The RecD Subunit of the RecBCD Enzyme from *Escherichia coli* Is a Single-stranded DNA-dependent ATPase*

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We have expressed the RecD subunit of the RecBCD enzyme from *Escherichia coli* as a fusion protein with a 31-amino acid NH₂-terminal extension containing 6 consecutive histidine residues (HisRecD). The overexpressed fusion protein can be purified in urea-denatured form by metal chelate affinity chromatography. The mixture of renatured HisRecD protein and the RecB and RecC proteins has a high level of ATP-dependent nuclease activity with either single- or double-stranded DNA, enhanced DNA unwinding activity, enhanced ATP hydrolysis activity in the presence of a small DNA oligomer cosubstrate, and χ-cutting activity. These are all characteristics of the RecBCD holoenzyme. The HisRecD protein by itself hydrolyzes ATP in the presence of high concentrations of single-stranded DNA (polydeoxythymidine). The activity is unstable at 37 °C, but is measurable at room temperature (about 23 °C). The HisRecD has very little ATPase activity in the presence of a much higher single-stranded DNA (oligodeoxythymidine)₁₂. HisRecD hydrolyzes ATP more efficiently than GTP and UTP, and has very little activity with CTP. We also purified a fusion protein containing a Lys to Gln mutation in the putative ATP-binding site of RecD. This mutant protein has no ATPase activity, indicating that the observed ATP hydrolysis activity is intrinsic to the RecD protein itself.

The RecBCD enzyme from *Escherichia coli* is an important enzyme in the DNA metabolism of the cell, acting in homologous recombination, resistance to UV irradiation and chemical DNA damaging agents, and degradation of foreign DNA (reviewed in Refs. 1–3). The enzyme catalyzes several reactions *in vitro*. ATP hydrolysis enables it to unwind double-stranded DNA (4–6), and the enzyme is a potent nuclease on double-stranded DNA in the presence of ATP and excess magnesium ion (7, 8). The double-strand nuclease activity of RecBCD is suppressed when it encounters a χ sequence (5’-GCTGGTGG) in the DNA (9–12). The enzyme continues to unwind the DNA past the χ sequence (11) and the unwound DNA is a substrate for recombination catalyzed by the RecA protein (13–15). RecBCD enzyme is also an ATP-stimulated nuclease with single-stranded DNA, and single-stranded DNA stimulates ATP hydrolysis (7, 8).

The first preparations of the RecBCD enzyme were thought to contain only the proteins encoded by the *recB* and *recC* genes (16–19). However, one experiment indicated the possible existence of an additional component, essential for enzymatic activity, which could be separated from both the RecB and RecC proteins by treating the enzyme with high salt concentrations (20). A later study showed that this was a third protein subunit, called RecD, encoded by a gene (recD) adjacent to the *recB* gene (21).

The function of the RecD subunit in catalysis by RecBCD enzyme is not clear, although it is implicated in several activities of the enzyme. The identification of the RecD subunit was made possible by the fact that it is required for high levels of nuclease activity with single- or double-stranded DNA (21, 22). The enzyme with a mutation in the ATP-binding site of RecD retains single-stranded DNA-dependent ATPase activity, which we concluded is catalyzed by the wild-type RecD subunit in that enzyme (23). The enzyme with a Lys to Gln mutation in the putative ATP-binding site of the RecD subunit (the RecBCD-K177Q enzyme) retained all the activities for which we tested (ATPase, nuclease, and helicase), although each was reduced in rate and other properties (25, 26). The effect of χ sequences on the nuclease activity has led to the proposal that χ causes inactivation of, and perhaps ejection of, the RecD subunit (2, 27–30). This is consistent with the fact that RecBC is a helicase but lacks significant nuclease activity (31–33), and that recombination in recD− cells is not affected by χ sequences (22).

Purification of the RecD protein has been difficult and has led to little insight into its function in the reactions catalyzed by RecBCD. No catalytic activity was found to co-purify with the RecD subunit isolated from the RecBCD holoenzyme (20, 21). The RecD protein was overexpressed and purified from inclusion bodies, and the isolated subunit restored nuclease activity and χ-specific cleavage to RecBC, but no activity was found in RecD alone (32). The difficulty of obtaining RecD protein in native form led us to prepare RecD fused to an NH₂-terminal peptide containing six consecutive histidine residues. The protein (HisRecD) can be purified by metal chelate affinity chromatography in either native or denatured form, and reconstituted with the RecB and RecC proteins to obtain high levels of ATP-dependent nuclease activity, and other activities characteristic of the RecBCD enzyme. The HisRecD protein alone catalyzes ATP hydrolysis in the presence of high concentrations of single-stranded DNA.

**EXPERIMENTAL PROCEDURES**

**Materials**

Isopropyl β-D-thiogalactopyranoside, guanidinium hydrochloride, and DTT were from U. S. Biochemical Corp.; Triton X-100 was from J. 1

1 The abbreviations used are: DTT, dithiothreitol; MOPS, 3-(N-morpholino)propanesulfonic acid; NTP, ribonucleoside diphosphate; TTP, 5′-dithiogalactopyranoside, guanidinium hydrochloride, and DTT were from U. S. Biochemical Corp.; Triton X-100 was from J.

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ATP Hydrolysis by Histidine-tagged RecD Protein

T. Baker, urea (electrophoresis grade) was from Fisher Scientific Corp.; and HEPES and imidazole were from Sigma. Ribonucleoside triphosphates (100 mM solutions), [γ-32P]ATP (3000 Ci/mmol), [α-32P]CTP, [α-32P]GTP, and [α-32P]UTP (each 800 Ci/mmol) were purchased from DuPont New England Nuclear. Oligodeoxyribonucleoside triphosphates were purchased from Pharmacia. 

Restriction enzymes, Taq DNA polymerase, and T4 DNA ligase were from Promega Corp., U. S. Biochemicals Corp., or New England Biolabs. Bovine serum albumin was from New England Biolabs. Exonuclease I was purchased from U. S. Biochemicals Corp. RecD exonuclease was a gift from Prof. Richard Kolodner, Harvard University. E. coli single-stranded DNA-binding protein was purified as described (26, 34). E. coli strain V186 (ΔrecBCD; Ref. 35) and pBR322-XH plasmid DNA (11) were gifts from Dr. Gerald Smith, Fred Hutchinson Cancer Research Center, Seattle. 12.5-Labeled ZfP75700 plasmid DNA (5001 base pairs, constructed by us for unrelated experiments) was prepared as described (25).

RecBCD, RecB, and RecC Purification—RecBCD enzyme was purified as described (36), and the concentration was determined from the absorbance at 280 nm, using ε<sub>280</sub> = 4 × 10<sup>5</sup> M<sup>-1</sup> cm<sup>-1</sup> (6). The RecB and RecC proteins were purified and quantitated, using ε<sub>280</sub> = 1.7 × 10<sup>5</sup> M<sup>-1</sup> cm<sup>-1</sup> (RecB) and ε<sub>280</sub> = 2 × 10<sup>5</sup> M<sup>-1</sup> cm<sup>-1</sup> (RecC) as described (32, 33). The concentration of each enzyme was determined under native conditions, and in the figure legends are those based on the absorbance readings, unless stated otherwise. However, each enzyme preparation apparently contained some inactive enzyme. The nuclease activity of RecBCD was maximal with about 2 enzyme molecules per DNA end (measured as in Ref. 25, with 2 and 4 nM ends). Thus the RecBCD enzyme was about 50% active. The rate of ATP hydrolysis by RecBC (prepared by mixing 1 μM RecB and 1 μM RecC) was maximal at about 4 RecBC per DNA end (measured as in Ref. 37), indicating that only about 25% of the RecB and/or RecC was active.

Construction of a Plasmid Expressing the Histagged RecD Protein (pHisRecD)—The histagged RecD protein (HisRecD) was expressed using the vector pTrcHisB (Invitrogen Corp.). The recD gene was transferred to the vector as follows. A 712-base pair fragment from the plasmid pVSnSm19, which contains the recD gene (36), was amplified by polymerase chain reaction. The downstream primer annealed across the NcoI site (bold) within the gene (primer number 1: 5'-GGGATCT-TCGGGAATG-3') and the upper primer annealed at the 5'-end of the gene (primer number 2: 5'-GGGAATCTGCGAAGACGTAATTGGGG-3'). Primer number 2 is partially complementary to the recD gene (underlined), to introduce a BglII site (bold) in the 5'-end of the gene. The amplified 712-base pair fragment was digested with BspEI and BglII and ligated into pVSn19 cleaved with the same enzymes (the BglII site in pVSn19 is within a portion of the recB gene contained in this plasmid). The recD gene was removed from this plasmid by cleavage with BglII and BamHI and ligated into pVSn19 cleaved with the same enzymes, to produce pHisRecD. The recD fragment is in the correct orientation for expression when the BglII sticky ends are joined to the compatible BamHI ends.

The resulting plasmid (pHisRecD, 6.6 kilobase pairs) encodes the RecD protein fused to a 31-amino acid leader peptide (3.4 kDa) containing six consecutive histidine residues. The protein is expressed from a tac promoter and a ribosome-binding site within the vector sequence. The RecD amino acid sequence was altered slightly during the subcloning procedure, since the first two residues in RecD, Met-Lys, are changed to a single Asp in the fusion protein. The 5′ terminus of the fusion gene was sequenced to confirm the structure of the junction between the vector and recD gene sequences.

The NH<sub>2</sub>-terminal leader peptide encoded by the vector also includes a sequence recognized by the protease enterokinase (−Asp−Asp−Asp−Asp−Asp−Asp−Lys). However, we have been unable to cleave the NH<sub>2</sub>-terminal peptide with enterokinase (Invitrogen Corp.), under a variety of reaction conditions, enterokinase concentrations, incubation times, etc. Consequently we have worked with the fusion protein itself, since it gives high levels of activity despite the additional peptide.

Construction of a Plasmid Expressing Histagged RecD-K177Q Protein (pHisRecD-K177Q)—The plasmid pVSnSm19-DK177Q, encoding a lys to Gln mutation in the putative ATP-binding site of RecD (36), was digested with BamHI, and the 442-base pair fragment containing the mutation was ligated into pHisRecD cleaved with the same enzyme. The presence of the mutation in the resulting plasmid, pHisRecD-K177Q, was also confirmed by DNA sequencing.

Methods

Purification and Renaturation of Histagged RecD Protein—The HisRecD protein was expressed in E. coli strain JM100 (recA recBCD) or V186 (ΔrecBCD) transformed with pHisRecD. Cells were grown to 25 ml of LB broth (38) at 37 °C. Isopropyl β-D-thiogalactopyranoside (1 mM) was added when the A<sub>550</sub> = 0.4, and growth was continued for 4 h. The cells were harvested and resuspended in either denaturing binding buffer (20 mM sodium phosphate, pH 7.8, 0.5 mM NaCl, 6 mM guanidinium hydrochloride) or native binding buffer (20 mM sodium phosphate, pH 7.5, 0.5 mM NaCl). For the denatured preparation, the resuspended cells were rocked gently for 10 min at room temperature, and then the lysates were cleared by centrifugation for 10 min at 17,000 × g. The lysate was applied to a 2-ml nickel-containing column (ProBond resin, Invitrogen Corp.) in denaturing binding buffer. The column was washed in 20 mM sodium phosphate, pH 6.0, 5 mM urea, 0.5 mM NaCl, and then the same mixture at pH 5.3. The HisRecD fusion protein was eluted in denaturing elution buffer (20 mM sodium phosphate, pH 4.0, 4.5 mM urea, 0.5 mM NaCl). Its concentration was determined from the absorbance at 280 nm, using ε<sub>280</sub> = 48,500 M<sup>-1</sup> cm<sup>-1</sup>, calculated for HisRecD (the NH<sub>2</sub>-terminal leader peptide contains a single tyrosine residue, and no tryptophans). The typical yield was about 0.5 mg from 25 ml of culture.

For preparation under native conditions, lysozyme (0.1 mg/ml) was added to the resuspended cells, followed by incubation for 10 min at 37 °C, 5 min on ice, and then the lysate was cleared by centrifugation as above. The lysate was applied to a 2-ml Protein G column which was washed with 20 mM sodium phosphate, pH 7.8, 0.5 mM NaCl, then with 20 mM sodium phosphate, pH 6.0, 0.5 mM NaCl, and finally the bound protein was eluted in a gradient of 50–500 mM imidazole in 20 mM sodium phosphate, pH 6.0, 0.5 mM NaCl.

The denatured HisRecD protein was renatured by first diluting with denaturing elution buffer to less than 50 μg/ml, and then dialyzing in a collagen membrane (25-kDa cut-off; Schleicher & Schuell) at 4 °C against renaturation buffer (10 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.1% Triton X-100, 0.5 mM NaCl, 20% glycerol). The denatured HisRecD protein was stable when stored at 4 °C. However, we have not found conditions under which the renatured HisRecD can be stored without substantial loss of renaturation activity. Thus, HisRecD was renatured as described.

Reconstitution of HisRecD with RecBC —The reconstitution method used for most experiments was as follows: the renatured HisRecD protein (in renaturation buffer) was mixed with RecC (in RecBCD dilution buffer: 10 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 0.1 mM dithiothreitol, 20% glycerol), and placed at room temperature for 4 h. RecB (in RecBCD dilution buffer) was then added and incubation was continued overnight at room temperature. The final reconstitution mixture usually contained 80 nM RecC and 300 nM HisRecD, in a 1:1 mixture of renaturation buffer and RecBCD dilution buffer. A few reconstitutions were done by adding HisRecD to RecB and incubating for 2–4 h at room temperature. The reconstituted RecBC(HisRecD) enzyme is relatively stable. The enzyme lost only 20% of its nuclease activity on double-stranded DNA after 2 months at −20 °C, in this solution.

Native Polyacrylamide Gel Electrophoresis—Native gel electrophoresis was carried out on 5% polyacrylamide gels (30:1, acrylamide:bis-acrylamide) prepared in imidazole/HEPES buffer (43 mM imidazole, 35 mM HEPES, pH 7.4) containing 0.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, and placed at 5.5 V/cm for 30 min before protein samples were loaded, and then the gels were run at 3.5 V/cm. The gels were silver-stained with Rapid Ag-Stain (ICN Biomedicals).

RecBCD Enzyme Assay Methods

The standard reaction conditions we used for these experiments were 43 mM imidazole, 35 mM HEPES buffer, pH 7.4, 10 mM MgCl<sub>2</sub>, 0.67 mM DTT, at 37 °C, unless indicated otherwise. The reconstituted RecBCD was compared as a 60°-fold dilution with a 1:1 mixture of renaturation buffer and RecBCD dilution buffer, and then diluted 10–100-fold further into the reaction mixtures. Thus, the components of the renaturation and reconstitution buffers were also present in the reaction mixtures. The other enzymes used in these experiments (RecBCD holoenzyme, RecBC, and HisRecD alone) were also diluted with the same buffer mixture, so that all enzymes were compared under the same reaction conditions.
Nuclease—Nuclease activity was determined by measuring the production of trichloroacetic acid-soluble oligonucleotide fragments (36), using \(^{3} \text{H}\) pTZpB700 DNA (25 \(\mu\)M nt), linearized by cleavage with PstI. Reactions with double-stranded DNA contained 50 \(\mu\)M ATP, while those with heat-denatured DNA contained 200 \(\mu\)M ATP.

Hydrolase of Ribonucleoside Triphosphates—Nuclease triphosphate hydrolase was measured by thin layer chromatography, as described (25), using \(\gamma\) or \(\alpha\)-\(^{32}\)P-labeled ribonucleoside triphosphate substrate. Polyethyleneimine chromatography plates (J. T. Baker) were developed in 1 M formic acid, 0.5 M LiCl\(_2\) for ATPase reactions, or 1 M formic acid, 1 M LiCl\(_2\) for CTP, GTP, and UTP. The relative amounts of \(\gamma\)-\(^{32}\)P-ATP and \(\alpha\)-\(^{32}\)P-P\(_i\), or \(\alpha\)-\(^{32}\)P-NTP and \(\alpha\)-\(^{32}\)P-NDP, on the developed plates were determined using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

DNA Unwinding—DNA unwinding was assayed by the conversion of \(^{3} \text{H}\) pTZpB700 DNA (21 \(\mu\)M nt), linearized with PstI, to a form sensitive to exonuclease I and Recf exonuclease, as described (37). Reaction mixtures contained 200 \(\mu\)M ATP, 10 mM MgCl\(_2\), and 8 \(\mu\)M \(E.\) coli single-stranded DNA-binding protein.

\(\chi\)-Specific Cleavage Reactions—\(\chi\)-specific cleavage reactions were done essentially as in Ref. 39 using \(p\)BR322-\(\chi\)-FH (11), containing two \(\chi\) sequences in opposite orientations. The plasmid was cleaved with ClaI, treated with calf intestinal alkaline phosphate, and 5'-end-labeled with polynucleotide kinase, and \(\gamma\)-\(^{32}\)P-ATP. Markers for \(\chi\)-specific cleavage were prepared by cleaving the labeled, ClaI-cut DNA again with AsuL.

The \(\chi\)-cutting reactions contained 20 mM MOPS-KOH, pH 7, 5 mM ATP, 3.5 mM magnesium acetate, 5 \(\mu\)M \(E.\) coli single-stranded DNA-binding protein, 1 mM DTT, and ClaI-cut [\(\gamma\)-\(^{32}\)P]pBR322-\(\chi\)-FH (3.1 \(\mu\)M DNA molecules; 26.7 \(\mu\)M nt), at 37 °C. Samples removed from the reaction mixtures were quenched by adding 0.2 volumes of a mixture containing 40% glycerol, 2.5% SDS, 0.1 M EDTA, 0.125% bromphenol blue, and analyzed by electrophoresis on 1% agarose gels. The radioactivity in the dried gel was visualized using the PhosphorImager.

RESULTS

Purification of HisRecD and Reconstitution with RecB and RecC—A protein of the expected size (slightly larger than RecD) was found in lysates of cells transformed with \(p\)HisRecD, after induction with isopropyl \(\beta\)-D-thiogalactopyranoside. We purified the histidine-tagged RecD protein (HisRecD) under both native and denaturing conditions, following the procedures recommended by Invitrogen Corp. The HisRecD could be highly purified in a single step (Fig. 1; some preparations contained a small amount of an impurity, probably the 21-kDa protein seen previously in His-tagged proteins purified from \(E.\) coli (40)).

The assay for RecD activity was the ability to restore ATP-dependent nuclease activity to RecBC, on linear single- and double-stranded DNA. The nuclease activities with both single- and double-stranded DNA (Fig. 2) are completely dependent on the presence of RecB, RecC, HisRecD protein, and ATP (not shown), as expected for RecBCD enzyme activity (8, 21, 32).

The reconstituted enzyme had a high level of activity in this assay. The activity of the reconstituted enzyme depends on: 1) the efficiency with which the three protein subunits assemble to make RecBC(\(\text{HisRecD}\)), and 2) the fraction of renatured HisRecD protein which is active. We estimated both quantities by comparing the nuclease activity of the reconstituted enzyme to that of the RecBCD holoenzyme. The nuclease activity on double-stranded DNA with RecBC(\(\text{HisRecD}\)) prepared by mixing RecB and RecC (2 \(\text{nM}\) each) with a large excess of HisRecD (45 \(\text{nM}\)), followed by 10-fold dilution into the assay, was the same as that of about 0.06–0.065 \(\text{nM}\) RecBCD in the assay (not shown). After accounting for the presence of inactive RecBCD and RecB/C protein (see “Experimental Procedures”), this suggests that about 60–65% of the RecBC could be converted to RecBC(\(\text{HisRecD}\)); 65% = (0.065/2 \(\text{nM}\) active RecBCD)/(0.2/4 \(\text{nM}\) active RecB/C) \(\times\) 100. The reconstituted enzyme used for the experiment in Fig. 2A was prepared by mixing HisRecD (290 \(\text{nM}\)) with an excess of RecB and RecC (400 \(\text{nM}\) each). The activity on double-stranded DNA, with 0.58 \(\text{nM}\) HisRecD, and 0.8 \(\text{nM}\) RecB and RecC in the assay mixture, was about the same as that of 0.17 \(\text{nM}\) RecBCD (Fig. 2A). This suggests that at least 0.17/2 /0.58 \(\times\) 100 = 15% of the HisRecD is active, or 23%, if only 65% of RecB and RecC assemble to RecBC(\(\text{HisRecD}\)).

The nuclease activity appeared when the proteins were re-
constituted at room temperature (about 23 °C), but little activity was found if the reconstitution was done on ice, under any conditions. The time required varied somewhat depending on the protein concentrations. Thus, the nuclease activity in a mixture of 3.5 nm RecBC and 45 nm HisRecD required about 2 h before reaching a maximal level. The rate of appearance of activity did not increase significantly if HisRecD was incubated overnight with either RecB or RecC and then the remaining subunit was added, as compared with adding HisRecD to RecBC. This suggests that the slow appearance of the nucleolytic activity is not due to slow assembly of HisRecD with only one of the other two proteins, since in that case overnight preincubation with that particular subunit would have allowed final assembly of the RecBC(HisRecD) complex to be rapid. (We did notice that slightly greater activity (about 2-fold) was obtained by adding the subunits sequentially (the method given under "Experimental Procedures") rather than incubating all three subunits together. Thus we used that procedure as the standard way to prepare RecBC(HisRecD) for further experiments.)

The HisRecD purified under native conditions had lower activity than the renatured HisRecD, but greater activity was obtained when this HisRecD protein was first dialyzed against renaturation buffer. This protein was also less pure after the affinity chromatography step than was the denatured HisRecD. We also purified HisRecD from V186 cells (ΔrecBCD) and obtained nuclease activity when the protein was added to RecB and RecC, indicating that the presence of the wild-type recBCD genes in JM109 did not affect the HisRecD preparation from those cells. Most further experiments were done with HisRecD purified under denaturing conditions from JM109.

We also tested several factors during renaturation for their effects on the yield of active HisRecD. Dialysis against renaturation buffer containing (NH₄)₂SO₄ (0.5 M) worked about as well as NaCl, and both gave greater activity than dialysis against the same concentrations of KCl, NH₄Cl, or potassium glutamate. Dialysis to lower the urea concentration in steps (4, 2, 1, 0 M) did not lead to significantly greater yield of activity. There was no effect of including ZnCl₂ (0.1 mM; see Ref. 32) in the dialysis buffer. Lower activity was observed when: 1) renaturation was done at room temperature rather than 4 °C; 2) renaturation buffer contained 0.2 M NaCl rather than 0.5 M NaCl; 3) the renaturation buffer was MOPS, pH 6.5, rather than Tris-HCl, 8.0.

Native Polyacrylamide Gel Electrophoresis—The nuclease activities shown above strongly indicate that the HisRecD is bound to RecBC to make a RecBC(HisRecD) complex. We sought to detect the RecBC(HisRecD) complex directly by analyzing mixtures of the proteins on nondenaturing polyacrylamide gels (32). There was a band present in the mixture of RecB, RecC, and HisRecD (lane 1, Fig. 3) which co-migrates with RecBCD (lane 3) and is presumably RecBC(HisRecD). The renatured HisRecD does not form a clear band (lane 4), indicating that it may not be a single discrete species. The HisRecD protein would also have little charge and thus low mobility in the gel (pH 7.4), since its estimated pI is about 6.8, given its content of charged residues. The RecBC(HisRecD) band was not seen in gels prepared without MgCl₂. We believe that Mg²⁺ mainly stabilizes the reconstituted enzyme in the gel, since the nucleolytic activity of the RecBCD(HisRecD) mixture was about the same whether or not MgCl₂ was present during the reconstitution (not shown).

Reconstitution of Other RecBCD Enzyme Activities

Unwinding Activity—The rate of DNA unwinding by the RecBCD enzyme, measured by the RecJ/ExoI coupled assay, is about 4-fold faster than that of the RecBC enzyme (37). Reconstitution of RecBC with HisRecD protein gave about a 2.5-fold increase in the rate of DNA unwinding in this assay (Fig. 4).

χ-Specific Cleavage—Reactions of RecBCD and RecBCD(HisRecD) with a χ-containing DNA molecule are shown in Fig. 5. Each enzyme produces a full-length single-stranded product, as well as DNA fragments which comigrate with the markers from AvaI digestion. Since the AvaI sites are only 75 nt away from χF, and 13 nt from χH, these fragments serve as markers for χ-specific cleavage. The χ-specific bands were not produced in reactions with [5-32P]pBR322, which lacks χ sites, nor by the RecBC enzyme. This experiment shows that the reconstituted RecBCD(HisRecD) enzyme is able to recognize and cleave double-stranded DNA near a χ sequence. χ-Specific cleavage has also been observed with RecBCD enzyme prepared by reconstituting RecB and RecC with native RecD protein (32).

ATP Hydrolysis Stimulated by pd(T)₁₂—I previously found (41) that RecBCD has much greater ATPase activity than does RecBC in the presence of low concentrations of pd(T)₁₂ oligomers (Kₘ for pd(T)₁₂ = 4.5 μM oligomers for RecBC versus 0.1 μM for RecBCD). We believe that the ATP hydrolysis is catalyzed by the RecB subunit in both enzymes, since the RecBCD-K177Q mutant enzyme also has a low Kₘ for pd(T)₁₂, close to that of RecBCD. The reconstituted RecBCD(HisRecD) enzyme also has much greater ATPase activity at 1.5 μM pd(T)₁₂ compared with RecBC alone (Fig. 6). This observation provides further evidence for physical association of the proteins, since we believe that it is the presence of RecD (wild-type, mutant, or, in this case, HisRecD) in the RecBCD holoenzyme, that
FIG. 5. \( \chi \)-Specific cleavage by the RecBC(HisRecD) enzyme. Reaction mixtures containing Clai-cleaved [\( ^{32} \)P]pBR322-\( \chi \)FH (1.2 nM molecules) or [\( ^{32} \)P]pBR322-\( \chi \) (1.8 nM molecules) were prepared as described under “Experimental Procedures.” Samples were removed at the times (min) indicated above the lanes, quenched, and analyzed by electrophoresis on a 1% agarose gel. Reactions contained: 26 nM RecB and 32 nM RecC (lanes 1–3); 12 nM RecB, 14 nM RecC, 12 nM HisRecD (lanes 4–6); 1 nM RecBCD (lanes 7–9) and 11–13. M, heat-denatured Clai-cut [\( ^{32} \)P]pBR322-\( \chi \)FH, cleaved again with AvaI. Numbers to the side indicate the sizes of the \( S \)-labeled fragments. ss, heat-denatured Clai-cut [\( ^{32} \)P]pBR322-\( \chi \)FH marker. Schematic below shows the structure of pBR322-\( \chi \)FH. The arrows indicate the direction from which RecBCD must approach the \( \chi \) sequence for recognition. The number of nucleotide residues from the \( \chi \) sequence to the labeled 5'-end, corresponding to the approximate sizes of the fragments produced by the enzyme, are also indicated.

stimulates the activity of RecB. The molecular basis of this effect is not known.

**ATP Hydrolysis by HisRecD Protein**

In the course of studying ATP hydrolysis by the reconstituted enzymes, we also incubated identical samples of RecBC and HisRecD separately, to be used as controls. ATP hydrolysis was then measured at 37 °C. Interestingly, the HisRecD protein alone catalyzed a small amount of ATP hydrolysis in these experiments with poly(dT) as the DNA co-substrate (Fig. 7, closed circles), but the reaction stopped in about 2 min. A second burst of ATP hydrolysis occurred if more HisRecD was added, but it also stopped in about 2 min (Fig. 7, open circles). Little ATP hydrolysis was observed if the HisRecD was diluted and placed at 37 °C for 2 min, and then ATP and other reaction components were added to initiate the reaction (Fig. 7, closed squares). There was no detectable ATP hydrolysis by HisRecD in the absence of DNA (data not shown). These experiments suggested that HisRecD is a DNA-dependent ATPase but that it is not very stable and loses activity quickly at 37 °C. Consistent with this conclusion, much greater ATP hydrolysis by HisRecD was observed when the reaction was done at room temperature (about 23 °C; Fig. 7, closed triangles). The ATP hydrolysis activity of HisRecD was not stabilized significantly at 37 °C by the presence of either bovine serum albumin or RecC (not shown; we did not add RecB, since it is itself an ATPase). The HisRecD protein must be more stable when it is assembled with RecBC, since the RecBC(HisRecD) enzyme is very active in the assays shown above, all carried out at 37 °C, and it is also more stable in long-term storage than is HisRecD itself.

These experiments provide direct evidence that HisRecD, and presumably also RecD, is a DNA-dependent ATPase. There is ample reason to believe that RecD should be an ATPase (see "Introduction"), and it is unlikely that a contaminant would copurify with the HisRecD prepared in this way. We nonetheless carried out the following control experiment to confirm that the activity is due to HisRecD itself. We prepared three cultures of JM109 cells, transformed with pHisRecD, pHisRecD-K177Q, or pTrcHisB (vector-only control). Cell growth, lysis, and metal-chelate affinity chromatography under denaturing conditions were carried out as described under “Experimental Procedures.” The fractions eluting from the columns were analyzed by SDS-polyacrylamide gel electrophoresis. HisRecD was found only in fractions from pHisRecD or pHisRecD-K177Q-containing cells, and not in the pTrcHisB control (not shown). All three protein preparations contained a small amount of an impurity (see above). Fraction number 3 from each preparation was then renatured, and either reconstituted with RecB and RecC and assayed for nuclease activity, or assayed for ATPase activity directly. Neither activity was found with the vector-only control, but addition of either HisRecD or HisRecD-K177Q to RecB and RecC gave nuclease activity (Fig. 8A). The nuclease activity was greater with RecBC reconstituted with the wild-type HisRecD than with the HisRecD-K177Q mutant, as expected based on previous results.
unknown, and probably was quite low. unit" in that work (21), but the protein concentration was
obtained under identical conditions (these conditions are not optimal for
renatured. However, RecD would be expected to have little
renatured eluate from cells containing pHisRecD-K177Q (3.2 nM His-
length: 221 nt), but much lower activity with 0.01 mM poly(dT)
substranded DNA (XhoI-cut pTZpPB700, 0.01–1 mM nt residues; data not shown). The RecB reaction rate with 1 mM poly(dT)
recognition of DNA unwinding, if the native RecD
Mg\(^{2+}\) (not shown). There was no detectable ATP hydrolysis
proteins was about the same at low Mg\(^{2+}\) but increased up to 10 mM Mg\(^{2+}\) (about 0.5 mM). Both
proteins also hydrolyzed Ca\(^{2+}\), although less efficiently than Mg\(^{2+}\) (Fig. 10).

Masterson et al. (32) found no ATP hydrolysis activity in
RecD protein purified under denaturing conditions and then
renatured. However, RecD would be expected to have little
activity in the reaction conditions apparently used in that work
(37 °C, 1.1 nM RecD, 30 µM denatured DNA), if the native RecD
protein behaves similarly to HisRecD. Lieberman and Oishi
(20) also did not find ATPase activity with RecD (the "a subunit" in that work (21)), but the protein concentration was
unknown, and probably was quite low.

Characterization of the ATP Hydrolysis Activity of
HisRecD and Comparison to RecB

We then studied some of the properties of the HisRecD
ATPase activity, and compared it to that of RecB, assayed
under identical conditions (these conditions are not optimal for
RecB, as that enzyme is somewhat more active at pH 7 than at
pH 7.4, and it is active at 37 °C). These experiments were done
to obtain comparative information as to the specificity of the
two proteins. These properties could of course change when the
subunits are in the RecBCD holoenzyme. For example, the ATP
hydrolysis activity of the isolated RecB subunit itself is much
lower than that of RecB in RecBC or the RecBCD holoenzyme
(32, 33, 41).

We initially used high concentrations of poly(dT) to test for
ATP hydrolysis catalyzed by HisRecD, based on the results of
previous experiments with the RecBCD enzyme (41). That en-
zyme exhibits ATP hydrolysis activity stimulated by poly(dT)
binding at two sites. ATP hydrolysis by RecB is stimulated by a
high affinity site, while DNA binding in a second site with
much lower affinity appeared to stimulate ATP hydrolysis by
RecD. Consistent with these observations, HisRecD has sig-
ficant ATPase activity with 1 and 0.1 mM poly(dT) (average
length: 221 nt), but much lower activity with 0.01 mM poly(dT)
and almost none with pd(T)\(_{12}\) (Fig. 9). There was also essen-
tially no ATP hydrolysis by HisRecD with linear double-
stranded DNA (XhoI-cut pTZpPB700, 0.01–1 mM nt residues;
data not shown). The RecB reaction rate with 1 mM poly(dT)
was about 30–40-fold greater than that of HisRecD, under the
reaction conditions of Fig. 9. The RecB-catalyzed reaction rate
was about the same with pd(T)\(_{12}\) and poly(dT), each at 1 mM
nucleotide residues (not shown). Thus ATP hydrolysis by RecB
was more than 1400-fold faster than that by HisRecD with
pd(T)\(_{12}\) as the DNA co-substrate.

The ATP hydrolysis activities of HisRecD and RecB depend
rather differently on the Mg\(^{2+}\) concentration (Fig. 10). HisRecD
was maximal at about 2 mM Mg\(^{2+}\), and then decreased slightly
at still higher concentrations (Fig. 10A). The activity of RecB
increased up to 10 mM Mg\(^{2+}\), the highest concentration we
tested (Fig. 10B). Thus, the ATP hydrolysis activity of the two
proteins was about the same at low Mg\(^{2+}\) (about 0.5 mM). Both
proteins also hydrolyzed Ca\(^{2+}\), although less efficiently than
Mg\(^{2+}\) (not shown). There was no detectable ATP hydrolysis
by HisRecD in the absence of DNA, with 2 mM Mg\(^{2+}\), under the
conditions of Fig. 10 (data not shown). The Mg\(^{2+}\) concentration
has significant effects on the activity of RecBCD, particularly
the relative levels of nuclease and helicase activity, and the
levels of \(\chi\)-specific versus nonspecific cutting (15, 39, 42).
We note that RecBCD can unwind DNA when ATP is in excess over
the Mg\(^{2+}\) (5, 43), conditions where neither subunit alone has
much ATP hydrolysis activity.

HisRecD hydrolyzes ATP most efficiently, with lower activity
with GTP and UTP, and almost none with CTP (Fig. 11A). ATP
is also the best substrate for RecB under these conditions, but
CTP, GTP, and UTP are hydrolyzed at a rate 35–50% that of

![Fig. 8. Enzymatic activity with His-tagged proteins purified from cells containing pTrcHisB, pHisRecD, and pHisRecD-K177Q. JM109 cells were grown, lysed, and the proteins which bound to and were eluted from a nickel column under denaturing conditions were prepared as described under "Experimental Procedures." A, renatured (dialyzed) protein mixture was reconstituted with RecB and RecC and analyzed for nuclease activity on double-stranded DNA. ● 0.2 nM RecB, 0.2 nM RecC + renatured nickel affinity column eluate from cells containing pHisRecD (2.8 nM HisRecD); ■ 0.2 nM RecB, 0.2 nM RecC + renatured eluate from cells containing pHisRecD-K177Q (3.2 nM His-RecD-K177Q); ▲ 0.2 nM RecB, 0.2 nM RecC + renatured eluate from cells containing pTrcHisB (vector-only control). B, ATP hydrolysis with poly(dT) was measured at about 23 °C as in Fig. 7. ● pHisRecD eluate (40 nM HisRecD); ■ pHisRecD-K177Q eluate (46 nM HisRecD-K177Q); ▲ vector-only control eluate.](http://www.jbc.org/content/10077/25/10077/F1.large.jpg)
ATP Hydrolysis by Histidine-tagged RecD Protein

**DISCUSSION**

The reconstituted RecBCD(HisRecD) enzyme has high levels of all the activities of RecBCD. The levels of activity show that the presence of the 31-residue amino-terminal peptide has little effect on the ability of the fusion protein to reconstitute with the other subunits and to influence the activity of the other subunits. We cannot be certain as to how the peptide might affect the activity and properties of RecD itself. In any case, the availability of this protein and the results we have so far will aid in further study of the function of RecD in RecBCD enzyme activity. HisRecD will also be a useful material for studying the RecD protein by itself, and this subunit reconstituted with the wild-type and with other mutant subunits.

The RecD subunit has been implicated to some degree in all activities of the RecBCD enzyme (ATP hydrolysis, helicase, nuclease, and the response to μ sequences). Indeed, it is difficult to assign separate functions to the subunits of the enzyme. Instead it appears that all three participate in the nuclelease reaction on single- and double-stranded DNA, and in unwinding double-stranded DNA. An exception is that RecB alone has some helicase activity, but it is much slower than that of RecBCD (6, 44).

The observation of ATP hydrolysis by HisRecD, and its properties, support the conclusion that RecD hydrolyzes ATP during the reaction catalyzed by RecBCD on double-stranded DNA (37). The specificity of HisRecD for single-stranded DNA is consistent with previous conclusions that RecD interacts with the partially unwound DNA produced by the helicase activity driven by ATP hydrolysis by RecB (24, 45, 46). The requirement of HisRecD for high poly(dT) concentrations may result from having isolated the subunit from its partners, since the local concentration of single-stranded DNA at the unwinding fork made by RecBCD would be relatively high, even at low total DNA concentration.

The nucleotide specificities of the two ATPase subunits are interesting in light of previous studies of the RecBCD enzyme. Wright et al. (7) observed double-strand nuclelease activity by RecBCD with all eight ribo- and deoxyribonucleoside triphosphates. The activity showed sigmoidal dependence on the nucleotide concentration, and the minimum concentration required for activity went in the order ATP < GTP < UTP < CTP.

We found later that the RecBCD-K177Q mutant enzyme required a slightly higher ATP concentration than did RecBCD before double-strand nuclease activity was observed (25), as did RecBC for detectable DNA unwinding (37). We interpreted these observations to indicate that ATP hydrolysis by both RecB and RecD, in RecBCD, contributes to the DNA unwinding and nuclease reactions, at low ATP concentrations. ATP hydrolysis by RecB alone is sufficient for these reactions (in either RecBC or RecBCD-K177Q), but a higher ATP concentration is required. The nucleotide specificity of HisRecD together with the observations of Wright et al. (7) are consistent with this earlier interpretation that ATP hydrolysis by both subunits contributes to the reaction (unwinding and/or nuclease) with double-stranded DNA.

ATP hydrolysis by RecD can have no essential role in RecBCD helicase activity, since RecB is a helicase (31, 32, 33). The protein may function to accelerate translocation and enhance processivity during unwinding, since RecBC and RecBCD-K177Q are slower and less processive than RecBCD (26, 33, 37). The dependence of ATP hydrolysis rate on DNA length can be diagnostic of a mechanism where ATP hydrolysis is coupled to movement along the DNA (47). Although further study of the kinetics of ATP hydrolysis by HisRecD (or RecD itself) is clearly necessary, our observation that poly(dT) stimulates ATP hydrolysis much more effectively than pd(T)_12 is

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*H.-W. Chen, D. E. Randle, and D. A. Julin, submitted for publication.*
consistent with this proposed function for RecD. Finally, although ATP is indispensable for helicase activity, it cannot be ruled out that ATP hydrolysis by RecD can be coupled to DNA unwinding. A possible mechanism is that RecB must begin to unwind the DNA, but ATP hydrolysis by RecD could also contribute to DNA unwinding once the process has begun.

Finally, RecD is required for the high nuclease activity of RecBCD with single- and double-stranded DNA (20, 21, 32). Several recent experiments indicate that RecD can suppress the effect of a χ sequence in one DNA molecule under some conditions, since a χ sequence in one DNA molecule can lead to protection of a non-χ-containing DNA in vitro (9) and in vivo (12, 29, 30). Overexpression of RecD protein in vivo and in vivo (12, 29, 30). Overexpression of RecD protein in vivo and in vivo and in vivo.

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