Conformational Fluctuations Coupled to the Thiol-disulfide Transfer between Thioredoxin and Arsenate Reductase in *Bacillus subtilis*

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Arsenic compounds commonly exist in nature and are toxic to nearly all kinds of life forms, which directed the evolution of enzymes in many organisms for arsenic detoxification. In bacteria, the thioredoxin-coupled arsenate reductase catalyzes the reduction of arsenate to arsenite by intramolecular thiol-disulfide cascade. The oxidized arsenate reductase ArsC is subsequently regenerated by thioredoxin through an intermolecular thiol-disulfide exchange process. The solution structure of the *Bacillus subtilis* thioredoxin - arsenate reductase complex represents the transiently formed intermediate during the intermolecular thiol-disulfide exchange reaction. Comparison of the complex structure with that of thioredoxin and arsenate reductase proteins in redox states shows substantial conformational changes coupled to the reaction process, especially arsenate reductase adopts an ‘intermediate’ conformation in the complex. Our current studies provide novel insights in understanding the reaction mechanisms of the thioredoxin - arsenate reductase pathway.

Thiol-disulfide exchange reactions between proteins are involved in many important cellular oxidation and reduction processes (1). Among these, the thioredoxin system, which includes NADPH, thioredoxin reductase (TrxR) and thioredoxin (Trx), has been extensively studied (2-6). Thioredoxin is a family of small proteins (~ 12 kDa) that exist ubiquitously in organisms from bacteria to human. Thioredoxin generally functions as protein thiol-disulfide oxidoreductase for maintaining the reducing environment in cells (2, 3). Upon interaction with its substrates, the intermolecular thiol-disulfide exchange reaction is involved, and finally an intramolecular disulfide bond is formed between the two cysteine residues in Trx. The regeneration of active Trx is accomplished by TrxR, which utilizes the reducing equivalents from NADPH. Trx catalyzes the reduction of a wide range of downstream protein targets, one of which is the well documented arsenate reductase
ArsC catalyzes the reduction of arsenate (As(V)) to arsenite (As(III)) and is the key enzyme involved in arsenic detoxification (7). Several families of ArsC have been identified and extensively studied (8). The thioredoxin-coupled ArsC family includes *Bacillus subtilis* ArsC on the chromosomal skin element and the *Staphylococcus aureus* ArsC on the plasmid pI258. Previous studies revealed that three redox active cysteine residues (Cys10, Cys82 and Cys89) are critical for the enzymatic activity of *S. aureus* ArsC (9), all of which are conserved in *B. subtilis* ArsC. A disulfide cascade mechanism was proposed based on structural, biochemical and mutagenesis studies (10-13). Residue Cys10 of ArsC is responsible for the initial nucleophilic attack on arsenate to form the ArsC-As(V) covalent intermediate. A subsequent attack by Cys82 releases the arsenite compound and introduces an intramolecular disulfide bond Cys10-Cys82. The final nucleophilic attack by Cys89 marks the completion of a single catalytic reaction cycle, converting the enzyme into its inactive form (oxidized form) and exposing an intramolecular disulfide bridge Cys82-Cys89 onto the protein surface. The active ArsC (reduced form) is subsequently regenerated by Trx through the intermolecular thiol-disulfide exchange reactions (14, 15).

The flow of reducing equivalents through the NADPH - TrxR - Trx - ArsC - As(V) pathway involves both intra- and inter-molecular thiol-disulfide exchange reactions, therefore providing a perfect model system. Up to date, the structures of both Trx and ArsC are extensively studied (10-13, 16-18). In addition, the interaction between TrxR and Trx has also been investigated by crystal structures (4-6).

However, the transiently formed intermediate state of Trx-ArsC interaction, which would provide essential information concerning the reaction mechanism, remains poorly understood. Attempts to crystallize the Trx-ArsC complex have failed thus far (15). The only structures available for the Trx-substrate complex linked by a disulfide bond are the structures of human Trx complexed with short peptides from its substrates NFκB and Ref-1 (19, 20). Essential questions regarding the Trx-ArsC interaction remain unanswered: What are the transient conformations of ArsC and Trx in the complex? And how are the conformational properties of the proteins in different stages coupled to the reaction processes?

We have previously reported the solution structures and backbone dynamics of *B. subtilis* ArsC in both reduced and oxidized forms (designated as re-ArsC and ox-ArsC) (13). For further understanding of the catalytic mechanisms of Trx-ArsC pathway, we have determined the solution structures of *B. subtilis* Trx in both reduced and oxidized forms (designated as re-Trx and ox-Trx), and particularly we solved the structure of the Trx-ArsC complex by NMR spectroscopy. The structure of Trx-ArsC complex in conjunction with those of Trx and ArsC proteins in different states presents an atomic resolution view of the conformational changes along the reaction pathway.

**EXPERIMENTAL PROCEDURES**

**Sample Preparations** - The *trxA* gene encoding the *B. subtilis* Trx was cloned into the pET21a(+) vector (Novagen) and the protein was expressed in *E. coli* strain BL21(DE3). The pET28a(+) *arsC* plasmid (13) and the pET21a(+) *trxA* plasmid were used as DNA templates in PCR.
amplification with primers designed to generate mutants of ArsC_C10SC15AC82S and Trx_C32S. Sample preparations of ArsC_C10SC15AC82S, Trx and Trx_C32S were similar to that previously reported (13). The purity of the proteins was determined to be greater than 95% as judged by SDS-PAGE. The Trx-ArsC mixed disulfide complex was made using the mutants Trx_C32S and ArsC_C10SC15AC82S by 5,5'-dithiobis (2-nitrobenzoic acid) incubation method following the reported protocol (15).

**Structure Calculations** - The details of NMR spectra collection, processing and analysis, as well as the structure calculations of the re-Trx and ox-Trx can be found in Supplemental Methods.

The structure of Trx-ArsC complex was calculated using the program CNS (21) and refined by AMBER (22). The inter-proton NOE derived distance restraints, the RDC derived long-range restraints, the hydrogen bond restraints based on hydrogen-deuterium exchange experiments and the dihedral angle restraints based on chemical shifts (23) were used for the structure calculation. Two hundred structures were calculated using CNS, and the 100 structures with the lowest energy were selected and further refined using AMBER. Finally, the 20 lowest-energy structures were selected as representative of the Trx-ArsC complex. The final structures were analyzed using the program packages MOLMOL (24) and PROCHECK_NMR (25).

**RESULTS AND DISCUSSION**

**Interaction between Trx and ArsC** - It has been demonstrated that the active *S. aureus* ArsC could be regenerated by Trx (14). To confirm that this pathway is conserved in *B. subtilis*, we performed *in vitro* experiments of the interaction between Trx and ArsC monitored by 2D 15N-edited heteronuclear single quantum coherence spectroscopy (HSQC) spectra. Unlabeled re-Trx was added into the NMR sample of the 15N-labeled ox-ArsC, and the 2D 15N-edited HSQC spectra showed that the conformation of ArsC switched from the oxidized form to the reduced form (13). A similar experiment in which unlabeled ox-ArsC was added into 15N-labeled re-Trx showed that the reduction of ArsC was coupled to the oxidation of Trx (Supplemental Fig. 1). In contrast, we performed titration experiments using unlabeled re-Trx and 15N-labeled re-ArsC. At Trx:ArsC molar ratio of 1:1 or higher, the 15N-labeled ArsC showed identical spectra to that of the re-ArsC HSQC, indicating that there is no interaction between re-Trx and re-ArsC. These results demonstrate the *in vitro* interaction and thiol-disulfide exchange between re-Trx and ox-ArsC. In addition, we performed *in vitro* enzymatic assay by measuring the oxidation of NADPH (14) and confirmed that the *B. subtilis* ArsC activity is also coupled to the NADPH-TrxR-Trx pathway.

**Solution structure of the Trx-ArsC complex** - To fully characterize the interaction between Trx and ArsC, especially the conformational changes coupled to the redox reactions, we have determined the solution structure of the *B. subtilis* Trx-ArsC mixed disulfide complex by NMR spectroscopy. The superimposed 20 structures with lowest energy, together with the ribbon representation of the complex are shown in Fig. 1A. During the Trx-ArsC interaction, residue Cys29 of Trx acts as the nucleophilic attacker and forms an intermolecular disulfide bridge with Cys89 of ArsC. Due to the transient nature of the Trx-ArsC interaction, a stable
Trx-ArsC complex with a mixed disulfide bond between Trx-Cys29 and ArsC-Cys89 was prepared using mutants Trx_C32S and ArsC_C10SC15AC82S following the 5,5'-dithiobis (2-nitrobenzoic acid) incubation procedures described by Messens et al (15). The structure of the complex is well determined based on both NOE-based distance restraints and residual dipolar coupling (RDC) restraints (Table 1). A total of 112 intermolecular NOE restraints were identified at the interface of the complex and the relative orientation of the two proteins was determined by RDC measurements. The backbone root mean square deviation (r.m.s.d.) from mean structure is 0.6 ± 0.2 Å for the secondary structures of the whole complex, while it is 0.23 ± 0.03 Å and 0.39 ± 0.07 Å for the individual Trx and ArsC molecules in the complex (designated as c-Trx and c-ArsC), respectively. The interaction between the two proteins buried a total of ~1350 Å² of solvent accessible area (~ 630 Å² for Trx and 720 Å² for ArsC), about 65% of which is contributed by non-polar amino acid residues. The residues that are directly involved in the interaction (with unambiguous NOE peaks) include Ala26, Trp28, Val57, Asp58, Gln61, Ala64, Gly65, Val69-Ile72 and Val88 of c-Trx, and residues Ile39, Ile67, Asn73 and Lys88-Pro94 of c-ArsC. Residues Lys88-Pro94 located in the extended loop of c-ArsC is sandwiched between two walls formed by residues Trp28, Asp58 and Gly65-Ile72 in c-Trx. Residues Met70 and Ser71 of c-Trx fit into a groove formed by residues Ile67, Asn73 and Lys88-Pro94 of c-ArsC in a similar fashion (Fig. 1B-D). The segment Lys88-Pro94 of ArsC interacts with Trx active site in the anti-parallel orientation similar to the human Trx-Ref-1 complex (20), and mainly involves hydrophobic interactions. Notably, the side chains of residue Met91 in c-ArsC and Met70 in c-Trx are both inserted into the other subunit, which may contribute to the stabilization of the complex.

**Conformational changes of Trx** - The detailed descriptions of structures of re-Trx and ox-Trx can be found in Supplemental Data and Supplemental Fig. 2. The overall structures of re-Trx, ox-Trx and c-Trx are similar, whereas subtle local conformational adjustments are observed in c-Trx (Fig. 2A-B). In particular, the short helix Gln61-Lys66 in c-Trx is slightly displaced from its original positions in the free forms. This local structural change orients the helix so that its C-terminal end is shifted toward the protein surface, which can be favorable for the interactions of residues Ala64 and Gly65 in c-Trx with ArsC. Furthermore, residue Val88 locates at the C-terminal end of the fifth β-strand in both re-Trx and ox-Trx, while in c-Trx it also moves away from its original position and interacts with ArsC. The conformational changes of the active site are not significant. However, the side chain of the active cysteine residue Cys29 appears to adjust its positions in different states (Fig. 2B). In c-Trx, the side chain of Cys29 moves slightly toward the protein surface to form the intermolecular disulfide bond as compared to re-Trx. After Trx switches to the oxidized form, the side chain of Cys29 moves back and further inward due to the formation of the intramolecular Cys29-Cys32 disulfide bond.

**The intermediate conformation of c-ArsC** - The overall structures of ArsC in free or complex states are similar, while the region from residue Thr80 to Glu99 shows an ‘intermediate’ conformation in c-ArsC (Fig. 2C-D). Particularly, segment Cys82-Cys89, which is involved in a helix to loop conformational transition between
re-ArsC and ox-ArsC (13), adopts an intermediate conformation in c-ArsC (Fig. 2D). The helical structure present in re-ArsC is not completely formed in the complex intermediate. However, the segment Cys82-Cys89 moves a considerable distance away from its position in the ox-ArsC and shows a high tendency to forming the helix. Most residues in this segment are located at intermediate positions between the reduced and oxidized forms, while some residues show conformations closer to the reduced form. The side chain position of Ser82 (mutated from Cys82) in c-ArsC is similar to Cys82 in re-ArsC, and the side chain of Lys88 in c-ArsC is also much closer to its position in re-ArsC than in ox-ArsC. Residue Cys89, however, locates at an intermediate position in the complex and points toward the protein surface to form the intermolecular disulfide bond with Cys29 of Trx. In contrast, the conformation of segment Pro90-Glu99 appears closer to ox-ArsC (Fig. 2D). This part is mostly extended in ox-ArsC structure, while it becomes mostly coiled and moves downward (as viewed in Fig. 2C-D) in re-ArsC. In c-ArsC, the segment Pro90-Glu99 closely resembles the conformation of ox-ArsC (Fig. 2D). In addition, the segment Lys88-Val96 is closer to the short helix Ser69-Ile72 in re-ArsC than in ox-ArsC, while it is also located at an ‘intermediate’ position in c-ArsC (Fig. 2C).

**Biological implications** - We present the solution structure of the Trx-ArsC complex from B. subtilis. In the complex structure, ArsC adopts an intermediate conformation compared to the reduced and oxidized forms, and represents a structural transition from the oxidized form to the reduced form. Extensive interactions between ArsC and Trx are observed in the covalently linked complex. Specially, a pair of methionine residues are observed to insert their side chains into the other subunit. Interestingly, the E. coli Trx-R-Trx interaction involves the hydrophobic side chains of a pair of arginine residues similar to that of the methionine residues in the B. subtilis Trx-ArsC interaction (5), which may represent a co-evolution of the specific residues.

In previous studies, a docking model of the S. aureus Trx-ArsC complex was proposed which placed the Cys82-Cys89 loop in the interacting surface (15). Our solution structure of the B. subtilis Trx-ArsC complex, however, shows that the loop Cys82-Cys89 itself does not contribute much to the Trx-ArsC interaction. Instead, the neighboring segment Cys82-Glu99 forms most of the intermolecular interaction with Trx.

In addition, the crystal structures of the corresponding triple mutant ArsC_C10SC15AC82S of S. aureus and its 5-thio-2-nitrobenzoic acid adduct are also available (26). Both structures show a conformation similar to the reduced form of the wild type ArsC, with an intact helix between Cys82 and Cys89. Since the 5-thio-2-nitrobenzoic acid adduct of S. aureus ArsC_C10SC15AC82S is able to react with Trx, it appears that the reactivity of ArsC-Cys89 may play a major role in the reaction between ArsC and Trx.

Previous studies suggested that S. aureus re-Trx may be able to discriminate between the folds of the reduced and the oxidized ArsC, and interacts only with the oxidized form (15). Our results also showed that there is no interaction between the B. subtilis re-Trx and re-ArsC. Although the ability of Trx to discriminate ox-ArsC from re-ArsC based on conformational differences is still debatable, it is possible
that the conformation of ox-ArsC may be preferable for interaction with Trx. In particular, the segment Lys88-Pro94 undergoes extensive conformational changes during the reaction processes, and shows the most extended conformation in the oxidized state (Fig. 2C and D). The structures of human Trx complexed with peptides of its substrates showed that the peptides adopt an extended conformation (19, 20). It is reasonable to suggest that the extended conformation of segment Lys88-Pro94 in ox-ArsC may facilitate its recognition and interaction by Trx. Furthermore, in re-ArsC, the closer distance between loop Lys88-Val96 and helix Ser69-Ile72 appears to close the groove on ArsC surface. In ox-ArsC, the loop Lys88-Val96 moves away and opens up the groove, which may help to properly dock the interacting residues of both proteins into the surface grooves. It is very likely that both the reactivity of ArsC-Cys89 and the conformation of ox-ArsC work cooperatively to facilitate the thiol-disulfide exchange reaction between Trx and ArsC in vivo.

Concluding remarks - We present the solution structure of the Trx-ArsC complex, the first protein-protein complex structure between the thioredoxin and arsenate reductase families reported thus far. The structure represents an intermediate conformation during the intermolecular thiol-disulfide exchange reaction that exists only transiently under natural conditions. The structural investigations demonstrate the changes on the conformational properties of the enzymes in different reaction stages, which probably contribute to the progression of the reaction. The Trx - ArsC - As (V) pathway involves a series of redox reactions, our structural and biochemical studies of the Trx-ArsC complex and the free proteins establish a dynamic picture of the conformational fluctuations of both enzymes coupled to the reaction processes.

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FOOTNOTES
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Data deposition: The atomic coordinates of the B. subtilis Trx-ArsC complex (code 2IPA) and
the reduced and oxidized forms of B. subtilis Trx (codes 2GZY and 2GZZ) have been
deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics,
Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
The abbreviations used are: TrxR, thioredoxin reductase; Trx, thioredoxin; ArsC, arsenate reductase; re-ArsC (Trx), reduced form of ArsC (Trx); ox-ArsC (Trx), oxidized form of ArsC (Trx); c-ArsC (Trx), ArsC (Trx) in the complex; HSQC, heteronuclear single quantum coherence spectroscopy.

FIGURE LEGENDS

**Fig. 1:** Solution structure of the Trx-ArsC complex. A) Superimposition of the 20 lowest energy structures and ribbon diagram of the Trx-ArsC complex. The sulfur atoms that form the intermolecular disulfide bridge are presented by yellow balls. The side chains of residues with unambiguously observed intermolecular NOEs are presented in stick in the ribbon representation. B) Enlarged view of the Trx-ArsC interface at two different angles. The backbone conformation of the residues at the interface is shown in stick representation. The side chains of ArsC-Met91 and Trx-Met70 are also shown and labeled. C, D) The molecular surface representations of c-Trx (C) and c-ArsC (D) with the interacting peptide segments of the other protein presented by stick. The N- and C-terminal ends of the long segments are labeled. The backbones of segments are colored in green; the side chains of positively and negatively charged residues are colored in blue and red, respectively; other side chains are colored in yellow. The sulfur atoms of the peptide segments involved in the disulfide bridge are also shown.

**Fig. 2:** Structural comparison of ArsC and Trx at different states. A) Ribbon representations of Trx structures, colored as follows: re-Trx, red; c-Trx, light green; ox-Trx, light blue; the segment Cys29-Cys32 (Cys29-Ser32 in c-Trx), violet; the segment that involves in interaction with ArsC (including the helix Q61-K66 and the following loop Met70-Ile72), yellow. The two active cysteines (serines in c-Trx) and residue Val88 are presented and labeled. B) The conformational changes near the active site (segments 24-34, 57-73) in Trx, colored as follows: re-Trx, red; c-Trx, light green; ox-Trx, light blue. The side chains of selected residues are presented in stick and labeled. The ends of the segments are labeled by numbers. The movements of selected residues are shown by arrows. C) Ribbon representations of ArsC structures, colored as follows: ox-ArsC, light blue; c-ArsC, light green; re-ArsC, red; the P-loop (Cys10-Cys15), violet; the extended segment containing Cys89 and involved in interaction with Trx, yellow. The three active cysteines (serines in c-ArsC) are presented and labeled. The short helix S69-L72 is also labeled. D) The conformational switches of the segment 80-99 in ArsC, colored as follows: ox-ArsC, light blue; c-ArsC, light green; re-ArsC, red. The side chains of selected residues are presented in stick and labeled. The ends of the segment are labeled by numbers. The movements of selected residues are shown by arrows. The proteins in different states are arranged in (A) and (C) from left to right following the stages of the enzymatic reaction.
Table 1. Structural statistics of the Trx-ArsC complex

| NMR distance & dihedral constraints | complex | c-Trx | c-ArsC |
|-------------------------------------|---------|-------|--------|
| Distance constraints                |         |       |        |
| Total unambiguous NOE               | 6782    | 3396  | 3386   |
| Intra-residue                       | 2595    | 1261  | 1334   |
| Sequential ([i-j] = 1)              | 1681    | 853   | 828    |
| Medium-range ([i-j] < 4)            | 1014    | 497   | 517    |
| Long-range ([i-j] > 5)              | 1492    | 785   | 707    |
| Intermolecular                      | 112     |       |        |
| Total ambiguous NOE                 | 2215    | 1137  | 1078   |
| Hydrogen bonds                      | 70      | 28    | 42     |
| Disulfide bonds                     | 1       | 0     | 0      |
| Total dihedral angle restraints     |         |       |        |
| phi                                 | 127     | 56    | 71     |
| psi                                 | 132     | 57    | 75     |
| RDC restraints                      | 154     | 79    | 75     |
| Restraint Violations                |         |       |        |
| Distance (> 0.3 Å)                  | 3       |       |        |
| Dihedral angle (> 5°)               | 0       |       |        |
| Energy                              |         |       |        |
| Mean Amber Energy (kcal/mol)        | -12545.51 ± 18.28 |       |        |
| NOE distance restraints violation energy | 71.52 ± 2.76 |       |        |
| Torsion angle restraints violation energy | 2.20 ± 0.49 |       |        |
| r.m.s.d. from mean structure (Å)    |         |       |        |
| Heavy                               | 1.14 ± 0.16 | 0.68 ± 0.05 | 1.12 ± 0.13 |
| Backbone                            | 0.80 ± 0.20 | 0.29 ± 0.04 | 0.67 ± 0.11 |
| Secondary structure heavy           | 0.98 ± 0.15 | 0.56 ± 0.05 | 0.94 ± 0.12 |
| Secondary structure backbone        | 0.65 ± 0.20 | 0.23 ± 0.03 | 0.39 ± 0.07 |
| Ramachandran statistics             |         |       |        |
| Residues in most favored regions (%)| 79.6    |       |        |
| Residues in additional allowed regions (%) | 17.3    |       |        |
| Residues in generously allowed regions (%) | 2.3     |       |        |
| Residues in disallowed regions (%)  | 0.8     |       |        |
Fig. 2
Conformational fluctuations coupled to the thiol-disulfide transfer between thioredoxin and arsenate reductase in bacillus subtilis
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