

Evidence that Phenylalanine 69 in *Escherichia coli* RuvC Resolvase Forms a Stacking Interaction during Binding and Destabilization of a Holliday Junction DNA Substrate*

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***Escherichia coli* RuvC resolvase is a specific endonuclease that recognizes and cleaves Holliday junctions formed during homologous recombination and recombinational repair. This study examines the phenotype of RuvC mutants with amino acid substitutions at phenylalanine 69 (F69L, F69Y, F69W, and F69A), a catalytically important residue that faces the catalytic center of the enzyme. F69Y, but not the other three mutants, almost fully complements the UV sensitivity of a Δ ruvC strain and substantially resolves synthetic Holliday junctions *in vitro*. In the presence of 100 mM NaCl, RuvC F69A and F69L are defective in junction binding, but F69Y and F69W retain near wild-type binding activity during a gel shift binding assay. KMnO₄ was used to probe synthetic Holliday junction DNA in a complex with wild-type and mutant RuvC; F69A and F69L did not induce disruption of base pairing at the crossover to the same extent as wild-type RuvC. Thus, the aromatic ring of Phe-69 is involved in DNA binding, probably via a stacking interaction with a nucleotide base, and this interaction may induce a structural change in junction DNA that is required to form a catalytically competent complex.**

Homologous DNA recombination is ubiquitous in all organisms. It promotes genetic diversity, plays an important role in DNA repair and helps maintain genome integrity. *Escherichia coli* RecA protein plays a central role in homologous recombination pathways and catalyzes strand exchange between homologous DNA molecules, leading to the formation of recombination intermediates (1, 2). In these intermediates, two homologous duplex DNA molecules are joined by a four-way DNA junction referred to as a Holliday junction (3). *E. coli* proteins RuvA and RuvB form a protein complex that promotes branch migration of Holliday junction and facilitates the extension of heteroduplex DNA region. *E. coli* RuvC protein resolves a Holliday junction by endonucleolytic cleavage (4, 5). Recently, it has been proposed that the three proteins RuvABC form a protein complex called a resolvosome that efficiently processes Holliday junctions (6).

Although Holliday junction-resolving activities have been found in extracts from a wide variety of organisms, *E. coli* RuvC is one of the best characterized resolvases. Genetic anal-

yses indicate that RuvC resolvase plays a defined role in processing recombination and repair intermediates (7–11). *In vitro* studies confirm that RuvC specifically interacts with and resolves Holliday junctions by endonucleolytic cleavage (12, 13). Using several model substrates, including synthetic Holliday junctions, the following has been shown. (i) RuvC is active as a stable dimer that binds a Holliday junction in the presence or absence of divalent metal ions (13–15). (ii) RuvC forms a complex with Holliday junctions in which the two continuous (non-crossover) DNA strands at the junction are hypersensitive to attack by hydroxyl radicals and permanganate; this indicates a distortion in the junction DNA and disruption of base pairing at the crossover. This is likely to be an important characteristic of the interaction between RuvC and its DNA substrate (14, 16). (iii) In the presence of metal ions such as Mg²⁺ and Mn²⁺, RuvC resolves the junctions by introducing symmetrical nicks in the continuous (noncrossover) strands (14, 15, 17), and (iv) cleavage occurs preferentially at the consensus sequence 5'-(A/T)TT↓(G/C), where ↓ represents a cleavage site close to the crossover point (18, 19). Following cleavage, the resolution process is completed by DNA ligase, which rejoins the 5'-P and 3'-OH termini in the nicked duplex products (13, 14).

Structural analysis by x-ray crystallography at 2.5 Å resolution combined with mutation studies also provided further insight into the reaction mechanism of RuvC-mediated Holliday junction resolution (20–23). The two subunits in the RuvC dimer are related by a dyad axis, and each subunit possesses a large cleft, which could accommodate double-stranded DNA at the Holliday junction (20). The catalytic center of RuvC includes the four acidic residues Asp-7, Glu-66, Asp-138, and Asp-141 located at the bottom of the cleft (20, 21). It was recently shown that the basic residues Lys-107 and Lys-118, located on the wall of the cleft near the catalytic center, are involved in DNA binding and play an important role in stabilizing the reaction intermediate during endonucleolytic cleavage by RuvC (22).

Phe-69 lies in the protruding loop preceding the second α -helix and faces the catalytic center of RuvC (20). A previously isolated F69L mutant of RuvC showed no detectable cleavage activity, but formed a dimer that bound a Holliday junction in the same manner as wild-type RuvC, as judged by a gel shift assay in which no salt was added to the reaction mixtures (23). This result suggests that Phe-69 does not directly participate in DNA binding activity detectable by a salt-free gel shift assay, but that it must play an important role in RuvC function. We speculated that this residue makes a stacking interaction with a nucleotide base near the cleavage site (20, 23).

This study examines the role of Phe-69 of RuvC in greater detail. A series of mutants of Phe-69 were constructed and analyzed *in vivo* and *in vitro*. The results provide direct evi-

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dence that Phe-69 interacts with DNA and contributes to disruption of base pairing in the RuvC-DNA complex. This function is critically important for formation of a catalytically competent complex between RuvC and the Holliday junction. A model is proposed for RuvC-mediated endonucleolytic cleavage and resolution of Holliday junctions.

EXPERIMENTAL PROCEDURES

***E. coli* Strains and Plasmids**—*E. coli* strain HRS1200 (Δ ruvC200::Km^r) is a derivative of AB1157, a *ruv*⁺ control strain (21). HRS777 is a Δ ruvC200::Km^r derivative of BL21 (DE3) and was used for overproduction of mutant RuvC proteins (21). Plasmid pRC100 (21) carries the *ruvC*⁺ gene in pET-8c, a T7 expression plasmid (24). The mutant *ruvC* genes were constructed based on the method of Kunkel (21) using a site-directed mutagenesis kit (Takara Shuzo, Kyoto). The codon for Phe-69 (TTT) was altered to that of Tyr (TAC), Trp (TGG), and Ala (GCT). *ruvC* gene mutations were confirmed by sequencing. The F69L mutant had been isolated in a previous study (23). Each mutant *ruvC* gene was carried in pET-8c with the same construction as pRC100. Bacteria were routinely cultured in Luria-Bertani medium at 37 °C. If necessary, ampicillin was added to the medium at the final concentration of 50 μ g/ml.

UV Light Sensitivity Test—Exponentially growing HRS1200 (Δ ruvC) cells harboring *ruvC*-expression plasmids, which were suspended in M9 buffer ($\sim 2 \times 10^8$ cells per ml), were irradiated with various doses of UV. Cells were plated on Luria-Bertani plates containing ampicillin (50 μ g/ml), and the surviving colonies were scored after incubation for 15 h at 37 °C in the dark.

Purification of Wild-type and Mutant RuvC Proteins—Phe-69 mutant proteins were purified by the same procedure as wild-type and other mutant proteins, which was described in our previous report (22).

Synthetic Holliday Junctions—Synthetic Holliday junctions were prepared by annealing four synthetic oligonucleotides as described (21). The sequences of synthetic Holliday junctions, X12 and HJ2, were described elsewhere (22). The synthetic Holliday junction HJ2L consists of four oligonucleotides, which are ST-1 (5'-GACGCTGCCGAATCTTACCAGTGCCTGGCTTTGACCTCTTTGCCACCTGCAGGTTCA-CCC-3'), ST-2 (5'-TGGGTGAACCTGCAGGTGGGCAAAGAGGTCAATCGGTTGTAAACGTCAAGCTTTATGCCGTT-3'), ST-3 (5'-GAACGGCATAAAGCTTGACGATTACAACCGATTCTGGAGCTGTCTAGAGGATCCGACTATCGA-3'), and ST-4 (5'-ATCGATAGTCGGATCTCTAGACAGCTCCAGAAAGCCAGGCACTGGTAGAATTCGGCAGCGT-3'). The sequences underlined are the same junction core sequences as those of HJ2. HJ2L was used as a substrate for a potassium permanganate footprinting experiment.

Cleavage and Gel Shift Assays—Holliday junction cleavage and gel shift assays were performed essentially as described previously (21). In brief, reaction mixtures (20 μ l) contained 10 ng of a ³²P-labeled synthetic Holliday junction, 20 mM Tris acetate, pH 8.0, 10 mM (for cleavage assay) or 0.5 mM (for gel shift assay) magnesium acetate, 1 mM dithiothreitol, 100 μ g/ml bovine serum albumin, 5% (v/v) glycerol, and the indicated concentrations of RuvC. For a standard cleavage assay, the mixtures were incubated at 37 °C for 30 min, and then the samples were analyzed by 9% native polyacrylamide gel electrophoresis (PAGE)¹ in TAE (40 mM Tris acetate, pH 7.8, 1 mM EDTA) buffer. To determine the cleavage sites, samples which had been incubated at 37 °C for 60 min were analyzed by electrophoresis in 12% polyacrylamide gels containing 7 M urea. For a standard gel shift assay, the reaction mixtures were incubated on ice for 15 min, and the samples were analyzed by in 6% PAGE in TAM (40 mM Tris acetate, pH 7.8, 0.5 mM magnesium acetate) buffer. The radioactive materials were analyzed quantitatively using an image analyzer, Fuji BAS1000.

Potassium Permanganate Footprinting—Reaction mixtures (50 μ l) containing 3 ng of 5' ³²P-labeled junction DNA, 25 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 1 mM dithiothreitol, 100 μ g/ml bovine serum albumin, 5% (v/v) glycerol, and the indicated concentrations of RuvC were incubated for 10 min at room temperature. Two microliters of 50 mM KMnO₄ was added to the reaction mixtures and incubated for another 2 min. The reactions were stopped by addition of 2 μ l of 14.7 M β -mercaptoethanol. The products were extracted with phenol, precipitated with ethanol and dried. Pellets were dissolved in 50 μ l of 1 M piperidine, and the samples were heated at 90 °C for 30 min. The products were separated by

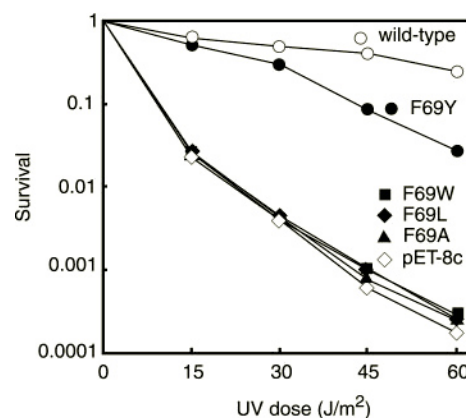


FIG. 1. DNA repair activity of Phe-69 mutants of RuvC. UV sensitivity test of HRS1200 (Δ ruvC) cells harboring *ruvC*-expression plasmids was performed as described under "Experimental Procedures." The values shown are the average of three independent experiments. Plasmids were as follows: *ruvC*⁺ (pRC100, ○), vector (pET-8c, ◇), F69Y (pRC501, ●), F69W (pRC502, ■), F69A (pRC503, ▲) and F69L (pRC504, previously named pRC226, Ref. 23; ◆).

electrophoresis in 12% polyacrylamide gels containing 7 M urea and analyzed using Fuji BAS1000.

RESULTS

DNA Repair Activities of Phe-69 Mutants of RuvC—RuvC mutants F69Y, F69W, and F69A were constructed by site-directed mutagenesis of a plasmid based on pET-8c (24). These mutant proteins and RuvC F69L (23) were tested for *in vivo* complementation of the UV repair deficiency of Δ ruvC strain HRS1200. RuvC F69W, F69A, and F69L did not complement the UV sensitivity of HRS1200 (Fig. 1). However, F69Y was active in repair and near fully complemented the repair defect. The expression level of these four mutants and wild-type RuvC was determined by Western blot analysis using anti-RuvC serum (data not shown). All four mutants were expressed at the same level as wild-type RuvC in HRS1200 cells under the conditions of the UV sensitivity test. Thus, it is likely that RuvC F69W, F69A, and F69L do not complement the repair deficiency because they are deficient in repair and not because they are poorly expressed or unstable proteins. Because RuvC F69Y complements the Δ ruvC deficiency but RuvC F69W, F69A, and F69L do not, it is likely that a benzene ring is required at this position of RuvC for proper enzymatic function.

In Vitro Junction Cleavage Activity of RuvC Phe-69 Mutants—Phe-69 mutant proteins were purified to greater than 99% homogeneity and characterized *in vitro*. These proteins were free of nonspecific nuclease activity (data not shown). RuvC D7N was also used as a negative control in these studies. This mutant has an amino acid change in the enzyme active site and binds to junction DNA with the same affinity as wild-type RuvC but is unable to cleave DNA (21). The cleavage activity of these mutants was examined using a synthetic Holliday junction, HJ2, as a substrate (21). Fig. 2 shows that RuvC F69Y is partially active as an endonuclease (63% of wild-type RuvC activity), but RuvC F69W, F69A, F69L, and D7N do not generate any detectable cleavage products with the synthetic Holliday junction substrate. During a longer incubation, F69W cleaved the substrate with low efficiency ($\sim 4\%$ cleavage product), but F69A, F69L, and D7N produced no cleavage product after 2 h (data not shown). Essentially the same results were obtained with another synthetic Holliday junction DNA substrate, X12, which was used previously by van Gool *et al.* (25), indicating that the results are not restricted to a particular substrate. RuvC F69Y cleaved HJ2 and X12 at the same sites as wild-type RuvC (data not shown). These results confirm the

¹ The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.

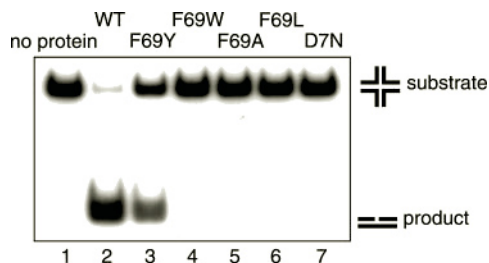


FIG. 2. Cleavage of synthetic Holliday junction by RuvC Phe-69 mutants. Reaction mixtures contained 10 ng of 32 P-labeled synthetic Holliday junction HJ2 and 15 ng of RuvC protein and were incubated for 30 min at 37 °C. The products were analyzed by 9% PAGE.

in vivo results and suggest that Phe-69 can be substituted with tyrosine but not with other amino acids that lack a benzene ring.

Binding Affinity of Phe-69 Mutant Proteins—The binding affinity of the RuvC Phe-69 mutants for a synthetic Holliday junction was examined using a gel-shift assay in the absence (Fig. 3A) and presence of 100 mM NaCl (Fig. 3B). All RuvC mutants formed a complex with HJ2 DNA as efficiently as wild-type protein in the absence of NaCl. This result is consistent with the previous result (23). In contrast, F69A and F69L did not bind junction DNA in the presence of 100 mM NaCl (Fig. 3B, lanes 11–16). This result indicates that substitution of Phe-69 by alanine or leucine reduces binding affinity when electrostatic interaction between RuvC and DNA is altered by a high concentration of monovalent salt. The fact that RuvC F69Y retains substantial cleavage activity (Fig. 2) and full binding activity (Fig. 3) suggests that the aromatic ring of the side chain of the residue 69 position is involved in a nonelectrostatic interaction with the Holliday junction DNA substrate and that this interaction is essential for cleavage by RuvC endonuclease. Interestingly, RuvC F69W has very low endonuclease activity but binds to the synthetic Holliday junction as efficiently as or more efficiently than wild-type RuvC even in the presence of 100 mM NaCl (Fig. 3B, lanes 8–10). This may indicate that the tryptophan residue interferes with cleavage of the Holliday junction because it is bulkier than phenylalanine or tyrosine; nevertheless RuvC F69W binds DNA similarly to wild-type and F69Y RuvC.

Aromatic Side Chain of Phe-69 Is Involved in Helix Destabilization at the Holliday Junction Crossover—The conformation of the synthetic Holliday junction substrate was previously characterized in the presence of Mg^{2+} (26, 27). The protein-free DNA junction folds into a stacked X-structure, which exhibits 2-fold symmetry. In this structure, two strands approximate B-form DNA, and the complementary strands are sharply bent as they pass from one helix to the other. Base pairing is maintained all around the junction (26, 27). When RuvC binds to the DNA substrate, the crossover is converted from a folded form into an unfolded form with 2-fold symmetry, and base pairing around the crossover is disrupted (14, 16). It has been proposed that disruption of base pairing is a crucial step in forming a catalytically competent complex between RuvC and the Holliday junction substrate (14, 16). These observations raise a possibility that Phe-69 is involved in a stacking interaction with a nucleotide base close to the crossover; formation of the catalytically competent complex may involve this interaction and the disruption of the base pairing associated with it. This hypothesis was tested by carrying out potassium permanganate ($KMnO_4$) analysis of junction DNA complexes in the presence of wild-type or mutant RuvC (Fig. 4). Potassium permanganate attacks the C-5=C-6 double bond in thymines that are unstacked, unwound, or unpaired (28). Thus, this method is

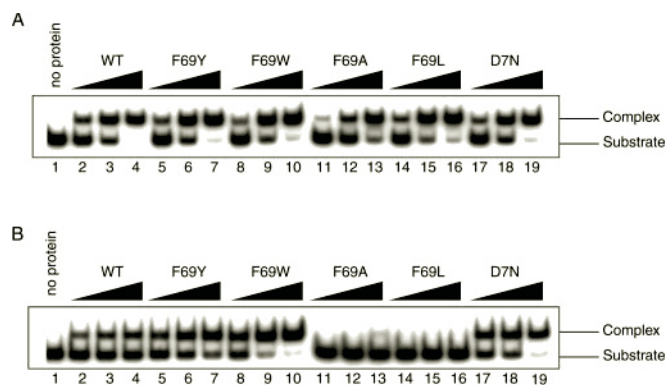


FIG. 3. Gel shift assay of binding of RuvC proteins to the synthetic junction HJ2 in the presence of NaCl at 0 mM (A) or 100 mM (B). Lane 1 contained no protein. Wild-type or mutant RuvC was added as indicated at increasing concentrations of 10, 20, and 50 nM.

useful for detecting base disruption around a Holliday junction crossover.

A synthetic junction called HJ2L was used in the $KMnO_4$ footprinting studies. HJ2L has longer arms but has the same central 10-base pair sequence as HJ2. HJ2L was used in this experiment, because it gives a piperidine-cleaved ladder with more similar intervals around the crossover than HJ2; its use would make it easier to evaluate the sensitivity of each band of the ladder. Wild-type RuvC cleaves the same sequences of HJ2 and HJ2L with equal efficiency (data not shown).

Each of the four strands of HJ2L was probed with $KMnO_4$ in the presence of Mg^{2+} . In the absence of protein, only background reactivity was observed. In the presence of wild-type RuvC, all thymine bases within the homologous region and two thymine bases outside the homologous region were hypersensitive to $KMnO_4$. The following thymines were highly reactive: three bases in the homologous region on strand 1 (Fig. 4A, lanes b–d); the thymine next to the homologous region on strand 2 (Fig. 4B, lanes b–d), and two thymines in the homologous region on strand 3 (data not shown). Strand 4 has no thymine bases near the crossover, and no hypersensitive bases were detected on this strand. A cleavage product was also detected on strand 1 produced by nicking at 5'-TTT ↓ G-3'. A DNA-RuvC complex with the active site mutant RuvC D7N produced the same $KMnO_4$ hypersensitivity as the complex with wild-type RuvC, but no cleavage product was observed (lanes m and n). The results of these control experiments were highly consistent with the results of earlier studies that used a different DNA substrate (16, 29). $KMnO_4$ sensitivity was also examined in complexes between RuvC Phe-69 mutants and HJ2L. Complexes with F69Y and F69W (lanes e–h) had the same $KMnO_4$ sensitivity as complexes with wild-type and D7N mutants of RuvC. In contrast, complexes with RuvC F69A and F69L demonstrated severely decreased sensitivity at the first thymine base on strand 1 (Fig. 4A, lanes i–l) and the thymine base on strand 2 (Fig. 4B, lanes i–l) although very similar sensitivity was observed for the two thymine bases on strand 3 (data not shown). These results strongly suggest that the aromatic side chain of Phe-69 interacts with particular nucleotide(s) and is involved in inducing an opened state of the crossover upon disruption of the base pairing. Fig. 5 summarizes the results of footprinting analysis of RuvC complexes with junction DNA.

DISCUSSION

This study presents genetic and biochemical evidence that the benzene ring of residue Phe-69 in RuvC is catalytically important for Holliday junction resolution. RuvC F69Y has substantial DNA repair activity *in vivo*, and it cleaves synthetic Holliday junctions with reasonable efficiency *in vitro*. In con-

FIG. 4. Analysis of Holliday junction HJ2L complex with wild-type and mutant RuvC proteins by potassium permanganate footprinting. Junction HJ2L with 32 P-labeled strand 1 (A) and strand 2 (B) was incubated with the indicated amount of RuvC in binding buffer. DNA-protein complexes were treated with permanganate and analyzed by denaturing PAGE as described under "Experimental Procedures." Lane a shows the ladder of the Maxam-Gilbert G+A reactions of the labeled strand. The DNA sequences are shown to the right. In each sequence, the *large bold T* is the thymine whose hypersensitivity is reduced in complexes with RuvC F69A and F69L. The corresponding thymine bands are labeled with arrows to the right of each gel.

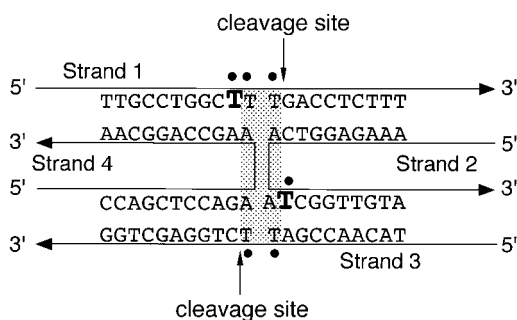
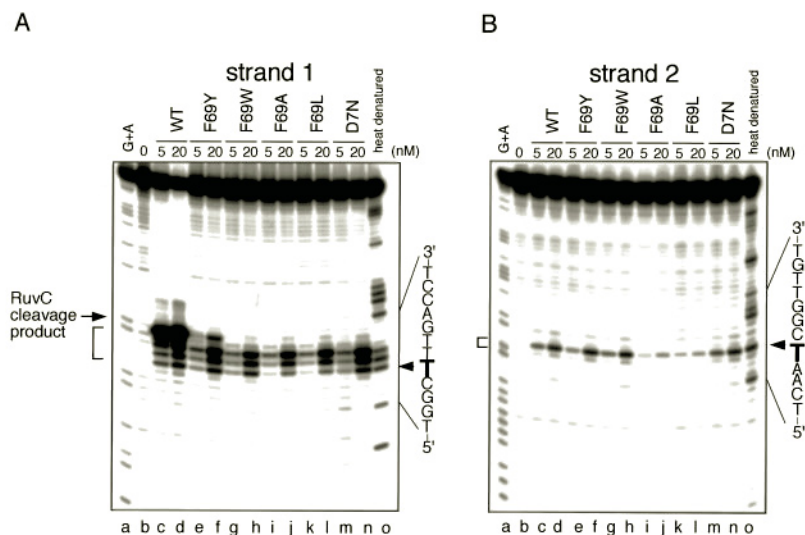


FIG. 5. Summary of the permanganate footprinting analysis. Shaded base pairs indicate the 4-base pair homology core region of HJ2L. Strands 1 and 3 are continuous strands and strands 2 and 4 are crossover strands (determined by hydroxyl radical footprinting and sensitivity to RuvC-mediated strand cleavage). RuvC cleavage sites are indicated by vertical arrows. The crossover point of the junction DNA was assigned as indicated. Dots indicate the nucleotides hypersensitive to permanganate when RuvC is bound. The two Ts in large bold letters are the nucleotides whose hypersensitivity is reduced in complexes with RuvC F69A and F69L.

trast, F69W, F69A, and F69L do not repair DNA damage *in vivo* or cleave synthetic Holliday junctions *in vitro*. RuvC F69A and F69L show reduced binding to junctions under high salt conditions, indicating that Phe-69 is involved in the interaction between RuvC and its DNA substrate. Indeed, the benzene ring of Phe-69 faces the catalytic center (20), which is a relatively uncommon orientation for hydrophobic residues such as phenylalanine. RuvC F69W binds DNA junctions with similar affinity to RuvC wild-type and F69Y, but F69W can not resolve Holliday junctions *in vivo* or *in vitro*. This suggests that precise interaction of the benzene ring at position 69 with the junction, that cannot be mediated by a bulky tryptophan residue, is required for junction resolution. Notably, orthologs of RuvC from eubacteria have phenylalanine or tyrosine but not tryptophan at the position equivalent to Phe-69 in *E. coli* RuvC (22).

Evidence indicates that RuvC binding leads to disruption of base pairing at the crossover junction. It is likely that this DNA conformational change is important in the mechanism of cleavage and resolution by RuvC (14, 16). A prominent wall is located between the two active centers in the RuvC dimer, and docking studies using RuvC dimer and junction DNA indicate that this wall disrupts base pairing of the junction DNA by penetrating the junction center (20). The wall consists of the protruding loop preceding the second α -helix, and the aromatic ring of Phe-69 faces the active center. A structure-based model

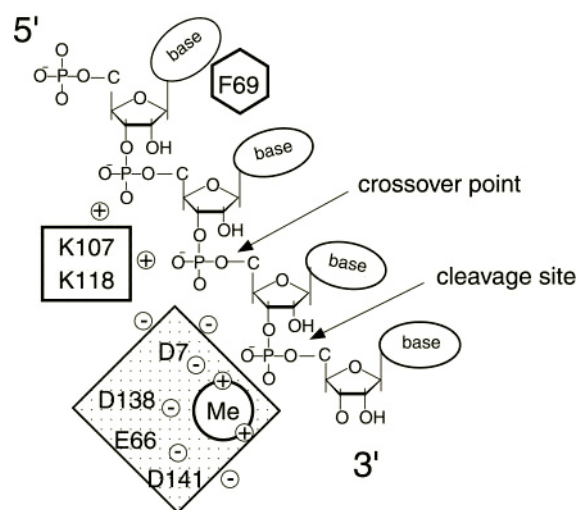


FIG. 6. Proposed mechanism of DNA hydrolysis by RuvC.

was proposed based on docking experiments which suggests that the aromatic ring of Phe-69 makes a stacking interaction with a nucleotide base close to the junction point (20).

Footprinting analysis with permanganate showed that the DNA substrate HJ2L contains thymine bases that become hypersensitive when wild-type RuvC binds. The hypersensitive bases include 3 thymine bases in strand 1, a single thymine in strand 2, and 2 thymine bases in strand 3 (Fig. 4). However, RuvC F69A and F69L did not fully induce hypersensitivity at the first thymine in strand 1 or the thymine in strand 2, whereas the hypersensitivity of the other thymine bases was not reduced in complexes with these RuvC mutants (Figs. 4 and 5). These observations suggest that Phe-69 is involved in disruption of a particular base pair near the junction crossover. We propose that the overall disruption of base pairing is induced when the wall penetrates the junction center (20), and that the aromatic ring of Phe-69 stabilizes an open structure by a stacking interaction with a nucleotide base, which is one of the requirements for catalytic competence. A catalytically important phenylalanine residue has been reported in the mismatch repair protein MutS, in which a specific phenylalanine forms a stacking interaction with a mismatched base (30, 31).

Hydroxyl radical footprinting analysis of the protein-free junction (data not shown) and identification of the strands cleaved by RuvC (Fig. 4) revealed structural information on the configuration of the HJ2L model junction (Fig. 5). Strands 1

and 3 are continuous (noncrossover) strands, and strands 2 and 4 are crossover strands. HJ2L contains a 4-base pair homologous core in the central region of the junction (a bimobile junction), which means that it can make two steps of spontaneous branch migration; thus, HJ2L has three possible junction points. Recently, we showed that RuvC preferentially nicks the phosphodiester bond one nucleotide 3' to the crossover point (32). The cleavage site is 5'-TTT↓G-3' in strand 1 (arrow indicates the cleavage site) and the crossover point is 5'-TT|TG-3' (vertical bar indicates crossover). These results are shown schematically in Fig. 5.

The permanganate footprinting analysis using RuvC mutants F69A and F69L suggest that Phe-69 affects the base pairings 2.5 nucleotides 5' to the cleavage site (Figs. 4 and 5). It is not possible to determine whether Phe-69 interacts with the continuous or the crossover strand of the substrate from the footprinting experiment. However, the model for the RuvC-junction complex based on docking experiments suggests that the base on the continuous strand is closer to Phe-69 in each RuvC monomer than the base in the crossover strand (20). Thus, the data indicate that each Phe-69 residue in the RuvC dimer forms a stacking interaction with a base 2.5 nucleotides 5' to the cleavage site on the continuous strand. This stacking interaction stabilizes an open state in the junction core and leads to formation of a catalytically competent protein-DNA complex.

Based on this information, a proposed catalytic mechanism for RuvC-mediated cleavage is presented in Fig. 6. When RuvC binds the junction DNA, it induces a change in the DNA configuration from a stacked X structure to an unfolded core structure (14, 16). The four acidic residues Asp-7, Glu-66, Asp-138, and Asp-141 constitute the catalytic center of RuvC (21). These residues coordinate divalent metal cation(s) such as Mg^{2+} or Mn^{2+} and make a pentacoordinated intermediate using the metal ion(s) and phosphodiester bonds at the cleavage site on the continuous strand (17, 21, 33). Two basic residues, Lys-107 and Lys-118, interact with the negatively charged phosphate of the DNA backbone via electrostatic interactions and stabilize the pentacoordinated intermediate in which negative charge accumulates during the transition state of hydrolysis (22). Phe-69 residue forms a stacking interaction with a base 2.5 nucleotides 5' to the cleavage site to stabilize the open state in the junction core, as described above. This model is well consistent with the results of *in vivo* and *in vitro* analyses of many mutant RuvC proteins and with feature of the RuvC structure. Further physicochemical analyses such as x-ray crystallography of RuvC-Holliday junction complexes will provide more

direct evidence concerning the mechanism of RuvC-mediated resolution of Holliday junctions.

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