and oxidized forms are shown in Fig. 3 (E and F, respectively).
Bone longitudinal relaxation rates (T2) and to obtain further functional insights into that of ArsC; the P-loop of the reduced (B. subtilis ArsC in solution (red) superimposed with chain A of the crystal structure (blue); D, the Cα trace of reduced B. subtilis ArsC (red) superimposed with that of bovine low molecular weight protein-tyrosine phosphatase (magenta); E, the Cα trace of reduced B. subtilis ArsC (red) superimposed with that of S. aureus ArsC (cyan); F, the Cα trace of oxidized B. subtilis ArsC (green) superimposed with that of S. aureus ArsC (yellow).

Solution Structures and Backbone Dynamics of ArsC

respectively). The r.m.s.d. between them are 1.38 and 1.51 Å for 130 pairs of Cα atoms determined using DALI (39). The conservation of the P-loop and the three essential cysteine residues (Cys10, Cys82, and Cys89) suggests that they may share a similar catalytic mechanism of arsenate reduction (18, 22). A previous study demonstrated that the arsenate reduction activity of S. aureus ArsC takes place via a triple disulfide cascade involving three cysteines (Cys10, Cys82, and Cys89) (18). The structural similarity supports the proposal that the B. subtilis and S. aureus ArsC proteins share a similar catalytic mechanism (17, 18, 22).

Relaxation Parameters—To investigate the dynamic properties of and to obtain further functional insights into B. subtilis ArsC, the backbone 15N longitudinal relaxation rates (R1), transverse relaxation rates (R2), and heteronuclear 1H-15N NOE values were obtained at a 1H frequency of 800 MHz for the reduced and oxidized forms. The experimentally determined R1, R2, and 1H-15N NOE values versus the amino acid sequence are shown in Fig. 4A. For the analysis of reduced ArsC, 114 of the 139 residues were used, whereas for the analysis of oxidized ArsC, 119 residues were used. The unanalyzed residues included five proline residues that have no amide protons, unassigned residues (seven residues for the reduced form and two for the oxidized form), and 13 residues that were either overlapped or too weak to be analyzed.

Overall, the entire enzyme is rigid, and similar 1H-15N NOE values for both forms were observed, except in the region from Cys82 to Val86, where Gly83, Asp84, and Ala85 are missing, and Cys89 and Met91 were excluded in the analysis due to the poor quality of correlation peaks in the reduced form. The R2/R1 ratios at the active site P-loop and the Cys82–Cys89 redox functional segment of reduced ArsC are systematically higher than the average values, which can be indicative of conformational exchanges on the millisecond time scale and/or motional anisotropy on picosecond-to-nanosecond time scales. For oxidized ArsC, residues in the Cys82–Cys89 segment showed R2/R1 ratios lower than the average values, suggesting fast internal motions on picosecond-to-nanosecond time scales.

Rotational Diffusion Anisotropy—Motional anisotropy, as described by rotational diffusion tensor, should always be considered in the analysis of any NMR relaxation data, especially in the characterization of chemical or conformational exchanges (45). Calculated from the solution structures, the ratios of principle components of the inertia tensors are 1.091:0.57 and 1.088:0.60 for reduced and oxidized ArsC, respectively. This suggests a strong anisotropy of the enzyme, which may significantly affect the analysis of motional properties. Rotational diffusion parameters were determined by minimizing the differences between the experimental and calculated R2/R1 ratios using isotropic, axially symmetric, and fully anisotropic models (46). We followed the common procedures to evaluate the rotational diffusion anisotropy of ArsC by excluding residues with conformational exchanges or, alternatively, internal motions (46). A total of 79 and 83 residues were used to determine the rotational diffusion tensors of reduced and oxidized ArsC, respectively. The diffusion tensors are best defined by the axially symmetric model for both forms, giving the global correlation time \( \tau_{\text{corr}} = 9.43 \pm 0.09 \) ns and \( 8.83 \pm 0.09 \) ns and the anisotropy of diffusion tensor \( D_p/D_c = 1.29 \pm 0.03 \) and \( 1.25 \pm 0.03 \) for reduced and oxidized ArsC, respectively. In addition, the experimentally determined global correlation times were compared with those calculated using the program HYDRONMR (47), which indicated that either form of the enzyme was in the monomeric state. The monomeric state of the
enzyme was further confirmed by analytical ultracentrifugation under the same sample conditions (data not shown).

Internal Dynamics—The amide $^{15}$N relaxation is dominated by dipolar coupling with the attached proton and $^{15}$N chemical shift anisotropy. The model-free analysis employs five semi-empirical forms of the spectral density function to fit the experimentally determined relaxation rates ($R_i$), transverse relaxation rate ($R_2$), and heteronuclear ($^1$H)$^{15}$N NOEs of the reduced (●) and oxidized (○) forms of $B$. subtilis ArsC versus the amino acid sequence. The spectra for determining the relaxation parameters were recorded on a Bruker Avance 800-MHz spectrometer at 25°C. Uncertainties were obtained using Monte Carlo simulations. $B$. the backbone dynamics parameters $S^2$, $\tau_e$, and $R_{ex}$ of the reduced (●) and oxidized (○) forms of $B$. subtilis ArsC versus the amino acid sequence. The secondary structural elements are shown at the top.

The calculations were performed using the experimentally determined relaxation rates, their uncertainties, and the coordinates of the NMR structures as input. The amide bond length was fixed at 1.02 Å, and a $^{15}$N chemical shift anisotropy value of $-175$ ppm was used in the calculations. Five increasingly complex models of internal mobility (M1, $S^2$; M2, $S^2$, $\tau_e$; M3, $S^2$, $R_{ex}$; M4, $S^2$, $\tau_e$, $R_{ex}$; and M5, $S^2$, $S^2$, $\tau_e$) were iteratively used to reproduce the experimental data until the confidence reached within 95% (50). The confidence levels were estimated using 300 Monte Carlo simulations/run in combination with $\chi^2$ and $F$ statistical analysis. The optimized internal mobility parameters of the generalized order parameter ($S^2$), fast internal motion on picosecond-to-nanosecond time scales ($\tau_e$), and millisecond time scale conformational exchange ($R_{ex}$) for both forms of ArsC are shown in Fig. 4B.

The residues in the secondary structural elements were described mainly by M1 (except residues in the Cys$^{82}$–Cys$^{89}$ segment), with an average $S^2 = 0.90 \pm 0.02$ and $0.93 \pm 0.02$ for reduced and oxidized ArsC, respectively. The residues near the N and C termini and the region between helix α1 and strand β2 were assigned to M2, with an average $S^2 = 0.80 \pm 0.02$ and $0.84 \pm 0.03$ and internal motions ($\tau_e$) on picosecond-to-nanosecond time scales for both forms. The residues that were described by M3 show an average $S^2 = 0.88 \pm 0.04$ and $0.91 \pm 0.03$ along with an average $R_{ex} = 4.5 \pm 0.8$ and $2.3 \pm 0.6$ s$^{-1}$ for the reduced and oxidized forms, respectively. Only one residue in the oxidized form was assigned to M4. Seven residues of the reduced form and five of the oxidized form were assigned to M5, with an average $S^2 = 0.65 \pm 0.04$ and $0.65 \pm 0.04$ and a various degree of flexibility on picosecond-to-nanosecond time scales ($\tau_e$).

Overall, the enzyme in both forms shows similar restricted motions on picosecond-to-nanosecond time scales as revealed by the generalized order parameter $S^2$, except the Cys$^{82}$–Val$^{96}$ segment. However, significant differences of motion on the millisecond time scale were observed between the reduced and oxidized forms, particularly in the catalytic P-loop and Cys$^{82}$–Cys$^{89}$ redox functional segment. For reduced ArsC, residues in the P-loop region have an average $S^2 = 0.89 \pm 0.05$ and an average $R_{ex} = 5.9 \pm 0.9$ s$^{-1}$. In contrast, for oxidized ArsC, residues in the P-loop region show restricted motions, with an average $S^2 = 0.94 \pm 0.02$, whereas residues in the Cys$^{82}$–Cys$^{89}$ segment have an average $S^2 = 0.86 \pm 0.02$ along with fast internal motions on picosecond-to-nanosecond time scales ($\tau_e$). In addition, Ala$^{85}$ also shows conformational exchange ($R_{ex} = 4.4 \pm 0.7$ s$^{-1}$).

**DISCUSSION**

The internal mobility parameters obtained from the model-free analysis correlate well with the three-dimensional structures of the reduced and oxidized forms of $B$. subtilis ArsC (Fig. 5). Most residues in the secondary structural elements of the enzyme in both forms have high $S^2$ values and lack contributions from the internal motions. On the other hand, residues near the N and C termini and the region between helix α1 and strand β2 show lower $S^2$ values and a certain degree of flexibility on picosecond-to-nanosecond time scales. Interestingly, residues in the catalytic P-loop region of the reduced form show conformational exchanges on the millisecond time scale, whereas they become rigid upon oxidation of the enzyme. For the reduced form, most residues in the Cys$^{82}$–Cys$^{89}$ segment are missing or unused due to the poor signal quality, whereas the remaining ones are involved in millisecond conformational exchanges. This observation suggests that the residues in the
Cys82–Cys89 segment have a substantial degree of conformational flexibility on the millisecond time scale, thus broadening the NMR signals in this region so as to be undetectable or unusable. In contrast, the residues in this segment become detectable and involved in sub-nanosecond time scale internal motions in the oxidized form. The backbone dynamics results demonstrated that, even though the Cys82–Cys89 segment forms a helix in reduced ArsC, it is not tightly structured and undergoes substantial conformational exchanges on the millisecond time scale. Thus, the helix can be readily dissociated as observed in chains C and D of the crystal structure. Apparently, the interchangeable conformations are supported by the embedded flexibility in this sequence.

Based on our observations and previous studies (18, 22), the proposed arsenate reduction mechanism of *B. subtilis* ArsC is as follows. The reaction starts with ArsC in the reduced state, and the residues in the active P-loop region undergo millisecond conformational exchanges. The conformational flexibility at the P-loop promotes enzyme-substrate interactions, which are essential for enzyme catalysis. We performed two-dimensional 15N-edited HSQC experiments in the absence and presence of sulfate in the sample to monitor its interaction with the enzyme. The residues in the P-loop region were undetectable in the absence of sulfate, but could be visualized in the presence of sulfate. The sulfate ion was undetectable directly by NMR spectroscopy, but was visualized in the crystal structure, supporting the binding of sulfate to the enzyme (22). Sulfate was used in the NMR experiments for structure determinations. Therefore, the solution structure of the reduced form mimicked the structure of a noncovalent enzyme-substrate complex in the first step of the reduction cascade, arsenate triggers a nucleophilic attack of the thiol of Cys10, followed by the formation of the ArsC Cys10-HAsO32− intermediate complex with the release of a water molecule (18, 51). In the following step, the thiol of Cys82 attacks Cys10 to form a Cys10–Cys82 disulfide bridge while arsenate is reduced to arsenite and dissociates from the enzyme. Finally, Cys10 is released from the Cys10–Cys82 disulfide bridge by a thiol-disulfide interchange with Cys89. A Cys82–Cys89 disulfide bridge is formed and becomes exposed to the enzyme surface. Therefore, one cycle of the catalytic reaction is finished with the concomitant oxidization of the enzyme. In reduced ArsC, the distance between the two sulfur atoms of Cys10 and Cys82 is 3.7 Å, which is very close to the sum of the van der Waals radii, whereas the two sulfur atoms of Cys82 and Cys89 are ~11 Å apart. Therefore, the significant conformational exchanges of the Cys82–Cys89 segment in the reduced form are required for the formation of the disulfide bridge between Cys82 and Cys89. On the other hand, the structural geometry and the sub-nanosecond flexibility of the Cys82–Cys89 segment in oxidized ArsC are favorable for the interaction with thioredoxin, which regenerates the reduced catalytically active form of ArsC for the next cycle of arsenate reduction (21). Moreover, Ala85 undergoes millisecond time scale conformational exchange in addition to sub-nanosecond flexibility in the oxidized form, which may facilitate the subsequent reduction of the enzyme by thioredoxin.

In conclusion, we have determined the solution structures of the reduced and oxidized forms of *B. subtilis* ArsC and demonstrated a correlation between the conformational switch and internal dynamics.
Solution Structures and Backbone Dynamics of ArsC

The Cys^{82}–Cys^{89} dynamic segment, as characterized by the backbone dynamics and titration data, supports the structural switch. The solution structures in combination with the backbone dynamics show an animated picture of the conformational switch associated with the catalytic reaction, providing further insights into the molecular mechanism of arsenate reduction.

Acknowledgments—We thank Dr. Xiao-Dong Su (Peking University) for discussions. We thank the present and former members of the Beijing Nuclear Magnetic Resonance Center for efforts in this project. We thank Dr. Jiyan Ma (Ohio State University) and Dr. Xiubei Liao (University of Illinois, Chicago, IL) for critical reading of the manuscript and suggestions. All NMR experiments were carried out at the Beijing Nuclear Magnetic Resonance Center.

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