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Overproduction, Purification, and ATPase Activity of the *Escherichia coli* RuvB Protein Involved in DNA Repair

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The *ruvA* and *ruvB* genes of *Escherichia coli* constitute an operon which belongs to the SOS regulon. Genetic evidence suggests that the products of the *ruv* operon are involved in DNA repair and recombination. To begin biochemical characterization of these proteins, we developed a plasmid system that overproduced RuvB protein to 20% of total cell protein. Starting from the overproducing system, we purified RuvB protein. The purified RuvB protein behaved like a monomer in gel filtration chromatography and had an apparent relative molecular mass of 38 kilodaltons in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which agrees with the value predicted from the DNA sequence. The amino acid sequence of the amino-terminal region of the purified protein was analyzed, and the sequence agreed with the one deduced from the DNA sequence. Since the deduced sequence of RuvB protein contained the consensus sequence for ATP-binding proteins, we examined the ATP-binding and ATPase activities of the purified RuvB protein. RuvB protein had a stronger affinity to ADP than to ATP and weak ATPase activity. The results suggest that the weak ATPase activity of RuvB protein is at least partly due to end product inhibition by ADP.

ruv mutants of *Escherichia coli* are sensitive to various DNA-damaging agents, such as UV light, ionizing radiation, and mitomycin, and form multinucleate filaments after treatment with such agents (8–10). Although *ruv* single mutants are proficient in conjugal recombination, *ruv* derivatives of *recBC sbcB* and *recBC sbcA* strains were shown to be deficient in recombination (4, 5). *recBC sbcBC ruv* strains were more sensitive to radiation than *recB ruv* or *recC ruv* strains, which had a level of sensitivity similar to that of *ruv* single mutants (4). From these results, it was suggested that *ruv* is involved in repair of damaged DNA by the RecF recombination pathway (4).

Expression of the *ruv* gene was shown both in vivo and in vitro to be regulated by the LexA repressor (13, 14). The *ruv* region has been cloned and shown to be an operon of two genes regulated by the SOS system (1, 2, 13). Benson et al. (2) suggested that the promoter-proximal gene (*orf1*) and the distal gene (*ruv*) be designated *ruvA* and *ruvB*, respectively, although no evidence for the involvement of the proximal gene in DNA repair has been provided. We constructed a strain with a deletion of the entire *ruv* operon. The strain was sensitive to UV even after introduction of low-copy-number plasmids carrying either *ruvA* (*orf1*) or *ruvB* (*ruv*), but UV resistance was restored by introduction of a plasmid carrying both *ruvA* and *ruvB* (unpublished data). These results demonstrate that *ruvA* as well as *ruvB* is involved in DNA repair, and therefore we have adopted the nomenclature proposed by Benson et al. (2).

Since the sequence of RuvB protein deduced from the DNA sequence contains the consensus sequence shared by ATP-binding proteins (2, 13), we wanted to examine the nucleotide-binding and ATPase activities of the purified protein. For this purpose, we constructed a plasmid that greatly overproduced RuvB protein and purified the protein to near homogeneity. RuvB protein showed weak ATPase activity and bound ADP more strongly than ATP.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* BE5036 (*ruv-9*) (8) was used for complementation tests. AB1157 (9) was used as a *ruvB*$^+$ control strain. The Ruv phenotype was identified by sensitivity to nitrofurantoin (1.5 μg/ml), UV light (30 J/m^2), or both. Plasmid pHS203 (13) is a pUC19 (15) derivative which expresses the *ruvB* gene under the control of the *lac* promoter. JM103 (7) was used as a host strain harboring pHS203 for overproduction of RuvB protein. LB medium (6) containing ampicillin (100 μg/ml) was used for overproduction. Induction of RuvB protein synthesis was done with isopropyl-β-D-thiogalactoside (IPTG) (1 mM).

DNA manipulation. In vitro DNA manipulations and transformation were done by standard procedures as reported before (6).

Chemicals and materials. [α-^{32}P]- and [γ-^{32}P]ATP were purchased from Amersham Japan (Tokyo, Japan). ATP, GTP, CTP, and UTP were from Pharmacia (Uppsala, Sweden). dATP, dGTP, ADP, and AMP were from Sigma Chemical Co. (St. Louis, Mo.). Adenosine and guanosine were from Seikagaku-kogyo (Tokyo). Polyethylenimine (Polymin P) was from Bethesda Research Laboratories (Rockville, Md.). DEAE-Biogel and hydroxylapatite (Biogel HT) were from Bio-Rad Laboratories (Richmond, Calif.), and phosphocellulose (P11) was from Whatman (Kent, England).

Purification procedure for RuvB protein. *E. coli* JM103 carrying plasmid pHS203 was grown at 37°C to an OD_{600} of ~0.5 in 3 liters of LB medium containing ampicillin (100 μg/ml). IPTG was added to the culture to a final concentration of 1 mM to induce synthesis of the RuvB protein, and the culture was incubated for 6 h. The cells were harvested by centrifugation. Subsequent steps were carried out at 4°C, and fractions containing RuvB protein were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The wet cell paste was suspended in 50 ml of R-buffer (20 mM Tris hydrochloride [pH 7.5], 0.1 mM

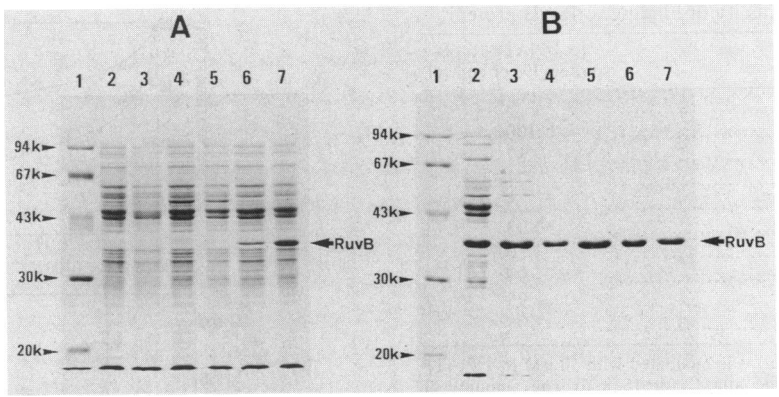

FIG. 1. Overproduction (A) and purification (B) of RuvB protein. Samples were run in 12.5% SDS-PAGE and stained with Coomassie brilliant blue. The positions of RuvB protein are indicated by arrows. (A) Lane 1, Molecular weight markers: phosphorylase b (94,000 M_r [94K]), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), and soybean trypsin inhibitor (20K). Lane 2, JM103. Lane 3, JM103 induced by IPTG. Lane 4, JM103(pUC19) (vector plasmid). Lane 5, JM103(pUC19) induced by IPTG. Lane 6, JM103(pHS203) (*ruvB*$^+$). Lane 7, JM103(pHS203) induced by IPTG. (B) Successive fractions in the purification of RuvB. Lane 1, Molecular marker proteins. Lane 2, Induced lysate. Lane 3, Fraction eluted with R-buffer plus 1.1 M NaCl from Polymin P precipitate. Lane 4, Pellet with 40% ammonium sulfate. Lane 5, Fraction obtained by DEAE-Biogel chromatography. Lane 6, Fraction obtained after separation on hydroxylapatite column. Lane 7, Fraction obtained by Superose 12 gel filtration.

EDTA, 2 mM 2-mercaptoethanol, 10% glycerol) containing 100 mM NaCl, and the cells were disrupted by sonication. The suspension was centrifuged at 40,000 × *g* for 1 h. Polymin P (10% [vol/vol], pH 7.9) was added to the supernatant to a final concentration of 0.6%. After being stirred for 1 h, the suspension was centrifuged at 25,000 × *g* for 20 min. The pellet was resuspended in 40 ml of R-buffer containing 400 mM NaCl, stirred for 30 min, and centrifuged at 25,000 × for 20 min. The pellet was suspended again in 40 ml of R-buffer containing 1.1 M NaCl, stirred for 1 h, and centrifuged at 25,000 × *g* for 20 min. To this supernatant, ammonium sulfate was added to a final concentration of 40% saturation, and the mixture was stirred for 1 h and centrifuged at 20,000 × *g* for 20 min. The pellet was resuspended in 15 ml of R-buffer containing 100 mM NaCl and dialyzed against the same buffer. After the precipitate formed during the dialysis was removed by centrifugation at 25,000 × *g* for 20 min, the supernatant was applied to a DEAE-Biogel column (2.5 by 15 cm), preequilibrated with R-buffer containing 100 mM NaCl, and developed with 500 ml of a linear gradient of 100 to 250 mM NaCl in R-buffer. The fractions that eluted at about 180 mM NaCl contained RuvB protein; these fractions were pooled and dialyzed against P-buffer (10 mM potassium phosphate [pH 6.8], 30 mM NaCl, 2 mM 2-mercaptoethanol). The dialyzed solution was applied to a phosphocellulose column preequilibrated with P-buffer. The passthrough fraction was applied to a hydroxylapatite column (15 ml) preequilibrated with buffer P. This column was washed with 1 column volume of P-buffer and developed with 100 ml of a linear gradient of 10 to 150 mM potassium phosphate in P-buffer. The fractions containing RuvB protein were eluted at about 80 mM potassium phosphate. These fractions were pooled and dialyzed against storage buffer (20 mM Tris hydrochloride [pH 7.5], 30 mM NaCl, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, 50% glycerol). For further purification, the dialyzed sample was applied to a Superose 12 column (HR 10/30, Pharmacia), and proteins were eluted with R-buffer containing 30 mM NaCl. The RuvB protein-containing fractions, as judged by UV absorption and SDS-PAGE, were dialyzed against the storage buffer and stored at −20°C.

Analysis of amino acid sequence of the protein. The amino acid sequence of the purified RuvB protein was analyzed by an automated gas-phase amino acid sequencer, ABI 477A (Applied Biosystems).

Measurement of protein concentration. The concentration of RuvB protein was measured by the biuret reaction with bovine serum albumin as the standard (3). RuvB protein at low concentrations (10 to 1,000 μg/ml) was measured with a Bio-Rad protein assay kit according to the manufacturer's protocol. In this case, the concentration was corrected by a standard factor (0.87) that was chosen by comparison with the buiret reaction.

ATP filter-binding assay. [α-^{32}P]ATP bound to RuvB protein was assayed by adsorption to nitrocellulose membrane filters (Millipore HA; 0.45-μm pore size, 24-mm diameter) as described previously (12). Standard reaction mixtures (50 μl) contained 20 mM Tris hydrochloride [pH 7.5], 50 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, 0.5% glycerol, [α-^{32}P]ATP, and 25 pmol of RuvB protein. After incubation at 0°C for 15 min, 40 μl of the solution was filtered through a nitrocellulose membrane presoaked in washing buffer (20 mM Tris hydrochloride [pH 7.5], 50 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM 2-mercaptoethanol). The filter was washed with 6 ml of ice-cold washing buffer and dried with an air-blower. Radioactivity retained on the filter was measured in a liquid scintillation counter.

ATPase assay. The ATPase activity of RuvB protein was

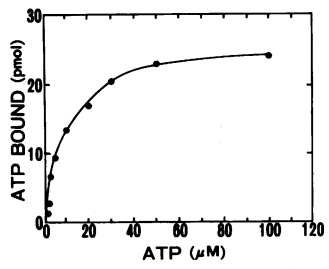

FIG. 2. Binding of ATP to RuvB protein. RuvB (25 pmol) was incubated with various concentrations of [α-^{32}P]ATP (10^3 cpm/pmol) in 50 μl of reaction mixture at 0°C for 15 min, and the [α-^{32}P]ATP-RuvB protein complex in the 40-μl sample was measured as described in Materials and Methods.

TABLE 1. Nucleotide specificity of binding to RuvB protein[a]

Competitor nucleotide	[α-^{32}P]ATP-RuvB protein (% of control)
ATP	9[b]
GTP	100
CTP	87
UTP	71
dATP	5
dGTP	93
ADP	0.7
AMP	95
Adenosine	95
Guanosine	95

[a] Purified RuvB protein (25 pmol) was incubated with 50 μM [α-^{32}P]ATP and 500 μM competitor nucleotide at 0°C for 15 min. The amounts of ATP-RuvB protein complex were measured by membrane filtration. Average values of two experiments with duplicates are presented.

[b] This value agrees with that expected for competition by dilution.

measured essentially as described previously (16). Standard reaction mixtures (10 μl) contained 20 mM Tris hydrochloride [pH 7.5], 50 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, 0.5% glycerol, [α-^{32}P]ATP or [γ-^{32}P]ATP, and RuvB protein as noted. Reaction mixtures were incubated in plastic Eppendorf tubes at 30°C. The reaction was stopped by adding 5 μl of 20% trichloroacetic acid. Samples (1 μl) of the reaction mixture were put onto polyethyleneimine (PEI)-cellulose plates (Merck) and developed in 1 M formic acid–0.4 M LiCl. [^{32}P]ATP, $^{32}P_i$, and [^{32}P]ADP in the plate were identified and counted with a β-scanner (AMBIS System; Automated Microbiology Systems, Inc.).

RESULTS

Overproduction and purification of RuvB protein. In order to overproduce RuvB protein, we constructed a plasmid, pHS203 (13), which contained nucleotides 753 to 2543 (the *Aha*III-*Bgl*II fragment) of the *ruv* region in the *Hin*cII-*Bam*HI site of pUC19. In pHS203, the *ruvB* coding region is inserted in the same orientation as the *lac* promoter so that *ruvB* is transcribed from the *lac* promoter. As a qualitative test for the function of the RuvB protein encoded by the plasmid pHS203 was introduced into strain BE5036 (*ruv-9*). The plasmid restored resistance to nitrofurantoin (1.5 μg/ml) (data not shown).

The amount of RuvB protein in *E. coli* JM103 carrying pHS203 reached about 20% of total cell protein, as judged by densitometric tracing of the stained gel after incubation with IPTG for 6 h (Fig. 1A). Starting with this cell lysate, the purification of RuvB protein was monitored by SDS-PAGE (Fig. 1B). The induced lysate (Fig. 1B, lane 2) was fractionated by precipitation with Polymin P (Fig. 1B, lane 3), an additional precipitation step with 40% ammonium sulfate (Fig. 1B, lane 4), and chromatography on DEAE-Biogel (Fig. 1, lane 5) and a hydroxylapatite (Fig. 1B, lane 6) column. The last step of purification was gel filtration (Fig. 1B, lane 7). We estimated that the final fraction was about 98% RuvB protein.

The amino acid sequence from the amino terminus of the purified protein was analyzed by a gas-phase amino acid sequencer. The 20 residues from the amino terminus were Met-Ile-Glu-Ala-Asp-X-Leu-Ile-Ser-Ala-Gly-Thr-Thr-Leu-Pro-Glu-Asp-Val-Ala-Asp. The sixth residue could not be identified because the unique peak was not found on the chromatogram. This result agreed with the amino-terminal sequence predicted from the DNA sequence (2, 13) and proved that the purified protein was indeed the product of the *ruvB* gene.

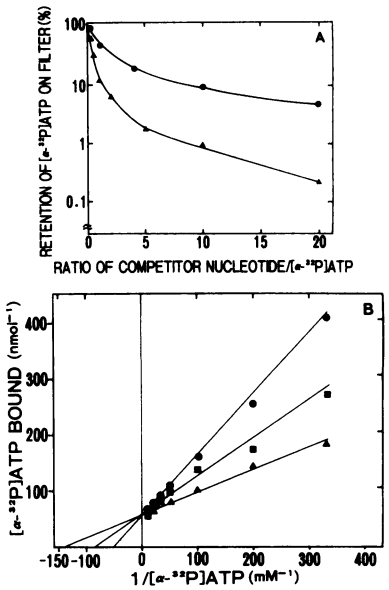

FIG. 3. Competition of ADP with ATP for binding to RuvB protein. (A) RuvB protein (20 pmol) was incubated with 50 μM [α-^{32}P]ATP (4 × 10^3 cpm/pmol) and various concentrations of unlabeled ATP (●) or ADP (▲) as a competitor. [α-^{32}P]ATP-RuvB complex was measured as described in Materials and Methods. (B) RuvB protein (20 pmol) was incubated with various concentrations of [α-^{32}P]ATP (4 × 10^3 cpm/pmol) without ADP (▲) or with 0.4 μM (■) or 1 μM (●) ADP. [α-^{32}P]ATP-RuvB complex was measured as described in Materials and Methods, and a double-reciprocal plot of the data is presented.

ATP binds to RuvB protein. We examined whether RuvB protein has ATP-binding activity, since the deduced RuvB protein sequence contains a consensus sequence for ATP-binding proteins (2, 13). When examined by binding of [α-^{32}P]ATP to RuvB protein on a nitrocellulose membrane, ATP bound to RuvB protein efficiently, with a K_d of 7.1 μM (Fig. 2). ATP binding to RuvB protein was also confirmed with [γ-^{32}P]ATP (data not shown). The number of ATP-binding sites per RuvB protein molecule was calculated to be 0.87 according to Scatchard plots (11), suggesting that 1 molecule of ATP binds to each RuvB monomer.

ADP binds to RuvB protein more effectively than ATP does. Using competition with ATP as a measure, we investigated

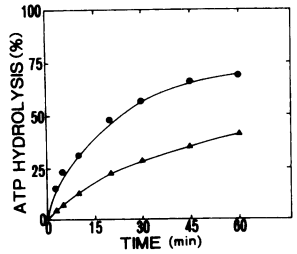

FIG. 4. Time course of ATP hydrolysis by RuvB protein. Reaction mixture (10 μl) containing RuvB protein (18 pmol) and [γ-^{32}P]ATP at 25 μM (●) or 100 μM (▲) was incubated at 30°C. At different times, 1 μl of reaction mixture was applied to a polyethyleneimine-cellulose thin-layer chromatography plate, and hydrolysis of ATP was measured as described in Materials and Methods.

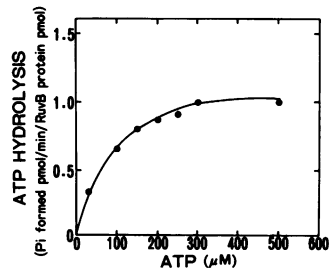

FIG. 5. ATPase activity of RuvB protein at various substrate concentrations. Reaction mixtures (10 μl) containing RuvB protein (18 pmol) and various concentrations of [γ-^{32}P]ATP were incubated at 30°C for 30 min. ATP hydrolysis was measured as described in Materials and Methods.

the nucleotide specificity for binding to RuvB protein. As shown in Table 1, ADP and dATP bound to RuvB protein efficiently, ADP about 10 times as effectively as ATP under our experimental conditions. To make sure that ADP was an effective competitor, the competition experiments were done with different concentrations of ADP (Fig. 3A). For the control, unlabeled ATP was used as a competitor. At all concentrations measured, ADP was about 10 times more effective than unlabeled ATP as a competitor. From the double-reciprocal plot of ATP binding of RuvB protein in the presence and absence of ADP (Fig. 3B), it was shown that the maximum ATP bound was unaltered, but the K_d value was increased. This result indicates a typical type of competitive inhibition. Therefore, we concluded that the ATP- and ADP-binding sites on RuvB protein are the same. The calculated K_i value for ADP was 0.65 μM. This agrees with the results of Table 1 and Fig. 3A and shows that ADP is a very effective competitor of ATP binding for RuvB protein as considered from the K_d value for ATP.

Hydrolysis of ATP to ADP and P_i by RuvB protein. Initially, we examined the ATPase activity of RuvB protein by measuring P_i complexed with molybdate with the use of unlabeled ATP and could not detect substantial ATPase activity in the protein, while the ATPase activity of RecA protein in the presence of single-stranded DNA could be assayed by this method (data not shown). Then we used a more sensitive method to measure hydrolysis of ATP by counting radioactivity of the reaction products of [^{32}P]ATP after thin-layer chromatography. The time course (Fig. 4) and the dependency on the ATP concentration (Fig. 5) of ATP hydrolysis were measured. At a lower concentration of the substrate (25 μM ATP), hydrolysis of ATP was slowed down as the time of the reaction proceeded. A similar phenomenon was observed when the ATP concentration was higher (100 μM). Although this concentration was about 15 times the K_d of ATP and should have been sufficient for the reaction to proceed, the velocity of the reaction at this concentration was not saturated (Fig. 5), and the reaction began to slow down after only 10 to 20% of the ATP was

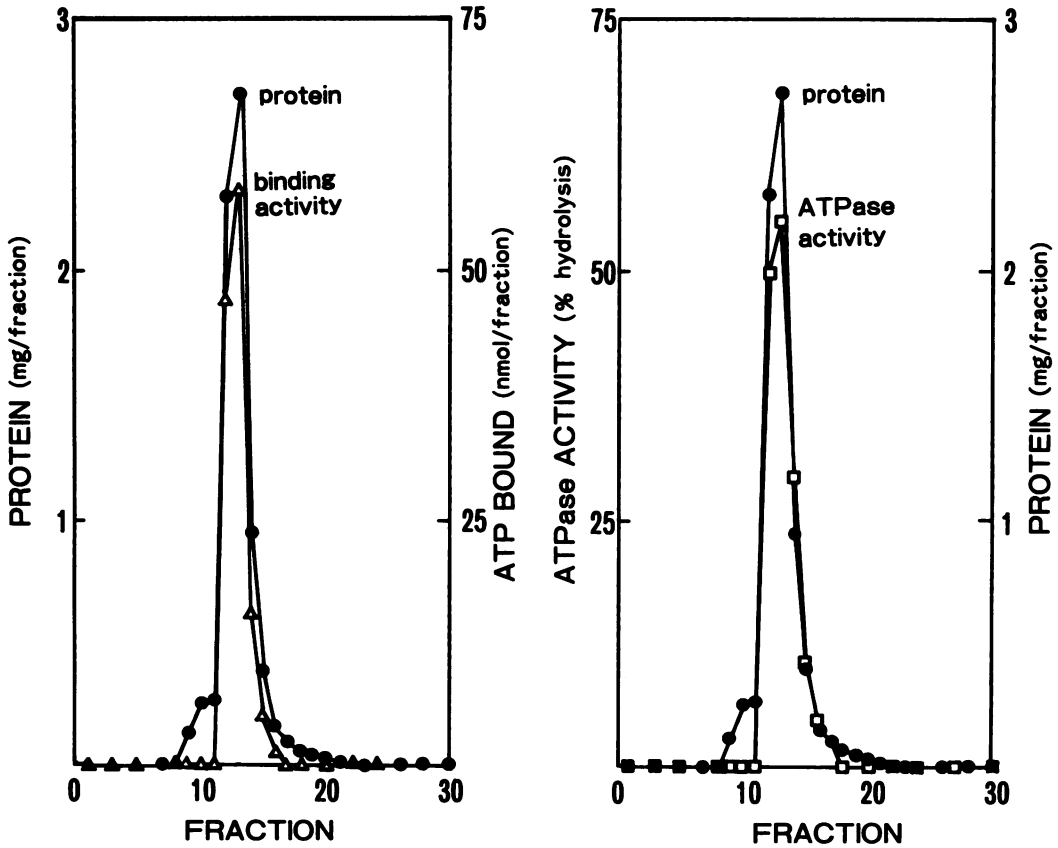

FIG. 6. Gel filtration of RuvB protein. Six milligrams of protein obtained after hydroxylapatite chromatography was applied to a Superose 12 gel filtration column, and the fractions eluted were tested for ATP-binding and ATPase activities. Standard assay conditions were used except for the ATP-binding assay, in which 5μl of each fraction was assayed, and for the ATPase activity assay, in which 1 μl of each fraction and 100 μM [γ-^{32}P]ATP were used. Symbols: ●, protein concentration; △, ATP-binding activity; □, ATPase activity.

hydrolyzed (Fig. 4). Since ADP competes with ATP for binding to RuvB protein and is produced by ATP hydrolysis, it may remain bound to the nucleotide-binding site on the protein and may inhibit ATP binding to the protein. To examine this possibility, the effects of ADP on ATPase activity were measured. After incubation of RuvB protein with unlabeled ADP (50 μM) or unlabeled ATP (50 μM) at 0°C for 5 min, [α-^{32}P]ATP (200 μM) was added, and the subsequent conversion of [α-^{32}P]ATP to [α-^{32}P]ADP was measured. The rate of [α-^{32}P]ATP hydrolysis was about 55% of the control (without addition of nucleotide) when preincubated with ADP, while it was 88% when preincubated with ATP. This result suggests that the low ATPase activity of RuvB protein is at least partly due to end product inhibition by ADP. However, the inhibition of ATPase activity by ADP was not as effective as we expected from the difference between the K_d of ATP (7.1 μM) and K_i of ADP (0.65 μM). This may be due to the difference in assay conditions for nucleotide-binding activity, which was measured at 0°C, and ATPase activity, which was measured at 30°C.

The pH dependence of the ATPase activity was measured, and the optimum was between pH 7 and 8, with a broad peak (data not shown).

ATPase activity is an intrinsic property of RuvB protein. Since the ATPase activity of RuvB protein was very low, we examined the possibility that the activity was due to a contaminating protein(s) in the RuvB protein preparation. We purified the protein extensively and tried to remove traces of impurities in the preparation (Fig. 1B). We measured ATPase activity, ATP-binding activity, and protein concentration in the peak fractions in the last step of purification, gel filtration chromatography. As shown in Fig. 6, the two activities cochromatographed with RuvB protein, and therefore we concluded that the two activities are intrinsic properties of the protein itself.

Other properties. The RuvB protein was eluted from the gel filtration column at the position expected for the monomeric size of the protein. It did not bind to either single-stranded or double-stranded DNA of bacteriophage M13 as examined by retardation of DNA mobility in agarose gel electrophoresis. It did not bind to DNA-cellulose linking either double-stranded or single-stranded DNA (Pharmacia). Incubation of RuvB protein with either double-stranded or single-stranded DNA of bacteriophage M13 did not affect the size of DNA as examined by agarose gel electrophoresis.

DISCUSSION

We have constructed a strain that greatly overproduces RuvB protein and described a simple purification procedure for the protein which allows preparation of a large amount of highly purified protein for biochemical studies. The purified protein had adenine nucleotide-binding and ATPase activities, which were expected for the protein from its possession of the consensus ATP-binding sequence (2, 13). The slow rate of ATP hydrolysis and slowing down of the hydrolysis rate in an early phase of the reaction may be partly due to the higher affinity of ADP than of ATP for RuvB protein.

RuvB protein did not bind to either single-stranded or double-stranded DNA, and the ATPase activity was not significantly affected by either form of DNA. Since genetic evidence indicates that the products of the *ruv* operon are involved in DNA repair and recombination, RuvA protein or RuvA protein complexed with RuvB protein may interact with DNA. Therefore, it would be necessary to study various conceivable activities for the Ruv proteins in their complexed form and as separate entities. We have constructed a strain that greatly overproduces RuvA protein and purified the protein (unpublished results). Our preliminary experiments suggest that the ATPase activity of RuvB protein is substantially enhanced by RuvA protein in the presence of single-stranded DNA.

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