Role of Walker Motif A of RuvB Protein in Promoting Branch Migration of Holliday Junctions

WALKER MOTIF A MUTATIONS AFFECT ATP BINDING, ATP HYDROLYZING, AND DNA BINDING ACTIVITIES OF RuvB*

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Escherichia coli RuvB protein, an ATP-dependent hexameric DNA helicase, acts together with RuvA protein to promote branch migration of Holliday junctions during homologous recombination and recombinational repair. To elucidate the role of the Walker motif A of RuvB (GXGKT; X indicates a nonconserved residue) in ATP hydrolysis and branch migration activities, we constructed four ruvB mutant genes by site-directed mutagenesis, altering the highly conserved Lys68 and Thr69 residues. K68R, K68A, and T69A mutants except T69S failed to complement UV-sensitive phenotype of the ruvB strain. These three mutant proteins, when overexpressed, made the wild-type strain UV-sensitive to varying degrees. K68R, K68A, and T69A were defective in ATP hydrolysis and branch migration activities in vitro. In the presence of Mg2+, K68R showed markedly reduced affinity for ATP, while K68A and T69A showed only mild reduction. K68A and T69A could form hexamers in the presence of Mg2+ and ATP, while K68R failed to form hexamers and existed instead as a higher oligomer, probably a dodecamer. In contrast to wild-type RuvB, K68R, K68A, and T69A by themselves were defective in DNA binding. However, RuvA could facilitate binding of K68A and T69A to DNA, whereas it could not promote binding of K68R to DNA. All of the three mutant RuvBs could physically interact with RuvA. These results indicate the direct involvement in ATP binding and ATP hydrolysis of the invariant Lys68 and Thr69 residues of Walker motif A of RuvB and suggest that these residues play key roles in interrelating these activities with the conformational change of RuvB, which is required for the branch migration activity.

Homologous DNA recombination involves multistep reactions that require many gene products. Much of our knowledge of the molecular mechanisms involved in recombination has been derived from studies of Escherichia coli (1, 2). The recombination intermediates called Holliday structures, in which two homologous duplex DNA molecules are held together by a single-stranded crossover (3), are formed by the functions of RecA and several accessory proteins. The Holliday intermediates are processed in a concerted and interactive manner by RuvA, RuvB, and RuvC proteins to give mature products (4–6).

RuvA, a Holliday junction-specific binding protein, is a tetramer in solution, forms a stable complex with RuvB, and facilitates the binding of RuvB to the junction DNA (7, 8). The crystal structure of E. coli RuvA has been determined at the atomic level and it reveals that the four subunits are arranged in a planar flower petal-like structure in the crystal (9, 10). More recently, crystal structures of the complexes of E. coli RuvA and a synthetic Holliday junction (11) and Mycobacterium leprae RuvA and a Holliday junction (12) have been reported. In the former structure, the four-way junction DNA was bound by a RuvA tetramer on one face, while in the latter, the junction DNA was sandwiched between two tetramers. It has not been determined which form of the RuvA-junction DNA complex represents the active form in vivo. The RuvA-RuvB complex catalyzes branch migration of Holliday junctions using energy derived from ATP hydrolysis (13, 14). RuvB is a helicase that catalyzes unwinding of DNA in a 5′ to 3′ direction with respect to single-stranded DNA (ssDNA) in an ATP hydrolysis-dependent manner (15). RuvB forms a hexameric ring structure in the presence of Mg2+ and ATP and binds DNA through the central holes of the ring (16, 17). However, under the conditions of low Mg2+ and/or low RuvB concentration, RuvB forms a stable dimer (18), suggesting that the dimer is the basic unit of RuvB in forming the hexamer. Thus, RuvB changes its tertiary and quaternary structures by allosteric interactions with various effectors such as Mg2+, ATP, RuvA, and DNA.

DNA helicases catalyze the unwinding of double-stranded DNA (dsDNA) to produce ssDNA using energy derived from nucleotide 5′-triphosphate hydrolysis (19). These enzymes play essential roles in a variety of processes in DNA metabolism, such as replication, recombination, repair, and transcription (20). A large number of helicases have been identified, and comparison of their amino acid sequences has revealed the presence of seven conserved sequence motifs in the majority of them (21), suggesting that many helicases share structural similarities.

Motifs I and II of the helicases correspond to the Walker A and B motifs (22), respectively, found in numerous NTP-binding proteins. The crystal structures of adenylate kinase (23), Ras (24), RecA (25), and F1-ATPase (26), for example, show that motif I binds the diphosphate or triphosphate moiety of nucleotides and that motif II is involved in binding nucleotides via Mg2+. Like all helicases so far characterized, the RuvB

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1 The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATP:S, adenosine-5′-O-(3-thiotriphosphate); DTT, dithiothreitol; BSA, bovine serum albumin; PCR, polymerase chain reaction; kb, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis.
family of helicases shares the consensus amino acid sequences for Walker motif A (GXXGTG) and B (DEXH) (where X indicates a nonconserved amino acid residue), while the other five common motifs are not clearly identifiable in RuvB. However, from a study of structure-based sequence alignments, the protein architectures of RuvB family helicases are suggested to be closely related to those of the clamp-loader proteins, such as the γ subunit of E. coli DNA polymerase III and human RF-C proteins, all of which are involved in the loading of clamp proteins to achieve high processivities of replicative DNA polymerases (27).

This suggests the possibility that RuvB, although it is an intrinsic DNA helicase, has a potential of protein folding and mechanism of DNA unwinding distinct from those of other typical DEXX DNA/RNA helicase family proteins. Therefore, we use Walker motifs A and B, rather than helicase motifs I and II, throughout this report to designate for the RuvB motifs.

In this study, we constructed four ruvB mutant genes with substitutions in the highly conserved amino acids Lys68 and Thr69 in Walker motif A by site-directed mutagenesis and demonstrated that these point mutations affected not only the RuvB activities of ATP hydrolysis and ATP binding, but also those of DNA binding, hexamer formation, and promotion of branch migration. The results suggest that amino acid residues directly involved in binding and hydrolysis of ATP play additional roles in interrelating other functions of RuvB.

**Experimental Procedures**

**Bacterial Strains and Plasmids—**E. coli HRS2500 (BL21(DE3)ΔruvABC100:cat) strain was constructed by 1 vitro transduction of ΔruvABC100:cat from HRS2303 (28) into BL21(DE3), a host strain for the T7-based overexpression plasmid (29), as described by Miller (30). HRS2301 (ΔruvB100:cat) was described previously (28). High copy number plasmid, pUC19 (Takara Shuzo), the expression vector using a T7 promoter, pET3a (29), and its derivative pAF101 (31), and the low copy number vector, pCH19 (28), were described previously.

**Media and Growth Conditions—**Bacteria were routinely grown at 37 °C in Luria broth medium (32). When needed, ampicillin and chloramphenicol were added to Luria broth at final concentrations of 100 and 37 °C in Luria broth medium (32). When needed, ampicillin and chloramphenicol were added to Luria broth at final concentrations of 100 and 37 °C in Luria broth medium (32). When needed, ampicillin and chloramphenicol were added to Luria broth at final concentrations of 100 and 37 °C in Luria broth medium (32). When needed, ampicillin and chloramphenicol were added to Luria broth at final concentrations of 100 and 37 °C in Luria broth medium (32).

**Site-directed Mutagenesis—**To remove the BglII cleavage site from pAF102, the plasmid was blunt-ended with mung bean nuclease. The 1.3-kb BglII fragment of pAF102 was digested with BglII and HindIII and cloned into the HindIII site downstream of the TAA termination codon of pUC19. The 2.7-kb HindIII fragment from pAF101, the plasmid was blunt-ended using mung bean nuclease after digestion with HindIII, was replaced by the BglII fragment of pAF102. A synthetic 27-mer (5'-CATCGTCCGACGCTCGATGTTGGCTTTGAGGTAACATATG-3') was used for PCR to generate a new Ndel site at the ATG initiation codon and a BamHI site downstream of the TAA termination codon of ruvB in pH5102 (33), respectively. The PCR products containing the ruvB gene were digested with Ndel and BamHI and cloned into the Ndel-BamHI site of pAF102. The KpnI-BglII region of ruvB, which was produced by PCR, was replaced by the KpnI-BglII region of ruvB in pH5102 to produce pTY311 (Fig. 1). The 1.3-kb XbaI-BamHI fragment from pTY311, containing the entire ruvB gene plus the flanking 5′ sequence, was subcloned into M13mp18 to yield M13mp18RVRP. Site-directed mutagenesis by PCR using the four appropriate synthetic 24-mer oligonucleotides and M13mp18RVP was carried out to alter codon 98 of ruvB from AAA (Lys) to AGA (Arg) or GCA (Ala) and to alter codon 69 from ACT (Thr) to TCT (Ser) or GCT (Ala). pTY317, pTY318, pTY319, and pTY320 were subsequently constructed by replacing the 0.8-kb KpnI-ClaI fragment of pTY311 with the 0.8-kb KpnI-ClaI fragment from M13mp18RV(R68R), M13mp18RV(R68A), M13mp18RV(T68R), and M13mp18RV(T68A). To construct low copy number plasmids containing the wild-type ruvB and four ruvB mutant genes, the 1.3-kb XbaI-BamHI fragments of pTY311, pTY317, pTY318, pTY319, and pTY320 were cloned into the XbaI-BamHI site of pSC19. The DNA sequences of the mutant ruvB genes were confirmed by sequencing the appropriate DNA regions (Applied Biosystems 373S DNA Sequencer).

**UV Sensitivity Test—**Sensitivity to UV irradiation of exponentially growing cells carrying the indicated plasmids was measured as described previously (28).

**Purification of Proteins—**RuvA proteins were purified as described previously (10). The wild-type and mutant RuvB proteins were over-produced in E. coli HRS2500 using the T7 expression system (29). E. coli HRS2500 strains carrying the plasmids containing wild-type or one of the mutant ruvB genes were grown at 37 °C to an A600 of about 0.4 in 1.5 liter of Luria broth medium containing ampicillin. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the cultures were incubated for 4 h. The RuvB proteins were purified by the procedures described previously until the step of DEAE-Sepharose chromatography (34). The fractions containing the peak fractions were pooled and dialyzed against R-buffer.

**ATPase Assay—**Reaction mixtures (50 μl) containing 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM DTT, the indicated concentration of ATP, 3 μCi of [γ-32P]ATP, 0.01% (w/v) BSA, 0.5 μg of form I pUC19 DNA, and 0.6 μg RuvA protein were preincubated at 37 °C for 5 min, and the reactions were started by addition of Ruvi (1 μM). Aliquots (5 μl) were sampled at the indicated times and immediately mixed with 5 μl of stop buffer (25 mM EDTA, 10 mM ATP). Samples (1 μl) were applied to polyethyleneimine-cellulose plates (Merck) and developed in a solution containing 1 M formic acid and 0.4 M LiCl. The amounts of [32P] and [γ-32P]ATP in each spot were determined by using a phosphorimager (Fuji BAS1500).

**Branch Migration Assay—**The ATP-dependent branch migration activity of the RuvA-RuvB complex was assayed by dissociation of synthetic Holliday junctions made by annealing four 72-mer deoxyoligonucleotides JY11, JY12, JY13, and JY14. The sequences of the four deoxyoligonucleotides were: CGAGGCGAGAACCCATCGAGAGAGCTT-CAATCGGCTCAAGAGCCATTTAGATGTTGGGGATCCGACTTACGGCATT-9 and TCGATGTCTCTGGGACGGTGTTACAGGTCGTTCTGGGAACGGACTTG-9 (JY11), and TCGATGTCTCTGGGACGGTGTTACAGGTCGTTCTGGGAACGGACTTG-9 (JY14). The standard reaction mixtures (20 μl) containing 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM DTT, 2 mM ATP, 0.01% (w/v) BSA, 70 mM KCl, 0.6 μg RuvA, and 1 μg pTY311 DNA were incubated at 37 °C for 30 min, and the reactions were stopped by the addition of 4 μl of stop buffer (50 mM EDTA, 5 mM/mg proteinase K, 2% SDS). The products were analyzed by 6% polyacrylamide gel electrophoresis and visualized by autoradiography.

**ATP Filter Binding Assay—**The amount of ATP bound to RuvB was measured essentially as described previously (34). The standard reactions were performed in 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 50 mM NaCl, and various concentrations of ATP and [γ-32P]ATP as noted, in the presence or absence of Mg2+ (10 mM). Reaction mixtures containing 2 μM RuvB were incubated on ice for 15 min and then passed through nitrocellulose filters (0.45 μm, Whatman). The filters were washed with the assay buffer and then dried, and the radioactivity of [γ-32P]ATP bound to RuvB on the filters was measured in a liquid scintillation counter (Beckman). The data obtained were fitted to the equation: [ES] = [E][S]Kᵣ/Kᵣ + [S], where [ES] is the concentration of RuvB-ATP complex, [E], is the concentration of RuvB, [S] is the concentration of ATP, and Kᵣ is the dissociation constant.

**Gel Retardation Assay—**DNA binding activity of RuvB was assayed in a solution (20 μl) containing 20 mM triethanolamine HCl, pH 7.5, 15 mM NaCl, 1 mM ATP, 0.1 mM BSA, and 0.5 μg of form I pUC19 DNA. Al the reactions were incubated for 20 min at 37 °C, glutaraldehyde was added to a final concentration of 0.25%. The solutions were further incubated for 30 min, and the reactions were stopped by addition of 10 μl of 1% SDS, and the samples were loaded on the gel and run for 2 h. The gel was stained with ethidium bromide and visualized by autoradiography.

**Gel Mobility Shift Assay—**DNA binding activity of RuvB was assayed in a solution (20 μl) containing 20 mM triethanolamine HCl, pH 7.5, 15 mM NaCl, 1 mM ATP, 0.1 mM BSA, and 0.5 μg of form I pUC19 DNA. The reactions were incubated for 20 min at 37 °C, glutaraldehyde was added to a final concentration of 0.25%. The solutions were further incubated for 30 min, and the reactions were stopped by addition of 10 μl of 1% SDS, and the samples were loaded on the gel and run for 2 h. The gel was stained with ethidium bromide and visualized by autoradiography.
RESULTS

Construction of the ruvB Mutant Genes with Substitutions of the Walker Motif A—Walker motif A (GXXGXXS) is highly conserved in many ATPases, including DNA and RNA helicases (21), and it is conserved in all bacterial RuvB homologs so far identified (28). To address the role of the Walker motif A of RuvB in the promotion of branch migration of Holliday junctions, we constructed four mutant ruvB genes (K68R, K68A, T69S, and T69A) with alterations at the Lys<sup>68</sup> or Thr<sup>69</sup> residues in the Walker motif A of RuvB by site-directed mutagenesis. The conserved residues in Walker motif A of RuvB are indicated by bold letters. The structure of the expression system for RuvB using the T7 phage promoter is shown.

Amino acid substitutions in the Walker motif A of RuvB. Highly conserved Lys<sup>68</sup> and Thr<sup>69</sup> residues were changed to Arg or Ala, and to Ser or Ala, respectively, by site-directed mutagenesis, as indicated by arrows. The conserved residues in Walker motif A of RuvB are indicated by <strong>bold letters</strong>. The structure of the expression system for RuvB using the T7 phage promoter is shown.

The amounts of mutant genes when overexpressed from the multicopy plasmids made the wild-type strain highly UV-sensitive (Fig. 2B). The mutant <i>ruvB</i> genes K68A and T69A in a low copy number plasmid (C) suspended in a multicopy vector, pAF102, B, UV sensitivities of wild-type strain, AB1157, harboring the mutant <i>ruvB</i> genes on a multicopy plasmid, pAF102. C, UV sensitivities of wild-type strain harboring the mutant <i>ruvB</i> genes on a low copy number plasmid, pSCH19. The symbols in A are: □, HRS2301/vector; ○, HRS2301/ruvB<sup>†</sup>; ●, HRS2301/K68R; ■, HRS2301/K68A; ▲, HRS2301/T69A; ▼, HRS2301/T69S; △, AB1157/vector and in B and C are: □, AB1157/vector; ○, AB1157/ruvB<sup>†</sup>; ●, AB1157/K68R; ■, AB1157/K68A; ▲, AB1157/T69A; ▼, AB1157/T69S.

The amounts of RuvB proteins expressed from the plasmids were estimated by Western blot analysis using a polyclonal anti-RuvB antibody (data not shown). In both the wild-type and <i>ΔruvB</i> strains, all mutant and wild-type RuvB proteins were synthesized at almost the same levels. The amounts of mutant and wild-type RuvB proteins expressed from the multicopy and low copy number plasmids were about 50- and 5-fold higher than the levels expressed from the chromosomal <i>ruvB</i> gene, respectively. RuvB protein is induced about 5-fold from the chromosomal gene by UV. These results suggest that the failure to complement the UV repair deficiency of the <i>ΔruvB</i> strain by K68R, K68A, and T69A alleles was not due to the lack of...
expression or instability of the gene products and that the dominant negative phenotype of the mutant genes for UV repair in vivo was due to the inhibition of the wild-type RuvB function by these mutant proteins coexpressed in the same cells.

The Lack of ATPase and Branch Migration Activities of the RuvB Mutant Proteins—To examine the biochemical properties of the mutant RuvB proteins, the wild-type and mutant ruvB genes were overexpressed in the HRS2500 (ΔruvABC::cat) strain using the T7 expression system. The proteins were purified to homogeneity as judged by SDS-PAGE (Fig. 3). To assess the contribution of the Walker motif A to the ATPase activity, we performed time course analysis of ATP hydrolysis using the purified mutant proteins. Reactions were carried out in the absence of RuvA and DNA. K68R, K68A, and T69A had no detectable ATPase activity (Fig. 4A). Since the ATPase activity of RuvB is synergistically stimulated by RuvA and DNA, we examined the effects of these cofactors on the ATPase activities of the mutant proteins. The ATPase activity of wild-type RuvB was stimulated by RuvA and DNA individually and synergistically (Fig. 4, B–D), as reported previously (16, 36). However, these cofactors did not enhance the ATPase activities of the mutant proteins (Fig. 4, B–D). These results suggest that the mutations altering the conserved Lys\(^{68}\) and Thr\(^{20}\) in Walker motif A inactivated the intrinsic ATPase activity of RuvB.

Dissociation of synthetic four-way junctions by the RuvAB complex has been used as a model system to study branch migration activity (14, 37). During a 30-min incubation in the presence of RuvA and ATP, wild-type RuvB dissociated 60% of the synthetic Holliday junctions, while K68R, K68A, and T69A showed no detectable branch migration activity (Fig. 5). These results show that the conserved residues in the Walker motif A are critically important for the branch migration activity, which reflects the importance of ATP hydrolysis for this activity.

ATP Binding Activity of RuvB Mutant Proteins—We next examined the ATP binding properties of the mutant RuvB proteins in the presence and absence of Mg\(^{2+}\) using a filter Binding assay (Fig. 6). Samples containing RuvB and ATP were incubated on ice to prevent ATP hydrolysis by wild-type RuvB and then passed through nitrocellulose filters. As shown in Fig. 6, the mutant RuvBs showed different degrees of defects in ATP binding activity. The double-reciprocal plots of the data obtained in the presence of Mg\(^{2+}\) showed that Mg\(^{2+}\) reduced the \(K_s\) value of wild-type RuvB about 2.5-fold, indicating that Mg\(^{2+}\) enhances the affinity of the RuvB for ATP. \(K_s\) values of K68R, K68A, and T69A were 66-, 8-, and 12-fold higher than that of wild-type RuvB, respectively (Fig. 6A). The differences in the \(K_s\) values between wild-type RuvB and the mutant proteins were less pronounced in the absence of Mg\(^{2+}\) than in its presence (Fig. 6B). In the absence of Mg\(^{2+}\), the \(K_s\) values of K68R and K68A were 5- and 3-fold higher than that of wild-type RuvB, respectively, and T69A had the same affinity as the wild-type protein. It is intriguing that Mg\(^{2+}\) increased the affinity of wild-type RuvB for ATP, while it decreased the affinities of K68R and T69A and did not significantly change the affinity of K68A.

DNA Binding Activity of RuvB Mutant Proteins—We examined whether mutations in Walker motif A affected the DNA binding activity of RuvB, which should be important for the RuvAB-catalyzed branch migration. The proteins were incubated with form I pUC19 DNA in the presence of 15 mM Mg\(^{2+}\) and 1 mM ATP\(_7\)S, conditions that favor the binding of wild-type RuvB to DNA (8). The resultant protein-DNA complexes were fixed with glutaraldehyde to stabilize the weak interaction between DNA and the proteins, and the reaction products were analyzed by agarose gel electrophoresis to determine the overall DNA binding (7). Gel retardation was observed when DNA was incubated with wild-type RuvB. The degree of mobility shift increased with the increasing RuvB concentration (Fig. 7A). In contrast, we could not detect retardation with K68R, K68A, or T69A under the same conditions (Fig. 7A), indicating that these mutants were defective in the ability to bind to dsDNA.

Because previous studies have shown that RuvA facilitates the loading of RuvB onto DNA (8), we examined whether RuvA could load the mutant RuvB proteins onto DNA. RuvA promoted the binding of K68A and T69A to dsDNA, as did the binding of wild-type RuvB, as shown by the presence of super-shift bands of the RuvAB-dsDNA complex (Fig. 7B). In contrast, RuvA could not load K68R onto dsDNA under the same conditions (Fig. 7B). Therefore, although the three mutant proteins are defective in loading onto DNA by themselves, K68A and T69A, but not K68R, can be loaded onto DNA with the help of RuvA.

Complex Formation between the Mutant RuvB Proteins and the RuvA Protein—To directly examine the ability of the mutant RuvB proteins to form complexes with RuvA, the proteins were mixed, incubated, and applied to a Superdex 200 gel filtration column in the absence of ATP and Mg\(^{2+}\). As shown in Fig. 8A, RuvA was eluted at a position indicating a mass of 105 kDa, corresponding to the tetramer, and wild-type RuvB was eluted at a position indicating a mass of 135 kDa, corresponding to the dimer. The Stokes radius of RuvB calculated from gel filtration was larger than that of a spherical protein with the molecular mass of RuvB (18). The mixture of RuvA and RuvB eluted at a peak position indicating a mass of 250 kDa. This peak contained both RuvA and RuvB, as shown by SDS-PAGE analysis, indicating formation of a RuvAB complex (Fig. 8A) (10, 16). Similarly, the formation of complexes between mutant RuvB proteins and RuvA was analyzed. In the absence of RuvA, K68R, K68A, and T69A were eluted at positions corresponding to 240, 140, and 155 kDa, respectively (data not shown). As shown in Fig. 8B, the mixtures of RuvA and mutant RuvB proteins eluted with molecular masses ranging from 250 to 330 kDa. SDS-PAGE analysis revealed that these peak fractions contained both RuvA and RuvB (data not shown). These results show that all of the mutant RuvB proteins retain the ability to form complexes with RuvA.
Oligomeric Structures of Mutant RuvB Proteins—To examine whether the mutations had any effect on the ability of RuvB to form hexameric rings, we investigated the oligomeric states by gel filtration chromatography. Since RuvB hexamer formation is dependent on high protein concentration and cofactors, RuvB proteins at 35 mM were applied to a Superdex 200 column in the presence of Mg$^{2+}$ and ATP (Fig. 9A). Wild-type RuvB was eluted at a position indicating a molecular mass of 230 kDa, corresponding to the RuvB hexamer, in agreement with a previous study (16). K68A and T69A eluted at positions corresponding to molecular masses of 260 and 230 kDa, respectively, indicating that these mutant proteins also formed hexameric ring structures under these conditions. K68A eluted with a broader peak than wild-type RuvB and T69A, suggesting that it contained higher oligomeric species in addition to hexamers. K68R eluted at a position indicating a molecular mass of 430 kDa, corresponding to the dodecamer. We also analyzed the oligomeric states of these mutant proteins in the presence of Mg$^{2+}$ or EDTA. In the absence of Mg$^{2+}$ and ATP and the presence of EDTA, K68A and T69A were eluted at a position corresponding to the dimer of RuvB, as was wild-type RuvB (18). However, K68R was eluted at a position corresponding to a molecular mass of 380 kDa (data not shown). In the presence of Mg$^{2+}$, wild-type RuvB and all the mutant RuvB proteins were eluted at a position indicating a molecular mass of 430 kDa (Fig. 9B), which corresponds to the size of the RuvB dodecamer, consistent with the findings of a previous study with wild-type RuvB (16). In summary, all the mutant proteins except K68R changed oligomeric states, as did the wild-type protein, in response to the presence of the cofactors ATP and Mg$^{2+}$ in solution. In contrast, K68R was refractory to the effect of the allosteric cofactor ATP.

**DISCUSSION**

In this work, we studied the roles of the conserved residues in the Walker motif A of RuvB in the ATPase and branch migration activities by constructing mutants. One of these, T69S, could fully complement the UV-sensitive phenotype of the ruvB strain, while others, K68R, K68A, and T69A, failed to complement. Overexpression of K68R, K68A, and T69A made the wild-type strain UV sensitive to similar degrees. However, at a lower level of expression, the K68R made the wild-type strain less UV-sensitive than the other two mutants. This may be due to the defective binding of K68R to DNA even in the presence of RuvA, which may make the mutant RuvB-RuvA complex less competitive than the wild type RuvB-RuvA complex for the junction loading.

The three mutant proteins showed markedly reduced ATPase activity in vitro. RuvA and supercoiled DNA stimulated the ATPase activity of wild-type RuvB, but had no stim-
ululating effect on the low, intrinsic ATPase activities of these three mutant proteins. The $K_d$ values of K68R, K68A, and wild-type RuvB for ATPase in the presence of RuvA and DNA were 0.11, 0.16, 0.6, and 30.2 min$^{-1}$, respectively. Thus, the mutations in Walker motif A of RuvB virtually eliminated the fundamental ability to hydrolyze ATP (Fig. 4), as has been shown for many other ATPases (38–40).

We demonstrated that the mutations in Walker motif A affected the affinity of RuvB for ATP (Fig. 6). In the absence of Mg$^{2+}$, K68R and K68A had reduced ability to bind ATP. Mg$^{2+}$ stimulated the ATP binding ability of wild-type RuvB (2.5-fold decrease in $K_d$), while it greatly reduced the ATP binding ability of K68R, compared with that of wild-type RuvB (66-fold increase in $K_d$). Meanwhile, the $K_d$ of K68A was unaffected by the addition of Mg$^{2+}$. These results suggest that Lys 68 in RuvB plays a key role in the interaction with ATP. X-ray crystallographic analysis of the RecA-ADP complex and F$_1$-ATPase indicate that the corresponding lysine residues in the P-loop directly interact with the $\beta$ and $\gamma$ phosphates of ATP (25, 26). The reduced ATP binding of K68A may be due to the loss of positive charge required for the direct interaction with the $\beta$ and $\gamma$ phosphates of ATP resulting from the substitution of lysine by alanine. K68R not only exhibited reduced ATP binding activity, similarly to K68A, in the absence of Mg$^{2+}$, but it also showed a further reduction in ATP binding activity in the presence of Mg$^{2+}$. This was surprising because the positive charge of the side chain was maintained. To account for this result, we speculate that some change in tertiary structure is caused by the substitution of lysine with arginine such that the bulky side chain of arginine sterically hinders the ATP binding, and this hindrance may be further enhanced by a Mg$^{2+}$-coordinated conformational change around the ATP-binding site of RuvB. Indeed, gel filtration analysis revealed that K68R formed higher oligomeric states (hexamers and dodecamers) under all conditions we studied, unlike the wild-type RuvB. T69A had the same ability to bind ATP as wild-type RuvB in the absence of Mg$^{2+}$, consistent with the x-ray crystallographic studies of the RecA-ADP complex and F$_1$-ATPase, which revealed that the threonine residue of Walker motif A (GKT) interacts with a magnesium ion that bridges the $\beta$ and $\gamma$ phosphates of ATP. However, T69A had reduced ability to bind ATP in the presence of Mg$^{2+}$ (5.5-fold increase in $K_d$). Gel filtration analysis revealed that T69A was able to undergo conformational change in response to Mg$^{2+}$ (Fig. 9B), indicating that T69A can bind to Mg$^{2+}$ even without the Thr69 residue. These results suggest that coordination of Mg$^{2+}$ not mediated by Thr$^{69}$ induces a conformational change that affects the topology around the P-loop. Thus, these equilibrium ATP binding experiments strongly suggest the roles for the lysine and threonine residues of the Walker A sequence of RuvB in ATP binding, which is in good agreement with the x-ray crystallographic data of the Walker A regions in other proteins such as adenylate kinase (23), Ras (24), RecA (25) and F$_1$-ATPase (26).

Protein-DNA cross-linking assays revealed that K68R, K68A, and T69A were defective in DNA binding in the presence of ATP$\gamma$S. Similar substitutions in the hexameric helicase T7 grammar protein (39) also caused defective DNA binding in the presence of dTMP-PCP. However, RuvA could facilitate the loading of K68A and T69A onto DNA. As an explanation for the DNA binding defects of K68A and T69A by themselves, these mutant proteins may not bind ATP$\gamma$S or may be defective in the conformational change induced by the ATP$\gamma$S, which is necessary for the DNA binding of wild-type RuvB. RuvA may restore
these defects of K68A and T69A. Consistent with this idea, it has been shown that RuvA reduced the $K_m$ of the RuvB ATPase and enhanced the intimate and continuous contact of RuvB with DNA (36). Electron microscopic and gel filtration studies have shown that RuvB forms a hexameric ring structure as an active form of RuvB that assembles in solution in the presence of ATP and Mg$^{2+}$. In this study, gel filtration analysis revealed that K68A and T69A as well as wild-type RuvB could form hexamers in the presence of Mg$^{2+}$ and ATP and could form complexes with RuvA. These results suggest that these two mutants are capable of undergoing the nucleotide-induced conformational change in the presence of saturating amounts of ATP.

On the other hand, K68R could not bind DNA even in the presence of RuvA, although K68R could form a complex with RuvA (Fig. 7B). Recently, Mezard et al. (41) showed D113N, with a substitution in Walker motif B, was defective in DNA binding both in the presence and absence of RuvA. D113N was also formed hexamers or dodecamers even in the absence of Mg$^{2+}$ at higher RuvB concentrations (41). K68R, like D113N, formed higher oligomeric states, such as hexamers and dodecamers, under all conditions we studied. Thus, K68R protein may self-aggregate to form hexameric or dodecameric rings in the absence of DNA, which prevents loading onto DNA. These results suggest that the change in oligomeric states induced by nucleotide binding is important for the assembly of RuvB onto DNA.

Although the ATPase activity of RuvB is required for the branch migration activity, the mechanisms by which RuvB protein transmits the energy derived from ATP hydrolysis into the movement necessary for the branch migration are still unclear. It is likely that a conformational change caused by the energy derived from ATP hydrolysis must be coupled to DNA unwinding and translocation. Indeed, nucleotide-induced conformational changes have been reported for several helicases such as E. coli DnaB, Rep, and bacteriophage T7 gene 4 proteins (42–44). In this study, we have shown that all of the substitution mutations in the conserved lysine and threonine residues in Walker motif A, except T69S, not only affect the ATP binding affinity and inactivate the ATPase activity, but also affect the DNA binding. In addition, K68R affected the oligomer formation. These results suggest that these residues may play key roles in interconnecting ATP binding and ATPase activities with DNA binding and oligomerization through nucleotide-induced conformational changes, all of which are required for the branch migration activity.

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