Cloning, Overexpression, Purification, and Characterization of the Escherichia coli RuvC Holliday Junction Resolvase*

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The ruvC gene has been cloned into the plasmid pT7-7 under the control of the T7 φ10 promoter. Following induction with isopropyl-1-thio-β-D-galactopyranoside, the 19-kDa RuvC protein was overexpressed to 20–30% of total cell protein. RuvC has been purified to homogeneity by a simple procedure involving precipitation from the crude lysate, followed by three chromatographic steps. The purified protein resolves synthetic Holliday junctions (80 nucleotides in length) by cleavage at the 3'-side of a phosphate group, to produce nicked duplex DNA. Under the same conditions no cleavage of linear duplex or single-stranded DNA was detected. However, low levels of cleavage were observed with supercoiled form I and single-stranded circular DNA substrates, consistent with the interaction of RuvC with secondary structures. Using synthetic Holliday junctions, we show that RuvC-mediated resolution requires Mg2+ (10 mM) and exhibits an alkaline pH optimum (pH 9.0). No energy cofactors are needed. When RuvC was analyzed by gel filtration and polyacrylamide gel electrophoresis, monomeric and dimeric forms of the protein were observed.

Homologous recombination occurs via an intermediate structure, known as a Holliday junction, in which two DNA molecules are linked by a crossover (1). To complete the recombination event, resolution of the Holliday junction is required to restore the DNA to two discrete molecules. In Escherichia coli, an activity capable of performing Holliday junction resolution in vitro was identified using cell-free extracts (2), and this activity was later found to be absent in ruvC mutants (3).

The ruvC gene is located within the ruv locus, at 41 min on the E. coli chromosome (4–6). The gene forms an operon with orf-26, which encodes a 26-kDa protein of unknown function. A second operon lying downstream of ruvC encodes two genes, ruwA and ruwB (7, 8). The ruwA and ruwB genes are LexA-regulated and induced as part of the SOS response to DNA damage (7–10). Cells carrying mutations in one of the three ruv genes have similar phenotypes, with an increased sensitivity to UV light, ionizing irradiation, and chemical mutagens (4, 11–14). In addition, in a recBCΔ recA, recBCΔΔΔ, or recG genetic background, ruw mutants are deficient in homologous recombination (11, 15–18). In certain cases, the effect of ruw on the phenotype of these multiple mutants is suppressed by recA mutations (15), suggesting that the ruwA, ruwB, and ruwC gene products are involved in a late step of recombination and the recombinational repair of damaged DNA.

The ruvA and RuvB proteins have been purified (19–21) and shown to promote the branch migration of Holliday junctions in vitro (20, 22–26). The product of ruvC, the 19-kDa RuvC protein, resolves Holliday junctions in vitro (27). Resolution was demonstrated using: (i) synthetic Holliday junctions; (ii) recombination intermediates made by the RecA protein; and (iii) cruciform structures extruded from supercoiled plasmids (27, 28). Cleavage occurred by the introduction of two symmetrical nicks close to the junction point (3, 27, 29). In the present work, we describe the cloning of the ruvC gene into a high level expression plasmid and detail the purification of RuvC protein. We investigate the substrate specificities and cofactor requirements of the cleavage reaction catalyzed by RuvC and report the general biochemical and physical properties of the purified protein.

Experimental Procedures

Enzymes and Reagents

Restriction enzymes were obtained from New England Biolabs, AmpliTaq DNA polymerase from Perkin-Elmer-Cetus Instruments, calf intestinal phosphatase from Boehringer Mannheim, and T4 polynucleotide kinase from Pharmacia LKB Biotechnology Inc. T4 endonuclease VII (30) was a gift of Dr. Berries Kemper (University of Cologne). Terminal transferase, [α-32P]ATP, and [γ-32P]ATP were obtained from Amersham Corp.

Bacterial Strains and Plasmids

E. coli strain BL21 (DE3) pLysS (31), plasmid pT7-7 (32), and plasmid pGS760 (5) are described elsewhere. The construction of plasmid pGS775 is described under "Results."

Polymerase Chain Reaction

A standard reaction contained oligonucleotides A and B (100 pmol of each molecule), pGS760 (60 ng), and AmpliTaq DNA polymerase (5 units). 30 cycles were set up as follows: 40 s denaturing at 92°C, 1 min of annealing at 45°C, and 2.5 min of extension at 72°C. The product was isolated by gel purification (GeneClean II, Stratatech).

DNA Substrates

The synthetic Holliday junction (4-X12) was prepared by annealing four partially complementary oligonucleotides (each approximately 60 nucleotides in length). Duplex DNA was prepared by annealing two complementary oligonucleotides. Annealing was performed as described (33), and the oligonucleotide sequences are listed in the accompanying paper (34). The annealed substrates were 32P-end labeled in the common strand (oligonucleotide 2). Labelling was performed using T4 polynucleotide kinase and [γ-32P]ATP to give a 5'-end labeled or terminal transferase and [α-32P]dideoxy ATP to give a 3'-end label. Single-stranded and replicative form I (RFI) DNA of phiX174 were purchased from New England Biolabs. 32P-Labeled single-stranded phiX174 DNA was prepared as described (35). Unless stated otherwise amounts of DNA are expressed in mol of nucleotide residues.

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Buffers
Lysis buffer contained 0.1 M Tris-HCl, pH 8.0, 2 mM EDTA, and 5% (v/v) glycerol. R buffer contained 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 10% (v/v) glycerol. TAE buffer contained 40 mM Tris base, 1.1% (v/v) acetic acid, and 1 mM EDTA. TBE buffer contained 89 mM Tris base, 1.84% (v/v) acetic acid, and 2 mM EDTA. SDS-sample loading buffer contained 0.125% Tris-HCl, pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue, and 10% (v/v) β-mercaptoethanol. Cleavage buffer for RuvC and T4 endonuclease VII was 25 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 100 μg/ml bovine serum albumin, and 1 mM dTT.

Time Course of Induction of RuvC Expression
A 200-ml culture of BL21 (DE3) pLYS3 pGS775 was grown with aeration at 37 °C in Luria broth containing 100 μg/ml carbenicillin and 50 μg/ml chloramphenicol. At a cell density corresponding to an OD₆₀₀ = 0.5, synthesis of RuvC protein was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to 0.4 mM. At various times, 1.5-ml samples were removed and the cells pelleted by centrifugation. The cells were resuspended in 100 μl of SDS-sample loading buffer and boiled for 3 min before analysis by SDS-PAGE.

Chromatography
Reactive Blue 4-agarose and single-stranded DNA-cellulose were obtained from Sigma and phosphocellulose (P11) from Whatman. Column chromatography was carried out at 4 °C.

Resolution Assay
Reactions (20 μl) containing 5'-32P-labeled junction DNA (150 nM), cleavage buffer, and RuvC protein were incubated for 30 min at 37 °C. They were stopped by the addition of EDTA to 25 mM, and the DNA was analyzed by neutral or denaturing PAGE followed by autoradiography.

Nonspecific Endonuclease Assays
Single-stranded or RF1 4X174 DNA (76 μM) was incubated in cleavage buffer (40 μl) at 37 °C for 15 or 30 min, respectively, in the presence of various amounts of RuvC. Reactions were stopped by the addition of EDTA to 25 mM, and the DNA was analyzed by electrophoresis following 1% agarose.

Analysis of Cleavage Termini
3'-Termini—Reactions (60 μl) containing 5'-32P-end-labeled 4-X12 DNA (approximately 0.15 μM) and RuvC (0.67 μM) or T4 endonuclease VII (300 units) were incubated in cleavage buffer for 30 min at 37 °C. The DNA products were denatured by heating to 100 °C for 2 min and the reactions split into two 30-μl aliquots. To each aliquot we added terminal transferase buffer (solution 1, Amersham 3'-end labeling kit). After a 20-min incubation at room temperature in the presence or absence of terminal transferase (10 units) and 2 mM dATP (total volume of 50 μl), reactions were stopped by the addition of EDTA to 20 mM, and the 32P-labeled products were analyzed by denaturing PAGE.

5'-Termini—3'-32P-End-labeled 4-X12 DNA (0.15 μM) and RuvC (0.17 μM) were incubated in cleavage buffer (80 μl) for 30 min at 37 °C. The reaction was stopped and the DNA denatured by heating to 100 °C for 2 min. Aliquots (20 μl) were incubated in the presence or absence of calf intestinal phosphatase (0.1 unit). After 40 min at 37 °C, EDTA was added to 50 mM. Following ethanol precipitation, DNA was kinased using T4 polynucleotide kinase (36) in a reaction containing 100 μM ATP. The 32P-labeled products were analyzed by denaturing PAGE.

Gel Filtration
RuvC (30 μg) was dialyzed for 3 h against R buffer supplemented with 10 mM MgCl₂ and 150 mM NaCl and applied to a Superose 12 HR 10/30 fast protein liquid chromatography column (Pharmacia). The column was precalibrated using molecular mass standards (Bio-Rad) consisting of bovine thyroglobulin (670,000), bovine γ-globulin (158,000), chicken ovalbumin (44,000), horse myoglobin (17,000), and vitamin B₁₂ (1,350). Protein was detected by measuring the absorbance at 280 nm.

Gel Electrophoresis
SDS-PAGE was carried out according to standard procedures using 13.5% gels (36). Unless stated otherwise, SDS-sample loading buffer was added to samples that were boiled for 4 min prior to loading. The standards (Bio-Rad) were rabbit muscle phosphorylase b (97,000), bovine serum albumin (66,200), hen egg white albumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400). Gels were stained with Coomassie Brilliant Blue or Rapid-Ag-Stain (ICN).

Gel electrophoresis was performed using TAE buffer and the DNA visualized by staining with ethidium bromide. For neutral PAGE, samples were electrophoresed through 5% polyacrylamide gels. For denaturing PAGE, the DNA products were heated to 95 °C for 3 min in TBE buffer containing 90% (v/v) formamide. Denatured samples were subjected to electrophoresis through 12% polyacrylamide gels containing 7 M urea. In both cases TBE buffer was used. The DNA was visualized by autoradiography.

RESULTS
Construction of RuvC Overexpression Plasmid—In previous studies, we purified RuvC protein from plasmid pG762 in which the ruvC gene was under control of the lac promoter (27). Induction of E. coli strain BL21 carrying pG762 by IPTG led to low level overexpression of RuvC protein (3-4% of total cellular protein). To facilitate greater overexpression of RuvC, the ruvC gene was cloned into the plasmid pT7-7. A clone was generated by site-directed mutagenesis of ruvC, in which the upstream and downstream sequences were altered. Two oligonucleotides were constructed as primers for a polymerase chain reaction (Fig. 1B). Oligonucleotide A corresponds to 34 bases of the 5' sequence of ruvC. It differs from the wild-type sequence at 6 residues giving an EcoRI restriction site and an improved ribosome binding site. Oligonucleotide B corresponds to 22 bases of the 3' sequence of ruvC and differs from the wild-type sequence at 2 residues giving an HindIII site. Using the two oligonucleotides a polymerase chain reaction product was generated from the plasmid pG760 (ruvC*) (5). The product was digested with EcoRI and HindIII and inserted into the plasmid pT7-7 (32). The resulting plasmid, pG775, carries the ruvC gene under the control of the T7 φ10 promoter (Fig. 1A) and is able to restore complete UV resistance to the four available ruvC mutants. Confirmation that pG775 carried the wild-type ruvC gene was obtained by DNA sequencing.

Plasmid pG775 was transformed into BL21 (DE3) pLYS3. A time course of the induction of pG775 following IPTG treatment is shown in Fig. 1C. Overexpression of the 19-kDa RuvC protein was apparent after 60 min of induction (Fig. 1C, lane d). Maximal expression was achieved after 180-240 min, with RuvC expressed to 20-30% of total cellular protein (Fig. 1C, lane g; Fig. 2B, lane c; and data not shown).

Purification of RuvC Protein—A summary of the purification procedure is shown in Fig. 2A. Two 1-liter cultures of E. coli strain BL21 (DE3) pLYS3 carrying pG775 (ruvC*) were grown, with aeration at 37 °C in Luria broth containing 100 μg/ml carbenicillin and 50 μg/ml chloramphenicol. At a cell density corresponding to an OD₆₀₀ = 0.1, IPTG was added to 0.4 mM and incubation continued for 1.5 h. After chilling on ice, induced cells (Fig. 2B, lane c) were collected by low speed centrifugation and resuspended in 20 ml of lysis buffer, giving a total volume of 30 ml. The cells were fast-frozen on dry ice/ethanol and stored at -20 °C.

When required, the cells were thawed, and 0.2 volume of 5 M NaCl, 0.01 volume of 100 mM DTT, and 0.01 volume of 10% (v/v) Triton X-100 were added. The high salt concentration was nec-

2. F. P. Hagan and G. J. Sharples, data not shown.
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*Fig. 1. Overexpression of RuvC protein.* Panel A shows the plasmid pGS775, in which the ruvC gene was placed under the control of the T7 φ10 promoter in pT7-7. Panel B, the ruvC clone was generated by polymerase chain reaction using two synthetic oligonucleotides. Oligonucleotide A (5' of ruvC) and oligonucleotide B (3' of ruvC) differ from the wild-type sequence at several bases (indicated by asterisks). These alterations produced EcoRI and HindIII sites and a modified ribosome binding site. Panel C shows the time course of the induction of pGS775 (ruvC+). IPTG was added at 0 min to a culture of BL21 (DE3) pLysS pGS775. At the times indicated, samples were taken and analyzed by SDS-PAGE (lanes b-g), as described under “Experimental Procedures.” Molecular mass markers (lane a) and the position of the RuvC protein are indicated. The gel was stained with Coomassie Brilliant Blue.

*Fig. 2. Purification of RuvC protein.* Panel A, summary of purification. Panel B, SDS-PAGE showing the purification of RuvC. Lane a, molecular mass markers; lane b, cellular proteins immediately before IPTG induction; lane c, cellular proteins 3.5 h after induction; lane d, resuspension of proteins precipitated from crude lysate (fraction I); lanes e-g, fractions eluted from Reactive Blue 4-agarose (fraction II), phosphocellulose (fraction III), and single-stranded DNA-cellulose (fraction IV), respectively. Lane g contains 6 μg of protein. The gel was stained with Coomassie Brilliant Blue. Panel C, silver-stained SDS-polyacrylamide gel. Lane h, molecular mass markers; lane i, purified RuvC (0.2 μg).

**Raw Text**

...ecessary to prevent precipitation of RuvC. Cells were lysed by three rounds of freezing and thawing, and the lysate was centrifuged for 60 min at 38,000 rpm in a Beckman Ti-45 rotor. The clear supernatant (36 ml) was dialyzed for 3 h against R buffer supplemented with 0.1 M KCl. During dialysis a heavy precipitate formed. The precipitate was collected by centrifugation at 15,000 rpm for 10 min, washed with R buffer containing 0.1 M KCl, and recentrifuged. The pellet was resuspended by addition of 90 ml of R buffer containing 0.5 M KCl. SDS-PAGE of the resuspended precipitate (fraction I) showed it was more than 90% pure (Fig. 2B, lane d). This fraction is sufficiently pure for physical analysis of RuvC.

To remove minor contaminants and obtain protein for biochemical analysis, RuvC was purified further by three chromatographic steps. Fraction I (90 ml; approximately 0.6 mg of protein/ml) was applied to a Reactive Blue 4-agarose column (1.6 x 14.0 cm, 28-ml bed volume) equilibrated with R buffer containing 0.5 M KCl. The column was eluted with a 400-ml gradient of 0.5-1.75 M KCl in R buffer. Fractions eluted from the column were assayed in two ways; (i) the peak of RuvC protein was identified by SDS-PAGE or (ii) the peak of resolvase activity was determined using 32P-labeled synthetic Holliday junctions. Resolution resulted in the formation of nicked duplex DNA, as detected by neutral or denaturing gel electrophoresis.

For the purification described here the lyophilized powder form of Reactive Blue 4-agarose (Sigma, R8754) was used. In subsequent purifications, the matrix was purchased as a suspension (Sigma, R2507) to which RuvC binds with a lower affinity. This makes it necessary to dialyze fraction I against R buffer containing 0.3 M KCl before loading and use a gradient of 0.3-1.25 M KCl in R buffer.
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Panel A, elution profile of RuvC from a phosphocellulose column as determined by SDS-PAGE. Lane a, molecular mass markers; lane b, sample applied to column (fraction II); lane c, column flow-through (FT); lanes d-m, fractions eluted from the column (not all fractions are shown). The gel was stained with Coomassie Brilliant Blue. Panel B, the same fractions were assayed for resolution of a 5'32P-end-labeled synthetic Holliday junction (4-X12), as described under "Experimental Procedures." The markers are PstI-linearized R6X174 DNA (lane f) and a 1-kilobase DNA ladder (lane g). Panel B, single-stranded (ss) R6X174 DNA (76 μm) was incubated with RuvC as indicated (lanes h-m). Lane o, RFI DNA. Reaction products were analyzed by electrophoresis through 1% agarose and visualized by ethidium bromide staining.

**Fig. 3. Coelution of RuvC protein with Holliday junction resolution activity.** Panel A, elution profile of RuvC from a phosphocellulose column as determined by SDS-PAGE. Lane a, molecular mass markers; lane b, sample applied to column (fraction II); lane c, column flow-through (FT); lanes d-m, fractions eluted from the column (not all fractions are shown). The gel was stained with Coomassie Brilliant Blue. Panel B, the same fractions were assayed for resolution of a 5'-32P-end-labeled synthetic Holliday junction (4-X12), as described under "Experimental Procedures." The markers are PstI-linearized R6X174 DNA (lane f) and a 1-kilobase DNA ladder (lane g). Panel B, single-stranded (ss) R6X174 DNA (76 μm) was incubated with RuvC as indicated (lanes h-m). Lane o, RFI DNA. Reaction products were analyzed by electrophoresis through 1% agarose and visualized by ethidium bromide staining.

**Fig. 4. Endonuclease assay.** Panel A, RFI R6X174 DNA (76 μm) was incubated with the indicated concentrations of RuvC (lanes a-e) as described under "Experimental Procedures." The markers are PstI-linearized R6X174 DNA (lane f) and a 1-kilobase DNA ladder (lane g). Panel B, single-stranded (ss) R6X174 DNA (76 μm) was incubated with RuvC as indicated (lanes h-m). Lane o, RFI DNA. Reaction products were analyzed by electrophoresis through 1% agarose and visualized by ethidium bromide staining.
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**Fig. 5. Cleavage specificity of RuvC.** Reactions (40 μl) containing 38 μM synthetic Holliday junction (4-X12) DNA (lanes a-d and m-p), 19 μM linear duplex DNA (lanes e-h and q-t), or 9.5 μM single-stranded linear DNA (lanes i-l and u-x) were incubated with the indicated amounts of RuvC. 5'-32P-End-labeled oligonucleotide 2 was common to all three substrates. After 60 min at 37 °C, the reactions were stopped with EDTA and the labeled products visualized by autoradiography following neutral PAGE or denaturing PAGE (panel A) or denaturing PAGE. By neutral PAGE, we observed that RuvC cleaved the junction to give products which migrated in the duplex position (Fig. 5A, lanes c and d). Denaturing PAGE of the same samples revealed that this occurred by specific nicking of the DNA resulting in a single labeled cleavage fragment (Fig. 5B, lanes o and p). Under identical conditions and RuvC concentrations, we were unable to detect cleavage or degradation of the duplex or single-stranded DNA by neutral or denaturing PAGE (Fig. 5, lanes e-l and q-x).

Fig. 5 indicates that RuvC has a high specificity for the junction DNA, with little or no activity on double- or single-stranded DNA. The apparent incongruity of these results with those presented in Fig. 4 can be explained in terms of secondary structure. Previously, Iwasaki et al. (28) showed that RuvC cleaves the cruciform structure in pUC4 by the introduction of nicks at the base of the cruciform to produce linear duplex DNA. Since RFI φX174 DNA contains a palindromic sequence that can extrude into a cruciform (37), the small amount of RFI φX174 DNA cleaved may be a result of interaction of RuvC with this structure. Cleavage of single-stranded circular φX174 DNA could also be structure-specific since interactions between regions of complementary DNA sequence could lead to the formation of junction-like structures.

**Buffer Requirements of RuvC**—To determine the buffer requirements of RuvC, cleavage of the synthetic Holliday junction was assayed under various reaction conditions. The results are shown in Fig. 6. RuvC activity was stimulated by alkaline pH, with optimum resolution occurring at pH 9.0 (Fig. 6A). The amount of cleavage obtained at more physiological pH (7.5) was about 17% of that observed at the optimum. RuvC showed a requirement for divalent cations (Mg2+) with an optimum at 5–10 mM (Fig. 6B). Mg2+ could be replaced by Mn2+ but not by Cu2+, Zn2+, or Ca2+. Resolvase activity was inhibited by salt, with 50% inhibition observed at 85 mM KC1 (Fig. 6C). We failed to observe any stimulation of activity by inclusion of ATP, spermidine or potassium glutamate (data not shown).

**Characterization of the Nick Introduced by RuvC**—To determine the nature of the 3'-terminal group created by RuvC at the site of resolution, the cleavage products of a 5'-32P-end labeled junction were heat denatured and treated with terminal transferase. Terminal transferase catalyzes the addition of deoxynucleotides to the 3'-end of DNA substrates only if a hydroxyl group is present. Upon incubation of the cleavage products (Fig. 7A, lane c) with terminal transferase, a ladder of DNA fragments was produced (lane d), demonstrating the presence of a 3'-hydroxyl group at the site of RuvC resolution. As a control, similar reactions were carried out using cleavage fragments produced by T4 endonuclease VII. This enzyme cleaved 4-X12 DNA at a number of sites throughout the homologous core to give three major and several minor products (Fig. 7A, lane e). Treatment with terminal transferase again resulted in a ladder of DNA fragments (Fig. 7A, lane f).

To determine the nature of the group at the 5'-terminus of the nick, the junction was labeled at the 3'-end of oligonucleotide 2. After incubation with RuvC, the DNA products were heat denatured and treated with calf intestinal phosphatase, which removes 5'-phosphate groups from DNA. Phosphatase treatment of a DNA molecule possessing a 5'-phosphate should result in a shift of mobility, as detected by denaturing PAGE, because of the removal of a charged group. As seen in Fig. 7B, treatment of the resolution products with calf intestinal phosphatase resulted in a slight reduction in the mobility of the...
applied to a gel filtration fast protein liquid chromatography column in R buffer supplemented with 10 mM MgCl2 and 150 mM NaCl. Measurement of the absorbance at 280 nm resulted in the elution profile seen in Fig. 8A. By comparison with protein standards analyzed in parallel (Fig. 8B), it was determined that the protein peak corresponded to a molecular mass of around 14 kDa. Upon SDS-PAGE of fractions collected during gel filtration, we observed that the elution of RuvC protein coincided exactly with the peak seen in the absorbance profile (data not shown). Since the predicted mass of RuvC is 18.7 kDa (6, 6), the gel filtration data indicate that RuvC is monomeric under these conditions.

SDS-PAGE analysis of RuvC led to the observation of multiple bands on a gel, as seen in Fig. 9. The three major bands have been termed I, II, and III. When denatured by dilution in 2% SDS and 10 mM DTT, RuvC ran as a single band (II) of about 20 kDa (Fig. 9, lane a). However, after dilution in R buffer lacking DTT, we observed two additional major bands (Fig. 9, lane b). Band I corresponds to a molecular mass of around 39 kDa, consistent with the predicted mass of a RuvC dimer. Band III ran slightly ahead of the RuvC monomer, at around 17 kDa, presumably because of incomplete unfolding. We also observed several minor bands (70 and 56 kDa and a doublet at 36 kDa). When RuvC was diluted in the same buffer containing 1 mM DTT, we observed the unfolded and partially unfolded forms of monomeric RuvC, with only a trace of the 39-kDa dimer (band I) (Fig. 9, lane c). From these results we conclude that in the presence of a reducing agent, the majority of the RuvC protein is monomeric. However, in its absence, RuvC protein is found in both monomeric and dimeric states.

**DISCUSSION**

In this paper we describe the construction of a plasmid in which the ruvC gene was placed under the control of the powerful T7 φ10 promoter. Following induction, RuvC protein was expressed to 20–30% of total cell protein. By lysing the cells in the presence of 1 M salt we were able to keep most of the RuvC in solution and allow its isolation from the crude lysate. The first step in the purification procedure, precipitation of RuvC by dialysis of the crude lysate to low salt, yields protein that is >90% pure. At this stage, after a very simple and quick procedure, the protein is sufficiently pure for physical analysis. Further purification through three chromatographic steps yielded approximately 6 mg of >99% pure RuvC from 2 liters of culture. The method described here provides a significant improvement over our previous purification scheme, which utilized a comparatively poor expression system (27), and that of Iwashaki et al. (28), in which over 70% of the RuvC was not recovered from the cells.

Using small synthetic DNA molecules, made by annealing oligonucleotides, we investigated the substrate specificity of RuvC. We found that the purified protein cleaves synthetic Holliday junctions to form nicked duplex products. At similar RuvC concentrations, we were unable to detect cleavage of duplex or single-stranded DNA substrates. The resolution activity was optimal at alkaline pH and required divalent cations (5–10 mM Mg2+). RuvC resolved the synthetic junctions by cleavage at the 3' side of a phosphate, leaving 5'-phosphate and 3'-hydroxyl groups at the incision termini. The resulting nicks may therefore act as substrates for DNA ligase (29).

In addition to the cleavage of Holliday junctions, we observed that RuvC cleaved RFI 4X174 DNA with low efficiency to produce linear duplex DNA. Since φX174 DNA contains short palindrome sequences capable of cruciform extrusion (37), it is likely that linearization results from resolution of a cruciform structure as described previously (28). Similarly, the fragmentation of single-stranded circular φX174 DNA by RuvC is likely...
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A

| Incubation Conditions | Product Formation |
|-----------------------|-------------------|
| RuvC alone           | - + + + +         |
| RuvC with T4 endo VII| + + + + +         |

RuvC cleavage product

dephosphorylated product

cleavage product

Fig. 7. Analysis of cleavage termini. Panel A, 3'-termini. 5'-32P-End-labeled synthetic Holliday junction DNA (4-X12) was incubated alone (lanes a and b), with RuvC (lanes c and d), or with T4 endonuclease VII (lanes e and f) as described under “Experimental Procedures.” The products were heat denatured, and aliquots were treated with terminal transferase (lanes b, d, and f) or left untreated (lanes a, c, and e) as indicated. The major cleavage products formed by RuvC and T4 endonuclease VII are indicated. Panel B, 5'-termini. 5'-32P-End-labeled junction DNA (4-X12) was incubated alone (lane g) or with RuvC (lanes h-k) as described under “Experimental Procedures.” The products were heat denatured and aliquots incubated in the presence (lanes j and k) or absence (lanes g-i) of calf intestinal phosphatase. DNA was precipitated and some samples (lanes i and k) treated with T4 polynucleotide kinase in the presence of ATP. Reaction products were analyzed by denaturing PAGE and visualized by autoradiography.

A

Absorbance

280 nm

Fraction Number

8

670 kD 158 kD 44 kD 17 kD 1.35 kD

B

Molecular mass (x 10^3)

Ve/Vo

158 kD 44 kD 17 kD 1.35 kD

Fig. 8. Gel filtration. RuvC was applied to a Superose 12 fast protein liquid chromatography column in R buffer containing 10 mM MgCl2 and 150 mM NaCl, as described under “Experimental Procedures.” Panel A, elution profile; panel B, determination of the relative molecular mass of RuvC by comparison with molecular mass standards. Absorbance at 280 nm is expressed in arbitrary units.

to be caused by cleavage of junction-like structures formed by the interaction of complementary sequences. In the accompanying paper, we describe the substrate specificity of RuvC protein in further detail (34).

REFERENCES

1. Holliday, R. (1964) Genet. Res. Camb. 5, 292-304
2. Connolly, B., and West, S. C. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8476-8480

5 R. J. Bennett and S. C. West, unpublished data.
Characterization of RuvC Holliday Junction Resolvase

3. Connolly, B., Parsons, C. A., Benson, F. E., Dunderdale, H. J., Sharples, G. J., Lloyd, R. G., and West, S. C. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6063–6067.
4. Sharples, G. J., Benson, F. E., Illing, G. T., and Lloyd, R. G. (1990) Mol. & Gen. Genet. 221, 219–226.
5. Sharples, G. J., and Lloyd, R. G. (1991) J. Bacteriol. 173, 7711–7715.
6. Takahagi, M., Iwaseki, H., Nakata, A., and Shinagawa, H. (1991) J. Bacteriol. 173, 5747–5753.
7. Benson, F. E., Illing, G. T., Sharples, G. J., and Lloyd, R. G. (1988) Nucleic Acids Res. 16, 1541–1550.
8. Shinagawa, H., Makino, K., Amemura, M., Kimura, S., Iwaseki, H., and Nakata, A. (1988) J. Bacteriol. 170, 4322–4329.
9. Shurvin, C. E., and Lloyd, R. G. (1982) Mol. & Gen. Genet. 185, 352–355.
10. Walker, G. C. (1985) Annu. Rev. Biochem. 54, 425–455.
11. Lloyd, R. G., Benson, F. E., and Shurvin, C. E. (1984) Mol. & Gen. Genet. 184, 303–309.
12. Otsuki, N., Iyehara, H., and Hideshima, Y. (1974) J. Bacteriol. 117, 337–344.
13. Sargentini, N. J., and Smith, K. C. (1989) Mutat. Res. 215, 115–129.
14. Stacey, K. A., and Lloyd, R. G. (1976) Mol. & Gen. Genet. 143, 223–232.
15. Benson, F., Collier, S., and Lloyd, R. G. (1991) Mol. & Gen. Genet. 225, 266–272.
16. Lloyd, R. G. (1991) J. Bacteriol. 173, 5414–5418.
17. Lloyd, R. G., Buckman, C., and Benson, F. E. (1987) J. Gen. Microbiol. 133, 2631–2638.
18. Lusini-Deluca, C., Lovett, S. T., and Kolodner, R. D. (1989) Genetics 122, 269–278.
19. Iwaseki, H., Shiba, T., Nakata, A., and Shinagawa, H. (1989) Mol. & Gen. Genet. 219, 328–331.
20. Shiba, T., Iwaseki, H., Nakata, A., and Shinagawa, H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8445–8449.

21. Tsaneva, I. R., Illing, G. T., Lloyd, R. G., and West, S. C. (1992) Mol. & Gen. Genet. 235, 1–10.
22. Tsaneva, I. R., Muller, B., and West, S. C. (1992) Cell 69, 1171–1180.
23. Parsons, C. A., Tsaneva, I., Lloyd, R. G., and West, S. C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5452–5456.
24. Parsons, C. A., and West, S. C. (1993) J. Mol. Biol. 232, 397–406.
25. Muller, B., Tsaneva, I. R., and West, S. C. (1983) J. Biol. Chem. 268, 17179–17184.
26. Muller, B., Tsaneva, I. R., and West, S. C. (1993) J. Biol. Chem. 268, 17185–17189.
27. Dunderdale, H. J., Benson, F. E., Parsons, C. A., Sharples, G. J., and West, S. C. (1991) Nature 354, 506–510.
28. Iwaseki, H., Takahagi, M., Shiba, T., Nakata, A., and Shinagawa, H. (1991) EMBO J. 10, 4381–4386.
29. Bennett, R. J., Dunderdale, H. J., and West, S. C. (1993) Cell 74, 1021–1031.
30. Kosak, H. G., and Kemper, B. W. (1990) Eur. J. Biochem. 184, 779–784.
31. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 60–88.
32. Tauber, S., and Richardson, C. C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1074–1078.
33. Parsons, C. A., Kemper, B., and West, S. C. (1990) J. Biol. Chem. 265, 9285–9289.
34. Benson, F. E., and West, S. C. (1994) J. Biol. Chem. 269, 5195–5201.
35. West, S. C., Casade, E., Mushaln, J., and Howard-Flanders, P. (1986) Proc. Natl. Acad. Sci. U. S. A. 77, 2569–2573.
36. Sambrook, E. F., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
37. Lilley, D. M. J. (1981) Nucleic Acids Res. 9, 1271–1289.