Werner syndrome is a premature aging disease caused by mutations in the WS gene and a deficiency in the function of Werner protein (WRN). The lack of WRN results in a cellular phenotype of genomic instability. WRN belongs to the RecQ DNA helicase family, but unlike other RecQ family members it possesses a functional exonuclease domain. We determined the crystal structure of mWRNexo (residues 31–238) bound to Zn^{2+} and the sulfate ion. Compared with the structure of human WRNexo (hWRNexo), notable conformational changes were observed in several active site residues in an H5–H6 loop and in helices H6 and H7 of mWRNexo, presumably because of the presence of sulfate, which mimics the phosphate of substrate DNA. In particular, the side chains of Lys^{185} and Tyr^{200} were reoriented toward the Zn^{2+} ion, whereas the side chain of Arg^{190} pointed away from the active site center. Mutational analysis of these conserved residues abolished WRN exonuclease activity, suggesting that these residues play a critical role in the WRNexo activity. Based on substrate modeling and mutational analyses, we propose a mechanism by which WRNexo becomes activated upon substrate DNA binding. We also describe the low resolution trimeric structure of mouse WRNexoL (mWRNexoL, residues 31–330), as elucidated by small angle x-ray scattering (SAXS) analyses.

Werner syndrome is a human autosomal recessive disorder and is associated with premature aging and an increased incidence of cancer (1, 2). Werner syndrome patients exhibit an increased predisposition to arteriosclerosis, osteoporosis, type II diabetes mellitus, and a variety of tumors, which are normally observed during aging (2, 3). Fibroblast cultures from Werner syndrome patients show a reduced life span and a variety of chromosomal abnormalities including reciprocal translocations, deletions, and inversions (4), abnormalities in S phase initiation or transit (5), and attenuated replicative potential (6, 7). In addition, some Werner syndrome cell lines have shown aberrant mitotic recombination (8) and increased levels of homologous recombination (9). Werner syndrome cell lines are also hypersensitive to the DNA-damaging agent, 4-nitroquinoline-1-oxide (10). Together, these cellular phenotypes suggest that Werner syndrome is associated with one or more defects in DNA metabolism.

Most Werner syndrome cases have been causally related to mutations in a single gene, WRN, which encodes a multifunctional protein, WRN, a member of the RecQ family of helicases (11). In humans, this family includes proteins such as Bloom syndrome and Rothmund-Thompson syndrome proteins, whose germline mutations are responsible for diseases associated with genomic instability (12, 13).

The precise molecular function of WRN is unclear. However, WRN is known to interact with several key proteins that play critical roles in DNA replication, recombination, and repair, which suggests that WRN may maintain genomic integrity and life span by participating in DNA transactions in mammalian cells (13).

WRN contains an exonuclease domain that is unique among RecQ family members (14–16). Although the biological importance of the Werner exonuclease domain (WRNexo) has not been precisely defined, the facts that WRNexo cleaves diverse substrates including the long fork form of DNA and Holliday junctions, and that WRN interacts with proliferating cell nuclear antigen (PCNA) suggest that WRNexo may participate in the DNA replication process (16). Recent studies show that inactivation of WRNexo alters DNA end-joining in human cells (17). Moreover, Ku70/80, an important regulator of genomic stability, stimulates the exonuclease activity of WRNexo, implying that WRNexo may play an important role in DNA repair (17). Interestingly, Ku70/80 inhibits the 3’-5’ exonuclease of Klenow fragment (KFexo), a structurally conserved 3’-5’ exonuclease, which suggests that the Ku-mediated activation mechanism could be unique to WRNexo. Thus, elucidation of structure and function of WRNexo may provide important insights to understand the molecular mechanism by which...
WRN cleaves diverse types of substrates in DNA replication and repair process.

Two divalent metal ions are essential for WRN exonuclease activity, as they are for other members of the DnaQ exonuclease family (16, 18–20). Zn$^{2+}$, Mn$^{2+}$, and Mg$^{2+}$ ions are known to stimulate the exonuclease activity of WRN, and Zn$^{2+}$ and Mn$^{2+}$ are more efficient than the Mg$^{2+}$ ion (17, 18). Zn$^{2+}$-mediated activation occurs in a dose-dependent manner, but at Zn$^{2+}$ concentrations over ~500 μM, exonuclease activity is inhibited (18). In DnaQ family members, a metal ion and two conserved residues, Tyr and Glu, at the active site orient nucleophilic attack of substrate phosphorus and generate a transition state that is stabilized by the two metal ions (19, 20).

Despite extensive biochemical studies on the exonuclease activity of WRN both in vitro and in vivo (15, 18, 21–24), structural information is lacking. Recently, the structures of human WRN exonuclease domain (hWRNexo) in the absence and presence of Mn$^{2+}$ and Mg$^{2+}$ ions were determined (17, 18). In mWRNexo and hWRNexo, respectively, throughout the text), a sulfate ion was bound in the presence of Mn$^{2+}$ and Mg$^{2+}$ ions (Fig. 1 and Table 1). Interestingly, in both apoform and Zn-bound structures, we observed a sulfate ion in the active site, which might mimic the phosphate ion of a substrate DNA. This sulfate ion induced a conformational change of residues in the active site, which included rotation of Tyr206 and Lys185 toward the active site and the movement of Arg190 to the surface. Based on structural and biochemical analysis, we propose a substrate binding site and a Zn$^{2+}$-dependent activation mechanism for WRNexo.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification—DNA-encoding mouse WRN-(31–238) (mWRNexo) and mouse WRN-(31–300) (mWRNexoL) were amplified by PCR and inserted into a pET28a vector using NdeI and XhoI sites. Each construct was induced in Escherichia coli BL21(DE3) with 1 mM isopropyl-1-thio-β-D-galactopyranoside at an OD of 0.6. Cells were cultured at 18°C for 12 h and harvested by centrifugation and lysed by sonication in 25 mM NaH$_2$PO$_4$, pH 7.4, 300 mM NaCl, and 7 mM 2-mercaptoethanol. The lysate was clarified by centrifugation, and the protein was purified by a nickel column. The imidazole gradient with solutions containing 50–250 mM imidazole in 25 mM NaH$_2$PO$_4$, pH 7.4, 300 mM NaCl, and 7 mM 2-mercaptoethanol was used.

**Crystallization and Data Collection**—The mWRNexo crystals were grown by the hanging-drop vapor diffusion method. Briefly, the protein (20 mg/ml) in 5 mM Tris-HCl, pH 8.0, with 150 mM NaCl and 5 mM DTT was mixed with reservoir solution (1.26 M ammonium sulfate and 0.1 M LiSO$_4$ in 0.2 M Tris-HCl buffer, pH 8.0) at an equal ratio. 4 μl of these mixtures were equilibrated with 500 μl of each reservoir solution at 18°C. The orthorhombic crystals (up to 0.1 × 0.02 × 0.03 mm) appeared after 1–2 days. Crystals were soaked with the ZnSO$_4$ (50 mM...
ZnSO₄ with 1.26 m ammonium sulfate and 0.1 m LiSO₄ in 0.2 m Tris-HCl buffer, pH 8.0) for 8 h. X-ray diffraction data were collected for the apoform or ZnSO₄-soaked mWRNexo crystal at −170 °C from a crystal flash-frozen in crystallization buffer containing 30% sucrose using the 4A. Data were processed with the program HKL2000 (25). The crystals from two different conditions belong to the same space group P2₁2₁2₁ with the program GNOM (28) was used to compute the compactness of polypeptide chains. To obtain shape information, the program GNOM (28) was used to compute the distance distribution function \( p(r) \), which also gives the maximum diameter of the protein \( D_{\text{max}} \), as the distance where \( p(r) \) function approaches zero. The low resolution shapes of mWRN exo and mWRN exoL were reconstructed using an \textit{ab initio} shape-determination program, DAMMIN (29). A sphere of diameter \( D_{\text{max}} \) is filled by a regular grid of points corresponding to a dense hexagonal packing of small spheres (dummy atoms) of radius \( r_0 \ll D_{\text{max}} \). The structure of the dummy atoms model is defined by a configuration vector \( X \) assigning as index to each atom (0 corresponds to solvent and 1 to the solute particle). The method searches for a compact interconnected configuration \( X \) minimizing the discrepancy \( \chi \) between the calculated \( I_{\text{calc}}(q) \) and the experimental curves. Successive runs of DAMMIN (29) on the same data set starting from same initial configurations of dummy atoms may provide different models, and analysis of these models allows one to improve the reliability of the final solution. For each protein ten independent models were generated, all models were compared, and the most probable one determined using the DAMAVER package (30), which aligned all models with the most probable one, and averaged

**TABLE 1**

Statistics of data collection and refinement

| Data sets       | Apoform | Edge | Peak | Remote | Zn-bound |
|-----------------|---------|------|------|--------|---------|
| Wavelength (Å)  | 1.0000  | 1.2831| 1.2825| 1.2699 | 1.2835  |
| Resolution (Å)  | 50-2.0  | 50-2.7| 50-2.7| 50-2.7 | 50-2.2  |
| Measured reflections | 862403 | 85654 | 187162| 81836 | 377880 |
| Unique reflections | 14814  | 6046 | 6006 | 6191 | 11712  |
| Completeness (%) | 96.0 (94.1) | 97.7 (87.5) | 98.8 (95.5) | 98.1 (95.7) | 99.5 (97.7) |
| Average (Å) | 29 | 22 | 38 | 24 | 40 |
| r.m.s. deviation bond length (Å) | 0.006 | 0.005 | 0.006 | 0.005 |
| Overall figure of merit (30-2.7 Å) | 0.44 | |
| Refinement | | | | | |
| Resolution range (Å) | 30-2.0 | 30-2.0 | | | |
| Number of reflections (Å > 1) | 13095 | 10377 | | | |
| \( R_{\text{work}} \) (%) | 20.9 | 19.7 | | | |
| \( R_{\text{free}} \) (%) | 24.3 | 24.4 | | | |
| Number of water molecules | 125 | 150 | | | |
| r.m.s. deviation bond angles (Å) | 1.2 | 1.1 | | | |

**Structure Determination**—The Zn-bound-mWRNexo structure was solved using the multiwavelength anomalous dispersion method. The SOLVE program (31) identified two Zn²⁺ positions and provided the initial phasing with three wavelengths (\( \lambda_1 = 1.2831 \, \text{Å}, \lambda_2 = 1.2825 \, \text{Å}, \text{and} \lambda_3 = 1.2699 \, \text{Å} \)) for zinc multiwavelength anomalous dispersion data at 2.7-Å resolution. Solvent flattening by the RESOLVE program (32) was used to improve the accuracy of phases. The graphic program O (33) was used for model building, and CNS (34) was used for refinement. The model was refined against the 2.2-Å native data collected at a 1.2835-Å wavelength. During the refinement, the \( R_{\text{free}} \) value was monitored using 5% of the data. The structure of the Zn-mWRNexo has been refined to an \( R \)-factor of 19.7% and an \( R_{\text{free}} \) of 24.4% at 2.2-Å resolution. The apoform of the mWRNexo structure was determined by a different Fourier method using a Zn-mWRNexo structure (Table 1). The final model has 95% of the protein main chain angles in the most favored region and none in the disallowed region of the Ramachandran plot, calculated using PROCHECK (35). In both structures, most of the regions are clearly defined except residues 82–84.

**Small Angle X-ray Scattering Study**—The synchrotron radiation small angle x-ray scattering (SAXS) experiments were performed at the bending magnet SAXS beamline 4C1 of the Pohang Light Source (26). SAXS patterns were recorded by a two-dimensional charge-coupled detector (2D CCD) (MAR165). The magnitude of scattering vectors \( q = 4\pi\sin(\theta)/\lambda \) was 0.08 mm⁻¹ < \( q < 6 \, \text{nm}^{-1} \), where \( \theta \) is a half of the angle between the incoming x-ray and the scattered x-ray, and \( \lambda = 0.1608 \, \text{nm} \) is the wavelength of the x-ray. The scattering vector calibration was done with a precalibrated Silver Behenate of the periodic length of 5.8376 nm. The sample cell was 50 µl in volume with 10-µm thick mica windows and had a 0.7-mm x-ray path length. Exposure times were typically 10 min for individual measurements. Measurements were carried out at 25 °C. The processed data recorded at two sample-to-detector distances (2 and 50 cm) were merged to yield the final composite scattering curves. The radius of gyration \( R_g \) was determined using the Guinier approximation \( I(q) = I(0)R^2q^2/3 \) valid for \( qR_g < 1.3 \) (27). The Guiner plots of SAXS data give estimates of the radius of gyration \( (R_g) \), which is informative about size and compactness of polypeptide chains. To obtain shape information, the program GNOM (28) was used to compute the distance distribution function \( p(r) \), which also gives the maximum diameter of the protein \( D_{\text{max}} \), as the distance where \( p(r) \) function approaches zero. The low resolution shapes of mWRN exo and mWRN exoL were reconstructed using an \textit{ab initio} shape-determination program, DAMMIN (29). A sphere of diameter \( D_{\text{max}} \) is filled by a regular grid of points corresponding to a dense hexagonal packing of small spheres (dummy atoms) of radius \( r_0 \ll D_{\text{max}} \). The structure of the dummy atoms model is defined by a configuration vector \( X \) assigning as index to each atom (0 corresponds to solvent and 1 to the solute particle). The method searches for a compact interconnected configuration \( X \) minimizing the discrepancy \( \chi \) between the calculated \( I_{\text{calc}}(q) \) and the experimental curves. Successive runs of DAMMIN (29) on the same data set starting from same initial configurations of dummy atoms may provide different models, and analysis of these models allows one to improve the reliability of the final solution. For each protein ten independent models were generated, all models were compared, and the most probable one determined using the DAMAVER package (30), which aligned all models with the most probable one, and averaged
aligned models. The scattering computed from the averaged experimental data, but this model keeps the most probable common features of the solutions.

Mutagenesis—All the mWRNexo mutants used in this study were constructed by PCR-based methods (Stratagene). The mutant proteins were purified using affinity chromatography followed by Mono-Q and gel filtration chromatography, as described above.

Circular Dichroism Analysis—Structural changes of a wild-type and mutant mWRNexo (10 μM) were monitored by circular dichroism (CD) spectrometry (Jasco J-715) at various wavelengths (200–250 nm). All samples used here were prepared in 20 mM KH₂PO₄, 150 mM NaCl, pH 7.4.

Exonuclease Activity Assays—PAGE-purified oligonucleotides (FLAP26 and TSTEM25) were prepared with 5'-³²P end-labeled as described in a previous study (18). Exonuclease assay reactions (20 μl) contained 30 mM HEPES (pH 7.4), 5% glycerol, 40 mM KCl, 0.6 mM DNA fork substrate, and the indicated concentrations of ZnCl₂ (or other metal ion) and mWRNexo. The metal was preincubated with mWRNexo for 30 min on ice before initiating the 20-min reaction at 37 °C with a radiolabeled substrate. Each reaction was quenched with 10 μl of stop buffer (80% formamide, 0.5 × Tris borate-EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue) and heated at 95 °C for 10 min. Products were resolved on denaturing 15% polyacrylamide gels and visualized by a PhosphorImager.

Gel Filtration Analysis—To analyze the oligomeric state of mWRNexol, the protein was loaded on the Superdex 200 column equilibrated with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 5 mM DTT. Molecular mass standards comprising bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa) were used for calibration.

RESULTS

mWRNexoL Trimerizes Through Its C-terminal Region—To define the domain boundary of mWRNexo, we employed an approach that combined limited proteolysis using subtilisin and sequence alignment with other WRN family members. This characterization identified two conserved core fragments of mWRNexo (residues 31–238) and mWRNexoL (residues 31–300). Size exclusion chromatography revealed that mWRNexo forms a monomer, whereas mWRNexoL exists as a trimer (Fig. 2A). The trimeric character of mWRNexol in solution was further confirmed by small angle x-ray scattering (SAXS) (Fig. 2, B and C, see “Structure of mWRNexo and mWRNexoL in Solution”).

In Vitro Exonuclease Activity—To confirm the exonuclease activity of the two mWRNexo fragments, we measured the exonuclease activities of recombinant mWRNexo and mWRNexoL using a fork-formed DNA substrate. Fig. 3, A and B show that both mWRNexo and mWRNexoL exhibit Zn²⁺-dependent exonuclease activity. When Zn²⁺, Mn²⁺, and Mg²⁺ ions were compared, Zn²⁺ and Mn²⁺ ions were found to exhibit a strong preference over Mg²⁺ for WRN exonuclease activity, and 3'-5' nuclease activity was very similar for Zn²⁺ and Mn²⁺ at 0.1 mM of the metal concentration (Fig. 3C). Interestingly, as the Zn²⁺ ion concentration is increased over 0.5 mM, mWRNexo nuclease activity is significantly decreased.

Because mWRNexo did not exhibit any efficient exonuclease activity in the presence of Mg²⁺ ion in our assay, we then measured the nuclease activity in the presence of a Zn²⁺/Mg²⁺ or Mn²⁺/Mg²⁺ mixtures to see if either Zn²⁺ or Mn²⁺ can be used as a preferred metal ion over Mg²⁺. When 0.1 mM of Zn²⁺ or Mn²⁺ ion was added to the reaction mixture in the presence of up to 5-fold excess amount of Mg²⁺ ion (0.5 mM), the mWRNexo removes the terminal nucleotide almost as efficiently as mWRNexo in the presence of Zn²⁺ or Mn²⁺ ion alone (Fig. 3D). Thus, although it is unclear which metal plays an important role in vivo, small amounts of Zn²⁺ ion clearly provide strong nuclease activity. Fig. 3 also shows that the mWRNexoL cleaves the 3'-end of substrate DNA more efficiently than the monomeric form.

Overall Structure of mWRNexo—Both apoform and Zn-bound mWRNexo structures were virtually identical with an r.m.s.d. value of 0.21 Å for 198 aligned Ca atoms. We, therefore, focused on the structural description on the Zn-bound mWRNexo structure.

mWRNexo forms an α/β-fold that consists of a central β-sheet (strands S1–S6) sandwiched between helices H1 and H3 and helices H4 and H7 (Fig. 4A). The central β-sheet is highly twisted and arranged in the spatial order S6-S5-S2-S3-S4-S1. As shown in Fig. 4A, the right side of the β-sheet is tightly packed against helix H7, and its left side is packed against helices H1 to H3.

In the center of the protein, a large and deep cleft is formed at the active site (Fig. 4B). The right wall of this cleft is formed by the H5 helix and the H5–H6 loop, and the left wall is formed by the H3 helix. The base of the wall is formed by strand S2 and by loops S5-H3 and H4–H5. The two Zn²⁺ ions are coordinated with several conserved acidic residues, Asp¹⁷⁶ and Glu¹³⁸ (the S2 strand), Asp¹³⁷ (the H3 helix), and Asp²¹⁰ (the H7 helix), to form the catalytic core in this cleft (Fig. 4A).

A data base search using DALI (36) revealed that mWRNexo structurally closely resembles the DnaQ superfamily and RNase D (37). The key active site residues are conserved, and both the active site architecture and the core secondary structural elements are similar in these structures. Of the DnaQ family members, KFexo (which shares 17% sequence identity with mWRNexo (1KFS (20)) exhibits highest structural similarity to mWRNexo. The structures of mWRNexo and the equivalent residues of KFexo can be superimposed with an r.m.s. deviation of 2.5 Å for 166 Ca atoms. Despite the high degree of overall structural conservation between mWRNexo and KFexo, significant structural differences were observed in regions including the S2–S3, H4–H5, and H6–H7 loops, and the H6 helix. These regions are involved in the formation of the cleft and thus structural differences in these regions may influence substrate binding of mWRNexo (see “WRNexo Substrate Binding Site”). Compared with the structure of hWRNexo (17), differences are also apparent in the S2–S3 and H5–H6 loops and the H6 helix of mWRNexo (see “Structural Comparison with the Human WRNexo Domain”).

Active Site Geometry—The anomalous difference map showed two strong peaks, one with a height of over 12σ and the other of 8σ; these peaks were assigned to Mₐ and Mₐ, respectively. The two Zn²⁺ ions, which are separated by 3.9 Å, were refined with...
full occupancy with B-values of 26.1 Å² and 40.0 Å² at MA and MB sites, respectively, against the 2.2-Å data. The Zn²⁺ at the MA site had a distorted trigonal bipyramidal geometry, in which the carboxyl groups of Asp⁷⁶ (2.1 Å), Glu⁷⁸ (2.0 Å), and Asp²¹⁰ (2.1 Å) were in equatorial positions and the two water molecules, w1 and w2, were in axial positions (Fig. 5A). A side chain of Asp⁷⁶ and a water molecule bridged the two Zn²⁺ ions.

The Zn²⁺ at MB formed an approximate octahedral geometry with one empty coordinate. This Zn²⁺ ion was directly liganded to the carboxyl group of Asp⁷⁶ (2.1 Å) and four water molecules.
molecules and formed a water-mediated interaction with Asp^{137} (Fig. 5A). In DnaQ exonuclease, the primary role of metal in the M_A site is the nucleophilic attack of the phosphodiester bonds of DNA, whereas metal in MB site is important for stabilizing protein-DNA interactions (20). Whereas the geometries of the acidic residues bound to Zn^{2+}/H11001 or Mg^{2+}/H11001 ions were similar to that of ligands to Mn^{2+}/H11001 or Eu^{2+}/H11001 ions, the positions of the ligand water molecules differed, which resulted in metal geometry differences (Fig. 5, A–C). Also, the two Zn^{2+} ions in mWRNexo were slightly farther apart than the Mg^{2+}, Mn^{2+} ions (3.7 Å), or the two Eu^{2+} ions (3.4 Å) that inhibit nuclease activity.

In an active site, a sulfate ion is located 5.4 and 5.7 Å away from the Zn^{2+}/H11001 A and Zn^{2+}/H11001 B ions, respectively. This sulfate ion makes contact with the amide groups of Leu171 and Asn172 and is 4.3 Å away from the side chain (N/H9280) of Lys185. In the apo-mWRNexo, a sulfate ion is located at a similar position compared with that in Zn-bound mWRNexo.

mTyr206 is crucial for directing the water-mediated attack of phosphodiester bonds by exonuclease family members, and it is generally believed that its role is conserved in all family members (19, 20). In mWRNexo, mTyr206 makes a water-mediated interaction with the Zn^{2+}/H11001 A ion, a sulfate ion, and the carboxyl groups of Glu78. The Tyr206 ring is packed between the side chain of Asp210 and the main chains of Ile189 and Arg190 and is surrounded by the side chains of Trp194 and Glu78 and the main chain of Leu184.
Activation Mechanism of Exonuclease Domain of Werner Protein

FIGURE 5. Comparison of active site geometry. Two metal ions are coordinated by carboxylated groups of the conserved residues and water molecule in an active site. A, two Zn$^{2+}$ ions in mWRNexo; B, two Mn$^{2+}$ ions in hWRNexo (Ref. 17, PDB ID: 2FBY); C, two Eu$^{3+}$ ions in hWRNexo (Ref. 17, 2FBY). While the liganded acidic residues are conserved, the positions of water molecules are different in each protein, presumably because of the position of conserved mTyr$^{206}$/hTyr$^{212}$ residues and the properties of metal ions. Oxygen and nitrogen atoms are shown in red and blue, respectively. The dotted lines indicate interactions between the metal ion(s) and the acidic residues and H-bonds.

Boxyl group of hAsp$^{216}$ (mAsp$^{210}$), is rotated to the surface in mWRNexo and forms an H-bond with a carbonyl group of mLys$^{185}$, thus stabilizing the H5–H6 loop in mWRNexo. The guanidinium group of mArg$^{190}$ was also found to be in a position to interact with the 3’-end nucleotide of a modeled substrate (see below).

To validate the functional relevance of the conformational differences between mWRNexo and hWRNexo described above, we mutated mLys$^{185}$, mArg$^{190}$, and mTyr$^{206}$ to Ala or Phe. Fig. 6B shows that 3’-5’ nuclease activity was completely abolished in mK185A and mY206F, and significantly reduced in mR190A, suggesting that these residues are important for the nuclease activity of WRNexo. Circular dichroism analysis of these mutant proteins revealed that the mutations did not perturb the overall mWRNexo structure (Fig. 6C). We also mutated the two neighboring residues of mLys$^{185}$, mAsn$^{172}$, and mLys$^{176}$ (helix H5), to serine (for solubility reasons) to further analyze the importance of this region in nuclease activity. No conformational differences were observed for these two residues in hWRNexo and mWRNexo. Unlike those residues above which exhibited significant conformational differences between hWRNexo and mWRNexo, mutations of these two residues did not affect the nuclease activity of mWRNexo.

Second, in mWRNexo the S2–S3 loop is highly flexible and part of the region (residues 82–84) are disordered (Fig. 7A). Because of the flexible conformation of this loop, the active site cleft is more open than that of hWRNexo, in which the equivalent loop (residues 87–94) is well ordered. The findings that: (i) in KFexo the equivalent loop partly blocks the cleft and interact with the 3’-leaving group of a substrate (Fig. 7B), and that (ii) several residues in this loop are highly conserved suggest that this loop might be involved in substrate binding or catalysis. However, recent studies revealed that replacing the hLeu$^{88}$ (mLeu$^{82}$) within the S2–S3 loop of hWRNexo to alanine did not alter its nuclease activity (17). To resolve the importance of this loop in substrate recognition, we deleted residues 83–87 of mWRNexo. The deletion mutant exhibited similar nuclease activity compared with the wild-type mWRNexo, and together with the previous data (17), our mutational analysis suggests that this region is not a critical factor for the cleavage of a substrate (Fig. 7C). Nevertheless, we do not exclude the possibility that other residues within the S2–S3 loop might be involved in the substrate recognition.

Third, because of local movement of the S2–S3 loop, the side chain of mArg$^{86}$ is directed toward and occupies the position of the hArg$^{49}$ (mArg$^{43}$) side chain, which is redirected to hTrp$^{145}$ (mTrp$^{139}$). Compared with mTrp$^{139}$, the indole ring of hTrp$^{145}$ is rotated by ~100° (Fig. 7A). In previous study of hWRNexo, mutation of hTrp$^{145}$ to alanine was found to retain nuclease activity, but the processivity was altered (17), which suggests that this residue may be involved in substrate recognition.

WRNexo Substrate Binding Site—To understand how mWRNexo recognizes its substrate, we superimposed the structure of trinucleotide-bound KFexo onto the structure of a Zn-bound mWRNexo using all Cα atoms, two Zn$^{2+}$, and a sulfate ion. Based on this model, we propose several residues that participate in substrate binding.
that forms the right part of the cleft has half of the length of the equivalent region in KFexo. These structural differences open the cleft more near the 3'-leaving groups in mWRNexo.

The two proteins also have obvious structural differences near the H4–H5 loop in which the 5'-end of the trinucleotide binds. Although WRNexo does not interact with single strand DNA, it is known to bind a long fork duplex DNA (24), and a part of the single strand in a fork DNA may bind to this region. Structural differences between the two proteins in this region suggest two possible regions of WRNexo that may interact with the 5' part of the DNA (Fig. 7B). First, the H4–H5 loop is directed toward the H5 helix in KFexo, and substrate would bind between the H4–H5 loop and the H3 helix, like trinucleotide binding in KFexo. The S6 strand and the H4 helix could be also involved in substrate binding in this region. Second, in mWRNexo, the H4–H5 loop is directed more toward the H3 helix than the equivalent loop in KFexo, and this could create additional space between the H4–H5 loop and the H5 helix in which DNA could pass through. The H5–H6 loop could also contribute to substrate binding in this region.

To identify the region where the 5' portion of substrate binds, we created a set of mutants in the region of the H4 helix, the H4–H5 loop, and the H5 helix. We introduced a bulky group to the middle of the H4 helix or in the H4–H5 loop by replacing Thr156 or Thr168 with tyrosine (Figs. 1 and 7B). If a substrate DNA binds to this region, then the addition of bulky groups in this region would perturb the interaction between substrate and mWRNexo. Both of these mutations perturbed the nuclease activity of mWRNexo (Fig. 7C). We then mutated Gly173 to arginine to determine whether the H5 helix participates in substrate binding. Replacing this residue with Trp or Tyr produced an insoluble form of mWRNexo. The G173R mutant exhibits the similar nuclease activity compared with the mutant mWRNexo.

Overall, the residues that interact with substrate DNA are not conserved in mWRNexo and KFexo (Fig. 1). Furthermore, conformation of the loops near the 3'- and 5'-ends of the modeled trinucleotides are markedly different in the two proteins, which suggests that their substrate binding modes are somewhat different.

The S2–S3 loop of mWRNexo, which is near the 3'-leaving nucleotide, is highly flexible, whereas the equivalent loop of KFexo is packed tightly against the helix equivalent to helix H3 in mWRNexo (Fig. 7B). Also, the H6–H7 loop in mWRNexo
small q-range using the Guinier plot, which can provide information about protein size. The $R_g$ values of mWRNexo and mWRNexoL in solution were 2.44 ± 0.03 nm and 4.72 ± 0.05 nm, respectively, indicating that mWRNexoL exists as an oligomeric protein composed of more than at least two mWRNexoL molecules in solution. As a result, this observed $R_g$ increase could influence the overall molecular shape of mWRNexo in solution. In addition, similar results were obtained using the $p(r)$ function in the GNOM program (28). As shown in Fig. 2B, the $p(r)$ function of mWRNexo exhibits a symmetrical form with a short tail, which has a maximum diameter $D_{max}$ of 10 nm, indicating that the shape of mWRNexo approaches a spherical form. On the other hand, the $p(r)$ function of mWRNexoL appears to completely depart from that of mWRNexo. The symmetry of the $p(r)$ function is completely destroyed, and the tail part becomes extremely long ($D_{max} = 19$ nm).

To obtain more detailed information on the structure and structural differences between mWRNexo and mWRNexoL in solution, we reconstructed model-independent low-resolution structural models of mWRNexo and mWRNexoL obtained using the ab initio shape determination program DAMMIN (29). These were constructed inside the search volume of maximum diameter $D_{max}$ calculated using the $p(r)$ function in GNOM (28). For mWRNexo and mWRNexoL in this study, SAXS profiles computed from the reconstructed models well-matched experimental data but displayed some systematic deviations. To improve the reliability of the final solution, both were reconstructed ten times. The resulting 20 independent models were then regenerated as averaged models using DAMAVER (30). Fig. 2C shows the averaged low-resolution structural models of mWRNexo and mWRNexoL, respectively. The structural model for mWRNexo was reconstructed without imposing any symmetry restriction, whereas the structural model for mWRNexoL was reconstructed by imposing a P3 symmetry restriction, by assuming that mWRNexoL exists in a homotrimeric form that is primarily because of a tail-to-tail interaction in solution. However, we cannot completely exclude the possibility that a fraction of mWRNexoL exists in a homodimeric form in solution.

**DISCUSSION**

In the present study, we describe structural differences between hWRNexo and mWRNexo, and propose an activation mechanism for WRN exonuclease based on these structural differences. Residues in the active of hWRNexo and mWRNexo are highly conserved (Fig. 1), and the active site residues in hWRNexo complexed with Mg$^{2+}$, Mn$^{2+}$, and Eu$^{2+}$ ions had similar structures (17). Thus, it is unlikely that the structural differences between hWRNexo and mWRNexo around the
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active site are caused by the differences in protein sequence or metal ion in mWRNexo. Moreover, the fact that (i) the apo mWRNexo structure is identical to that of Zn-bound mWRNexo and (ii) our mutational analysis on conserved active site residues in mWRNexo further supports our idea described above. Instead, we presumed that the sulfate ion at the active site might induce these conformational changes, and hypothesized that the role of sulfate is similar to that of the phosphate group of substrate DNA. The most dramatic conformational differences between the two were observed around residues Arg\(^{190}\), Lys\(^{185}\), and Tyr\(^{206}\), which are conserved in WRN family members (Figs. 1 and 6A). Our structural and biochemical data suggest that in mWRNexo, the role of Tyr\(^{206}\) is to orient and stabilize the nucleophile, whereas the role of Lys\(^{185}\) is to recognize the phosphate group of substrate DNA. Arg\(^{190}\) appears to have multiple roles because: (i) the movement of the side chain of this residue is critical for providing space for the rotation of the Tyr\(^{206}\) ring, (ii) Arg\(^{190}\) could interact with the substrate, and (iii) its guanidinium group stabilizes the H6–H7 loop of mWRNexo. While mutation of Lys\(^{185}\), Arg\(^{190}\), or Tyr\(^{206}\) abolished nuclease activity, the mutation of neighboring residues Asn\(^{172}\) or Lys\(^{176}\) did not alter this activity, demonstrating the importance of these conserved residues in nuclease activity and supporting our structural observations.

What might be the functional relevance of the conformational differences in hWRNexo and mWRNexo to the mechanism of WRNexo? Despite their important role in the WRNexo family, Arg\(^{190}\) and Lys\(^{185}\) are not conserved in KFexo or other exonuclease members, and conformational changes in the H5–H6 loop and the H6 helix in WRNexo have not been observed in the equivalent regions in other DNAQ family members. Thus, the activation mechanism of WRNexo might be unique in DNAQ family members. Ku70/80, a component of DNA protein kinase, stimulates WRNexo activity, but inhibits KFexo activity (17). Ku70/80 is known to functionally interact with the N-terminal domain (residues 1–50) of WRN, which is near the H5–H6 loop and the H6 helix of mWRNexo (22). Thus, it is possible that Ku binding to WRNexo might stimulate conformational change of the H5–H6 loop and the H6 helix as displayed in our structure to activate WRN exonuclease activity.

WRNexo binds to long fork duplex and the 5′-overhang duplex, but does not stably interact with single strand DNA or blunt-ended duplex (24). WRNexo also cleaves 5′-overhang duplex DNA with a great preference over blunt-ended, double-stranded, or single-stranded DNA (16). Our modeling and mutational analysis clearly defines the binding site for the 3′-end of substrate DNA. The 5′-overhang region of the other strand of a substrate may be accommodated to a space created by the highly flexible loop, S2–S3.

Then how does WRNexo recognize the 5′-end of a substrate? The single-stranded region of the long fork duplex could pass the two regions, between the H4 helix and the H4–H5 loop or between the H4–H5 loop and the H5 helix in mWRNexo. Our mutational analysis revealed that the region spanning the helix H4 and the H4–H5 loop are involved in substrate binding (Fig. 7, B and C). However, none of our mutants showed dramatically reduced nuclease activity, suggesting that interactions between WRNexo and substrate DNA may not be limited to this specific region. Structural studies of WRNexo/DNA complexes should provide more detailed answers.

We also determined the low resolution trimeric structure of mWRNexoL. Although we do not know the importance of this trimerization, findings that the oligomeric form of mWRNexo showed enhanced nuclease activity and altered processivity suggest that the oligomerization of mWRNexo might be required for the processing of various types of DNA substrates. Our SAXS analysis revealed that despite the predominance of the trimeric form of mWRNexoL, a small fraction of the dimeric mWRNexoL form might be present.

In summary, our structural and biochemical study provides important insights into the understanding of the activation mechanism, substrate recognition, and oligomerization of WRNexo. Structural information on WRNexo/nucleotides complexes and on WRNexo/Ku70/80 should further elucidate the activation mechanism of WRNexo.

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