The Bacillus subtilis YkuV Is a Thiol:Disulfide Oxidoreductase Revealed by Its Redox Structures and Activity*

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The Bacillus subtilis YkuV responds to environmental oxidative stress and plays an important role for the bacteria to adapt to the environment. Bioinformatic analysis suggests that YkuV is a homolog of membrane-anchored proteins and belongs to the thioredoxin-like protein superfamily containing the typical Cys-Xaa-Xaa-Cys active motif. However, the biological function of this protein remains unknown thus far. In order to elucidate the biological function, we have determined the solution structures of the oxidized and reduced forms of YkuV by NMR spectroscopy and performed biochemical studies. Our results demonstrated that the reduced YkuV has a low midpoint redox potential, allowing it to reduce a variety of protein substrates. The overall structures of both oxidized and reduced forms are similar, with a typical thioredoxin-like fold. However, significant conformational changes in the Cys-Xaa-Xaa-Cys active motif of the tertiary structures are observed between the two forms. In addition, the backbone dynamics provide further insights in understanding the strong redox potential of the reduced YkuV. Furthermore, we demonstrated that YkuV is able to reduce different protein substrates in vitro. Together, our results clearly established that YkuV may function as a general thiol:disulfide oxidoreductase, which acts as an alternative for thioredoxin or thioredoxin reductase to maintain the reducing environment in the cell cytoplasm.

Thioredoxins are small proteins (~12 kDa) that are ubiquitously present in all kinds of life forms from archaeabacteria to human (1). Generally, they function as protein thiol:disulfide oxidoreductases for maintaining the reducing environment in cytoplasm, protecting cells against hydrogen peroxide (2) and oxidative stress (3). In addition to the general function as a thiol:disulfide oxidoreductase, some thioredoxins from different organisms are also involved in various specified biological processes and therefore have specific biological functions. For example, thioredoxin is an essential subunit of bacteriophage T7 DNA polymerase in Escherichia coli (4), and in eukaryotes, thioredoxins facilitate the refolding of disulfide-containing proteins (5) and modulate the activity of certain transcription factors (6, 7). Moreover, thioredoxins have been considered as a possible target for drug development because of their roles in stimulating cancer cell growth and as an inhibitor of apoptosis (8).

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‡§¶ The atomic coordinates and structure factors (codes 2B5X and 2B5Y) 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: TrxA, thioredoxin; TrxB, thioredoxin reductase; ArsC, arsenate reductase; HSQC, heteronuclear single quantum coherence; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; r.m.s.d., root mean square deviation; CMP, cytochrome maturation protein; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; HSQC, heteronuclear single quantum coherence; TOCSY, total correlation spectroscopy.

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ies, including kinetics, structure determinations, and protein dynamics. We have solved the solution structures of B. subtilis YkuV in the biologically relevant reduced and oxidized states by high resolution NMR spectroscopy. The solution structures reveal significant conformational changes between the redox states. In addition, we have also characterized the backbone dynamic properties of this protein, which provide further insights in understanding the low redox potential of the reduced YkuV. These results in conjunction with the in vitro experiments strongly suggest that YkuV may function as a general thiol:disulfide oxidoreductase in vivo.

EXPERIMENTAL PROCEDURES

Sample Preparation—The B. subtilis ykuV gene was cloned into pET21a (+) expression vector and expressed in E. coli strain BL21(DE3). The cell culture was grown in 1000 ml of LB medium, centrifuged, and resuspended in 500 ml of M9 minimal medium at 37 °C with ampicillin and 15NH4Cl in the presence or absence of 13C-glucose for the preparations of 13C/15N-labeled or 15N-labeled samples, respectively (28). YkuV was purified by anion-exchange chromatography (DEAE) and gel filtration (Superdex 75) with the AKTA FPLC system (Amersham Biosciences). The purity was determined to be greater than 95% as judged by SDS-PAGE.

NMR samples were prepared with 1 mM YkuV dissolved in 90% H2O, 10% D2O buffer containing 50 mM sodium phosphate and 100 mM NaCl (pH 7.4) and were flushed with argon. The reduced form of YkuV was obtained by adding excess DTT (50 mM). The oxidized sample was prepared by incubating YkuV with an excess oxidized form of glutathione (10 mM) at room temperature for 3 h, followed by gel filtration to remove the oxidants. The redox state of the thiol groups was monitored using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) incubations (29).

The B. subtilis ykuU gene was cloned into pET24a (+) expression vector with a C-terminal His tag. The tag was expressed in E. coli strain BL21(DE3) and purified by a His-Trap HP column (Amersham Biosciences). The reduced and oxidized forms of YkuU samples were obtained by adding excess DTT or H2O2, respectively. DTT or H2O2 were subsequently removed by gel filtration. The redox states of the thiol groups were confirmed by DTNB incubations. The protein was dissolved in the same buffer as for YkuV.

The B. subtilis trxB gene encoding the thioredoxin reductase (TrxB) was cloned into pET21a (+) expression vector. TrxB protein was expressed in E. coli strain BL21(DE3) and purified by using similar protocols as described for YkuV. The preparation of the oxidized B. subtilis arsenate reductase (ArsC) sample was reported previously (30).

The Midpoint Redox Potential—For determination of the redox potential, the reduced form of YkuV (5 μM) in deoxygenated buffer containing 50 mM sodium phosphate, 100 mM NaCl (pH 7.4) was incubated with 5 mM reduced DTT for 3 h at room temperature to generate the fully reduced YkuV. DTT was subsequently removed using gel filtration. We followed the common procedures for determination of the redox potential (24). Briefly, by varying the ratios of oxidized and reduced DTT while keeping the total concentration constant (5 mM), different potentials were generated, and each reaction was allowed to equilibrate for 4 h at room temperature. The fluorescence emission intensity at 337 nm was measured following the excitation at 280 nm. The measurements were repeated using the oxidized form of YkuV. During the calculations of the redox potential, the value of −330 mV for the standard redox potential of DTT (pH 7.0) and a correction coefficient of −59 mV per pH unit increment were used (31).

Protein Interactions—For YkuV-YkuU interaction, the oxidized YkuU containing the His tag was dissolved in 10 ml of deoxygenated buffer containing 50 mM sodium phosphate and 100 mM NaCl (pH 7.4). The reduced fraction of 15N-labeled YkuV was mixed with the oxidized YkuU. The mixture was allowed to equilibrate at room temperature for 1.5 h and subsequently loaded onto the His-Trap HP column with the AKTA FPLC system. The flow-through was collected and concentrated immediately for NMR analysis. A parallel control experiment was carried out while YkuU was absent in the reaction system.

The reaction between thioredoxin reductase (TrxB) and the oxidized form of YkuV was performed under the same conditions as that used for YkuV-ArsC, except that 1 mM NADPH was added to provide the reducing power. HSQC spectra were recorded to monitor the redox state of YkuV. An experiment without TrxB was also performed as a control.

NMR Spectroscopy—All NMR experiments were performed at 25 °C on Bruker Avance 500-MHz (equipped with cryoprobe) and 800-MHz spectrometers equipped with triple-resonance probes with pulsed field gradients. The spectra were processed with the software package NMRPipe (32) and analyzed by the program NMRView (33). The two-dimensional 15N-edited HSQC, three-dimensional triple-resonance spectra HNCA, CBCA(CO)NH, HNCO, HN(CA)CO, HNCa, and HN(CO)CA were collected for the backbone assignments (34). The three-dimensional spectra HBHA(CO)NH, HCH-COSY, (H)CH-COSY, H(CC)(CO)NH-TOCY35, and (H)(CC)(CO)NH-TOCSY were recorded to assign the side chain atoms (35–39), which were confirmed with the three-dimensional 15N-edited TOCSY-HSQC (mixing time 80 ms) spectrum. The three-dimensional 15N- and 13C-edited NOESY-HSQC (mixing times 50 and 100 ms) spectra were recorded to confirm the assignments and generate distance restraints for structure calculations. The three-dimensional HNHA experiment was performed to obtain the dihedral angle restraints (40). Hydrogen-deuterium (H-D)
exchange experiments were performed to obtain hydrogen-bonding information.

**Structure Calculations**—The structures of the reduced and oxidized forms of YkuV were calculated using the program CYANA (41) and refined by AMBER (42). The inter-proton nuclear Overhauser effect (NOE) was employed to generate the distance restraints from three-dimensional $^{15}$N- and $^{13}$C-edited NOE-HSQC spectra. The backbone dihedral angle restraints were determined using the program TALOS (43). Hydrogen bond restraints were generated from the H-D exchange experiments in conjunction with the intermediate range NOEs and the secondary structural information. The initial structures were calculated with the CANDID module of the CYANA program (41, 44). In the first step, 50 structures were calculated, and the 10 lowest energy structures were selected as models for SANE to extend the NOE assignments (45). In the next step, 200 structures were calculated with CYANA by the standard simulated annealing protocol using NOE distance restraints in conjunction with the dihedral angle and hydrogen-bonding information. In the following step, 100 structures with the lowest target function were selected and further refined using the program AMBER (42). Finally, the 10 lowest energy structures were selected as representative of the final structure and were further inspected by the program packages MOLMOL (46) and PROCHECK_NMR (47).

**Backbone Relaxation Parameters**—The $^{15}$N longitudinal relaxation rates $R_1$, transverse relaxation rates $R_2$, and steady-state heteronuclear [$^1$H]-$^{15}$N NOE values of the reduced and oxidized forms of YkuV were determined using conventional pulse sequences (48). The experiments were performed on a Bruker Avance 800-MHz NMR spectrometer at 25 °C. Spectral widths of 11160.7 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 per increment and a recycle delay of 2.5 s. The delays used for the $R_1$ experiments were 10 ($\times$2), 100, 300, 500, 800, 1000, 1200, 1600, 2000, 2500, and 2800 ms, and those used for the $R_2$ experiments were 8 ($\times$2), 32, 56, 80, 104, 128, 152, 176, 200, and 240 ms. The relaxation rate constants were obtained by fitting the peak intensities to a single exponential function using the nonlinear least square method as described previously (49). The [$^1$H]-$^{15}$N 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 (50). Forty eight transients were collected for each experiment.

**FIGURE 2.** Solution structures of the reduced and oxidized *B. subtilis* YkuV. Superimposition is shown of 10 representative structures of the reduced YkuV (A) and the oxidized YkuV (B) with $\alpha$-helices in red and $\beta$-strands in blue. Ribbon diagram representation is shown of the secondary structure elements of the reduced YkuV (C) and the oxidized YkuV (D). The sulfur atoms of Cys$^{41}$ and Cys$^{44}$ are indicated by yellow balls. The figure was generated using MOLMOL (46).
Accession Numbers—The chemical shift assignments of the reduced and oxidized forms of \textit{B. subtilis} YkuV have been deposited in the BioMagResBank data base under accession numbers 6603 and 6847. The coordinates of the corresponding structures have been deposited in the Protein Data Bank under accession numbers 2B5X and 2B5Y.

\textbf{RESULTS}

\textit{Cloning and Expression}—The \textit{B. subtilis} \textit{ykuV} gene has been reported to encode a putative 153-amino acid residue protein (19). We cloned \textit{ykuV} from a \textit{B. subtilis} cDNA library by PCR. DNA sequencing of the cloned \textit{ykuV} gene revealed a deoxythymidylate insertion near the 3’ terminus, which is not present in the \textit{ykuV} sequence in the data base. This insertion causes an open reading frameshift, which replaces the last 10 residues WLKRNRYLTK by five residues (LAETE). Sequencing of additional individual clones revealed identical DNA sequences with the deoxythymidylate insertion. In an effort to generate \textit{ykuV} sequences as reported in the data base, we deleted the extra deoxythymidylate. The resulting protein appeared in the inclusion body in \textit{E. coli} and could not be refolded. In contrast, the YkuV protein we cloned could easily be expressed in \textit{E. coli}. In addition, the new protein sequence containing this difference is locally well aligned with its homolog, the YkuV protein from \textit{Bacillus licheniformis}, which is considered the closest relative species of \textit{B. subtilis} (51). Furthermore, the secondary structure prediction in combination with the homology modeling indicated that the altered residues do not change the secondary structures, and the extended residues have no effect on the global fold. Therefore, all our analyses were performed with the new \textit{YkuV} protein sequence.

\textit{Redox Potential}—To determine whether YkuV belongs to the family of thioldisulfide oxidoreductases, we measured its redox potential as shown in Fig. 1. The redox potential of \(-332 \pm 5\) mV (pH 7.4) was obtained by curve fitting (24). The value decreased to \(-308\) mV (pH 7.0) when a correction of \(-59\) mV per pH unit was used, which is characteristic for the involvement of two protons and two electrons in the redox process as expected for a thiol:disulfide oxidoreductase. The low redox potential suggests that YkuV belongs to the family of low potential, cytoplasmic thioldisulfide oxidoreductases, such as TrxA from \textit{E. coli} (24, 52).

\textit{Solution Structures of the Reduced and Oxidized Forms of \textit{B. subtilis} YkuV}—The chemical shift assignments for the reduced YkuV have been reported previously (22). Briefly, nearly complete backbone and side chain resonance assignments were obtained, except for residues His\(^{52}\), Ser\(^{131}\), Met\(^{133}\), and Lys\(^{134}\). For the oxidized YkuV, more than 90% of the chemical shift assignments for backbone and side chain atoms were achieved with the exception of residues Cys\(^{41}\), Leu\(^{63}\), His\(^{62}\), Ser\(^{131}\), Met\(^{133}\), and Lys\(^{134}\).

The YkuV structures were calculated using inter-proton NOE-derived distance restraints in combination with the dihedral angle and hydrogen bonding information. The superimposed representative structures (10 each), together with the ribbon diagrams of the mean structures, are shown in Fig. 2.

The structural statistics for both the reduced and oxidized forms of YkuV is summarized in Table 1. For the reduced form, there are two distance restraint violations greater than 0.3 Å, and no dihedral angle restraint violations greater than 5°. From the PROCHECK_NMR analysis, 81.0% of residues are within the most favored regions of the Ramachandran plot; 17.5% of residues are in the additionally allowed regions, and 0.8% of residues are in the disallowed regions. For residues 1–148, the overall backbone root mean square deviation (r.m.s.d.) from the mean structure is 0.45 \pm 0.05 Å and that of the regular secondary structural elements is 0.35 \pm 0.05 Å.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Distance restraints} & \textbf{Reduced} & \textbf{Oxidized} \\
\hline
Intra-residue unambiguous NOEs & 1595 & 1445 \\
Sequential unambiguous NOEs & 882 & 726 \\
Medium range unambiguous NOEs & 606 & 425 \\
Long range unambiguous NOEs & 1613 & 1272 \\
Total unambiguous NOEs & 3952 & 3233 \\
Total ambiguous NOEs & 1160 & 993 \\
Dihedral angles (\(\psi\) and \(\phi\)) & 178 & 160 \\
Hydrogen bonds & 51 & 49 \\
S–S bond & 0 & 1 \\
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\begin{tabular}{|c|c|c|}
\hline
\textbf{Restrains violations} & \textbf{Reduced} & \textbf{Oxidized} \\
\hline
Distance (\(>0.3\) Å) & 2 & 7 \\
Dihedral angle (\(>5°\)) & 0 & 4 \\
Hydrogen bonds & 0 & 0 \\
\hline
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\begin{table}[h]
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\begin{tabular}{|c|c|c|}
\hline
\textbf{Ramachandran statistics} & \textbf{Reduced} & \textbf{Oxidized} \\
\hline
Residues in most favored regions (%) & 81.0% & 78.9% \\
Residues in additionally allowed regions (%) & 17.5% & 17.9% \\
Residues in generously allowed regions (%) & 0.7% & 2.8% \\
Residues in disallowed regions (%) & 0.8% & 0.4% \\
\hline
\end{tabular}
\end{table}

For the oxidized form of YkuV, there are seven distance restraint violations greater than 0.3 Å and four angle restraint violations greater than 5°. From the PROCHECK_NMR analysis, 78.9% of residues are within the most favored regions of the Ramachandran plot; 17.9% of residues are in the additionally allowed regions, and 0.4% of residues are in the disallowed regions. For residues 1–148, the overall backbone r.m.s.d. from the mean structure is 0.54 \pm 0.09 Å and that of the regular secondary structural elements is 0.40 \pm 0.06 Å.

\textit{B. subtilis} YkuV consists of five \(\alpha\)-helices and six \(\beta\)-strands. The overall structure contains a thioredoxin-like fold, consisting of a mixed four-stranded \(\beta\)-sheet surrounded by three \(\alpha\)-helices. In addition, YkuV contains an insertion from Ser\(^{72}\)–Ser\(^{89}\), which gives rise to an additional strand (\(\beta4\)) and helix (\(\alpha3\)) between \(\beta3\) and \(\alpha4\). A similar insertion has been reported recently in the structures of CcmG and TlpA from \textit{B. subtilis} (25). The sequence alignment of YkuV with its structural homologs ResA and the canonical thioredoxin from \textit{B. subtilis} is shown in Fig. 3.

\textit{Conformational Changes Around the Cys-Xaa-Xaa-Cys Active Motif}—Although the overall structures of the reduced and oxidized forms of YkuV are similar, significant localized differences are observed. The most notable difference between the two states is around the Cys-Xaa-Xaa-Cys active motif, especially the arrangement of two cysteine residues, Cys\(^{41}\) and Cys\(^{44}\). In the oxidized form the distance between the two sulfur atoms is \(-2.05\) Å, which is very close to the length of a disulfide bridge. The incubation of oxidized YkuV with DTNB did not release any dianionic TNB\(^{-2}\), demonstrating that the two cysteine residues, Cys\(^{41}\) and Cys\(^{44}\), were in the oxidized state. The formation of the disulfide bridge Cys\(^{41}\)–Cys\(^{44}\) was further confirmed by the NOE contacts from the three-dimensional NOESY-HSQC spectra.

However, in the reduced form of YkuV, the sulfur atom of Cys\(^{41}\) shifts toward the outside of the structure core and the distance between the two sulfur atoms increases to \(-4.5\) Å. The incubation of the reduced YkuV with DTNB released roughly 2.0 mol of dianionic TNB\(^{-2}\) per mol of YkuV, indicating that both Cys\(^{41}\) and Cys\(^{44}\) were in the reduced state. In addition, the NOE contacts from the three-dimensional NOESY-
HSQC spectra indicated that Cys41 and Cys44 are farther away from each other, consistent with the reduced state.

The comparison between the HSQC spectra of the reduced and oxidized forms of YkuV and the composite 1H and 15N chemical shift changes are shown in Fig. 4. There is significant difference near the active site between the two states, indicating considerable conformational changes between the reduced and oxidized states. An overlay of the C/H9251 trace of the reduced and oxidized forms of YkuV is shown in Fig. 5A. The r.m.s.d. between them is 1.03 Å for the 148 pairs of C/H9251 atoms. The open and closed conformations of the redox active motif corresponding to the reduced and oxidized states of YkuV, respectively, were clearly observed as shown in Fig. 5B.

Structural Comparison between B. subtilis YkuV and ResA—We have searched the Protein Data Bank for homologous structures of B. subtilis YkuV using the program DALI (55), and we identified the soluble domain of ResA from B. subtilis as the best fit structure (Protein Data Bank entry 1SU9). ResA is a membrane-anchored protein that may act as a reductant for apocytochrome c on the cell membrane and thus belongs to the CMP family (17). Other matches, including TlpA and CcmG, are also membrane-anchored thioredoxin-like proteins and belong to the CMP superfamily. The largest structural difference between CMPs and YkuV was observed near the N-terminal region. Residues at the N-terminal region of the soluble domains of CMPs form a small anti-parallel β-sheet with strand Å4. The comparisons of the C/H9251 trace of the reduced and oxidized forms of YkuV (energy-minimized mean structure using AMBER) with that of the corresponding crystal structures of ResA are shown in Fig. 5, C and D, respectively. Notably, YkuV and ResA share a similar structural change between reduced and oxidized forms, especially around the active site Cys-Xaa-Xaa-Cys motif, and they both show significantly low midpoint redox potential (−308 mV for YkuV and −340 mV for ResA).
ArsC is required for the next cycle of reduction. Thioredoxin has been proposed to act as the electron donor for the regeneration of the active ArsC (59). Our in vitro experiments showed that YkuV could regenerate the reduced form of ArsC from its oxidized form, which was associated with the oxidation of YkuV. The conformational switch was monitored by two-dimensional $^{15}$N-edited HSQC spectra, which showed identical spectra to that in Fig. 4A, corresponding to the reduced and oxidized states of YkuV, respectively. Meanwhile, the opposite conformational switch of ArsC from the oxidized to the reduced form was also observed by two-dimensional $^{15}$N-edited HSQC spectra (data not shown).

In addition, the thioredoxin reductase (TrxB), which is believed to reduce thioredoxin (TrxA) in vivo, can also regenerate the reduced form of YkuV from its oxidized form. The conformational switch of YkuV was monitored by two-dimensional $^{15}$N-edited HSQC spectra, which were identical to the corresponding spectra in Fig. 4A. This result indicated that YkuV is a substrate of TrxB in vitro. All together, these results clearly establish that YkuV may perform a similar thiol/disulfide oxidoreductase function as thioredoxin and may be similarly involved in the redox cascade as that of thioredoxin.

Internal Dynamics—In order to characterize the motional properties and to further obtain functional insights of B. subtilis YkuV, the backbone $^{15}$N relaxation parameters $R_1$ and $R_2$ and the $^{1}H-^{15}$N NOE values were determined for the reduced and oxidized forms, respectively. We found that the oxidized form of YkuV had a high tendency to oligomerize during the measurements and resulted in large experimental errors (data not shown). The reduced form, however, remained mostly in the monomeric state and allowed proper measurement of the relaxation parameters. The experimental data for the reduced form of YkuV are shown in Fig. 6A.

In the analysis of the reduced YkuV, 105 of the 148 residues were used. The unanalyzed residues included 8 proline residues that have no amide protons, 8 residues that were unassigned, and 27 residues that were either overlapped or too weak to be analyzed. Overall, the entire enzyme in the reduced form is rigid as reflected by the high NOE values (0.75) for most of the residues. However, residues Gly25, Glu28, and Glu39 and those in the N- and C-terminal regions show low NOE values (<0.75), indicative of fast internal motions on picosecond to nanosecond time scales. In addition, residues near the active site and those in the region from Ile90 to Val96 show larger than the averaged $R_2/R_1$ ratios, suggesting the conformational flexibility on microsecond to millisecond time scales and will be discussed below.

Characterization of the motional anisotropy is crucial in the analysis of any NMR relaxation data, especially in the characterization of chemical or conformational exchanges (60). The rotational diffusion tensor is used to describe the motional anisotropy. Calculated from the solution structures, the ratio of the principal components of the inertia tensor is $1:0.89:0.65$ for most of the residues. However, residues Gly25, Glu28, and Glu39 and those in the N- and C-terminal regions show low NOE values (<0.75), indicative of fast internal motions on picosecond to nanosecond time scales. In addition, residues near the active site and those in the region from Ile90 to Val96 show larger than the averaged $R_2/R_1$ ratios, suggesting the conformational flexibility on microsecond to millisecond time scales and will be discussed below.

Conformational Changes Coupled to the Redox Reactions with Protein Partners—Based on its low redox potential, it is not surprising that the reduced YkuV can reduce oxidized protein substrates. In order to identify possible protein substrates of YkuV, we searched the B. subtilis genome and identified protein YkuU as a potential candidate. The gene ykuU is located in the same operon with ykuV in the B. subtilis genome. It is annotated as a 2-Cys peroxiredoxin, which is believed to use thioredoxin to regenerate the reduced YkuU,15N-labeled YkuV in the reduced state was incubated with unlabeled oxidized YkuU. In the analysis of the reduced YkuV, 105 of the 148 residues were used. The unanalyzed residues included 8 proline residues that have no amide protons, 8 residues that were unassigned, and 27 residues that were either overlapped or too weak to be analyzed. Overall, the entire enzyme in the reduced form is rigid as reflected by the high NOE values (0.75) for most of the residues. However, residues Gly25, Glu28, and Glu39 and those in the N- and C-terminal regions show low NOE values (<0.75), indicative of fast internal motions on picosecond to nanosecond time scales. In addition, residues near the active site and those in the region from Ile90 to Val96 show larger than the averaged $R_2/R_1$ ratios, suggesting the conformational flexibility on microsecond to millisecond time scales and will be discussed below.

<FIGURE 5. Structural comparison. A, overlay of the Cα trace of the solution structures of reduced (red) and oxidized (blue) B. subtilis YkuV. B, comparison of the local structures of the active site motif of the reduced (left) and oxidized (right) YkuV. C, the Cα trace of the reduced B. subtilis YkuV in solution (red) superimposed with the crystal structure of B. subtilis ResA (cyan). D, the Cα trace of the oxidized B. subtilis YkuV (blue) superimposed with that of the crystal structure of B. subtilis ResA (green). The figure was generated using MOLMOL (46).>
Structures and Functional Insights of B. subtilis YkuV

FIGURE 6. The backbone relaxation data and internal mobility parameters of B. subtilis YkuV. A, the $^{13}$N longitudinal relaxation rates $R_1$, transverse relaxation rates $R_2$, heteronuclear ($^1$H-$^{13}$N) NOE, and $R_2/\tau_e$ values of the reduced B. subtilis YkuV versus the amino acid sequence. The spectra for the relaxation parameters determination were recorded on a Bruker Avance 800 MHz spectrometer at 25 °C. Uncertainties were obtained using Monte Carlo simulations. B, the backbone dynamic parameters $S^2$, $\tau_e$, $R_{ex}$, and the model selections for the reduced B. subtilis YkuV versus the amino acid sequence. The secondary structural elements are shown at the top.

plicity (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 for descriptions of the internal mobility and reproduction of the experimentally determined data until it reached the confidence within 95% (61). The optimized internal mobility parameters of generalized order parameter ($S^2$), fast internal motion on the picosecond to nanosecond time scales ($\tau_e$), and millisecond time scale conformational exchange ($R_{ex}$) for the reduced form of YkuV are shown in Fig. 6B.

Sixty seven residues mainly in the secondary structural elements were described by M1, giving the average $S^2 = 0.90 \pm 0.03$. Nineteen residues near the N and C termini and the loop regions were assigned to M2, with the average $S^2 = 0.84 \pm 0.03$, and internal motions ($\tau_e$) on the picosecond to nanosecond time scales. Thirteen that were described by M3 showed the average $S^2 = 0.83 \pm 0.09$, along with the average $R_{ex} = 4.6 \pm 1.8$ s$^{-1}$. Three residues were assigned to M4, with the average $S^2 = 0.76 \pm 0.08$ and $R_{ex} = 7.7 \pm 1.5$ s$^{-1}$. Three residues were assigned to M5, with the average $S^2 = 0.42 \pm 0.04$, and various degree of flexibility on the picosecond to nanosecond time scales ($\tau_e$).

Overall, the enzyme adopts a fairly rigid fold as reflected by the overall averaged generalized order parameter $S^2 = 0.86 \pm 0.04$. However, different internal motional properties were also observed. The residues near the active site and those in the regions around residue Glu$^{39}$ and residues Ile$^{99}$ to Val$^{100}$ exhibited significant conformational exchanges on the millisecond time scale. A closer examination of the dynamic results (Fig. 6B) revealed that many residues in the reduced form of YkuV showed smaller order parameter $S^2$ associated with various degrees of internal motion on the picosecond to nanosecond time scales.

DISCUSSION

The Strong Reducing Power—It was commonly observed in the thioredoxin-like proteins that the N terminus of the active site cysteines in the thiolate form is stabilized by interaction with the dipole of its following helix. In some cases, this interaction may reduce the pK$_a$ of the relevant cysteine and affect the redox potential of the enzyme (25). As YkuV maintains this conventional helix dipole-cysteine interaction, the pK$_a$ of Cys$^{41}$ may be reduced. Nevertheless, further experimental determination of the pK$_a$ is expected to yield definitive results.

Similar to ResA, the large distance (~4.5 Å) between the two sulfur atoms of the active cysteines in the reduced state of YkuV may be one reason for its low redox potential (~332 mV (pH 7.4)). In addition, for both YkuV and ResA, there were few identifiable interactions for the thiol(ate) group of the N-terminal cysteine (Cys$^{41}$ in YkuV and Cys$^{73}$ in ResA), such as hydrogen bond interactions in the structure of the reduced state. This character was quite different from DsbA, in which the active site thiolate was stabilized by four hydrogen bonds (25). A closer examination near the active site cavity of YkuV revealed that the side chain NH$_2$ of Arg$^{71}$ was close in space with the side chain CO of Asp$^{75}$ (~1.8 Å) in the oxidized state, an indication of the electrostatic interaction or hydrogen bonding. However, this interaction was broken in the reduced state, where the side chains of the two residues were more than 5 Å apart. Both Arg$^{71}$ and Asp$^{75}$ are spatially close to the active site. Their closest distances from the active cysteines (Cys$^{41}$ and Cys$^{73}$) in the reduced state are ~3–6 and 5–8 Å for Arg$^{71}$ and Asp$^{75}$, respectively. Therefore, this feature may also contribute to the destabilization of the active site of the reduced YkuV.

The results from the backbone dynamics further indicated that residues near the Cys-Xaa-Xaa-Cys active motif showed significant millisecond conformational changes in the reduced state. From residue Leu$^{39}$ to Cys$^{44}$, every observed residue showed significant conformational exchanges on the microsecond to millisecond time scales. Residues Ser$^{40}$ and His$^{42}$ did not show NH correlations in the HSQC spectrum, also an indication of conformational exchanges. The results suggest that the residues in this region have a substantial degree of conformational flexibility on the microsecond to millisecond time scales. Furthermore, the active residue Cys$^{41}$ also exhibited fast internal motion on the sub-nanosecond time scale as reflected by the lower than averaged order parameter ($S^2 = 0.6$), in addition to the millisecond flexibility. Notably, other residues in this region showed order parameters $S^2 \leq 0.80$, suggesting the lack of fast internal motions on the sub-
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nanosecond time scale. The large degree of motional flexibility occurring on the backbone of Cys\(^{41}\) may contribute to the destabilization of its side chain atoms, such as the thiol group. In contrast, the reduced form of DsbA from *E. coli*, which is a periplasmic thioredoxin-like protein and is required for disulfide bond formation *in vivo*, has a relatively high redox potential (−125 mV (pH 7.0)) (65, 66). The explanation of this fact was largely attributed to the formation of hydrogen bonds of the active thiolate with surrounding residues in addition to the well documented interactions in thioredoxins (25, 67). Based on this, it is not surprising that the reduced YkuV with a less stable structure exhibits a significantly lower redox potential (−308 mV (pH 7.0)), which is important for YkuV to function as an electron donor. A similar observation was reported recently for *B. subtilis* ResA (24). Combining our results with previous studies, it seems likely that the flexibility of the active site thiol group significantly affects the value of the midpoint potential.

A Docked Protein-Protein Complex Represents the Interaction between YkuV and Protein Substrates—In order to obtain insights into the interaction between YkuV and its protein substrates, we performed a docking using the solution structures of YkuV and ArsC (Protein Data Bank entry 1Z2E) by the program HADDOCK (68). The structures of a human thioredoxin in covalent complexes with peptides derived from NF-κB are the only available models representing the reaction intermediates of disulfide reduction by thioredoxin (69, 70). The structure of YkuV could be easily fit onto the structure of human TRX. The interaction network between human TRX and the substrates was used as the structural basis for docking the reduced YkuV and oxidized ArsC. Based on the solvent accessibility, Cys\(^{41}\) of YkuV and Cys\(^{89}\) of ArsC were assumed to form the first disulfide bridge. The disulfide bridge Cys\(^{82}\)–Cys\(^{89}\) of oxidized ArsC was removed, and the intermolecular disulfide bridge Cys\(^{41}\)–Cys\(^{89}\) was used as a restraint during the final steps of HADDOCK modeling. The structure with the lowest energy was selected as a representative model of YkuV–ArsC interaction, as shown in Fig. 7A. Based on the docking result, we proposed a redox mechanism for YkuV to reduce its substrate proteins (Fig. 7B). Here we discuss the redox procedures using the interaction between YkuV and ArsC. In the first reaction step, the thiol of YkuV-Cys\(^{41}\) attacks that of ArsC-Cys\(^{89}\) and cleaves the intermolecular disulfide bridge ArsC-Cys\(^{82}\)–Cys\(^{89}\), and subsequently an intermolecular disulfide bridge between YkuV-Cys\(^{41}\) and ArsC-Cys\(^{89}\) is formed. In the next step, the thiol of Cys\(^{44}\) of YkuV attacks that of YkuV-Cys\(^{41}\) and breaks the intermolecular disulfide bridge, and an intramolecular disulfide bridge YkuV-Cys\(^{41}\)–Cys\(^{44}\) is formed, resulting in the reduction of ArsC and oxidation of YkuV. It is well known that thioredoxin acts as the general electron donor to its substrate proteins such as ArsC *in vivo*. The *in vitro* experiment results in conjunction with the protein-protein docking model suggest that YkuV may also act as a general electron donor for its substrate proteins in cell cytoplasm.

In conclusion, the solution structures of the reduced and oxidized forms of *B. subtilis* YkuV in combination with the backbone dynamics of the reduced YkuV provide the structural and motional insights in understanding the low redox potential of YkuV. The *in vitro* interactions with the protein substrates reveal significant conformational changes at the active site motif associated with the redox transition of YkuV. The current studies demonstrate a good correlation among the structures, internal dynamics, and redox activity and thus provide information in exploring the biological functions of this protein at the molecular level. Our data strongly suggests that *B. subtilis* YkuV may act as a general thiol-disulfide reductase in the cell cytoplasm. Further investigations are required to confirm the thiol-disulfide oxidoreductase function of YkuV *in vivo*.

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FIGURE 7. Modeling of the *B. subtilis* YkuV–ArsC interaction. A, the reduced YkuV (red) is proposed to attack Cys\(^{82}\)–Cys\(^{89}\) of the oxidized ArsC (gray) through Cys\(^{41}\). The modeled complex structure represents the first intermediate of the reaction that involves the formation of an intermolecular disulfide bridge between YkuV-Cys\(^{41}\) and ArsC-Cys\(^{89}\). Cysteine side chains are represented by blue sticks and are labeled. The sulfur atoms of the cysteines are indicated by yellow balls. A black line joins the sulfur atoms of Cys\(^{41}\) and Cys\(^{44}\). The figure was generated using MOLMOL (66). B, a proposed general mechanism for YkuV reducing the protein substrates.
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