A DNA Helicase from Schizosaccharomyces pombe Stimulated by Single-stranded DNA-binding Protein at Low ATP Concentration*

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A DNA helicase named DNA helicase I was isolated from cell-free extracts of the fission yeast Schizosaccharomyces pombe. Both DNA helicase and single-stranded DNA-dependent ATPase activities copurified with a polypeptide of 95 kDa on an SDS-polyacrylamide gel. The helicase possessed a sedimentation coefficient of 6.0 S and a Stokes radius of 44.8 Å determined by glycerol gradient centrifugation and gel filtration analysis, respectively. From these data the native molecular mass was calculated to be 110 kDa, indicating that the active enzyme is a monomer. The DNA-unwinding and ATP hydrolysis activities associated with DNA helicase I have been examined. One notable property of the enzyme was its relatively high rate of ATP turnover (35–50 molecules of ATP hydrolyzed/ enzyme molecule) that may contribute to its inefficient unwinding activity at low concentrations of ATP (<0.2 mM). Addition of an ATP-regenerating system to the reaction mixture restored the DNA-unwinding activity of the enzyme. S. pombe single-stranded DNA-binding protein (SpSSB, also called SpRPA) stimulated the DNA helicase activity significantly at low levels of ATP (0.025–0.2 mM) even in the absence of an ATP-regenerating system. In contrast, SpRPA had no effect on ATP hydrolysis at any ATP concentration examined. These observations suggest that the stimulation of DNA unwinding by SpRPA is not simply a result of suppression of nonproductive ATP hydrolysis. Rather, the role of SpRPA is to lower the $K_m$ for ATP in the unwinding reaction, allowing the helicase to function efficiently at low ATP concentrations.

The unwinding of double-stranded DNA is required to provide single-stranded DNA (ssDNA) templates for DNA transactions such as those involved in recombination, repair, and replication. For this purpose, DNA helicases catalyze a DNA strand separation reaction by breaking hydrogen bonds that hold the DNA strands together. DNA helicases use energy derived from the hydrolysis of nucleoside triphosphates for bond breakage and translocation of the enzyme along the DNA duplex. The DNA-unwinding activity catalyzed by a helicase exhibits a specific directionality ($5' \rightarrow 3'$ or $3' \rightarrow 5'$) with respect to the DNA strand to which the enzyme is bound and moves.

DNA helicases are ubiquitous and have been isolated from a wide variety of sources from bacteria to humans as well as bacteriophages and animal viruses. The biochemical properties of a number of DNA helicases have been reviewed extensively (1–5). Since detection of the first DNA helicase (Escherichia coli DNA helicase I) (6), more than 60 distinct DNA helicase activities have been described (3, 4). Most organisms contain multiple helicases; for example, at least 12 different DNA helicases have been identified in E. coli (7). Eight distinct helicases have been identified in Saccharomyces cerevisiae (8) and more than 15 from various types of mammalian cells, including those from murine, bovine, and human (2, 4, 9–11, 30, 47, 48). Amino acid sequence comparisons have revealed that many DNA and RNA helicases contain 7 distinct conserved motifs (3, 12). The completed S. cerevisiae genome data base (13) revealed more than 80 open reading frames with such helicase motifs. Thus the number of identified DNA helicases should continue to grow as the proteins encoded by these open reading frames are studied biochemically.

The presence of such a large number of DNA helicases in individual cells probably reflects the variety and complexity of DNA metabolic reactions and the distinct structural template requirements for a given DNA helicase. The precise in vivo roles of specific helicases are only well understood when both genetic and biochemical approaches are available. The most well characterized helicases are prokaryotic and eukaryotic viral replicative helicases, including DnaB (14), PriA (15), T7 gene 4 (16), T4 gene 41 (17), SV40 T antigen (18, 19), BPV E1 (20, 21), and HSV-1 UL8/52 (22, 23). In addition, several gene products involved in nucleotide excision repair and basal transcription in mammalian cells have been shown to be DNA helicases (ERCC2 and ERCC3, two essential subunits of transcription factor TFIH) (24, 25). A common feature of the well characterized helicases is that each is a critical component of a multiprotein complex and thus, requires distinct protein-protein interactions to carry out its specific function. For example, the DNA helicase RuvB, in concert with another protein RuvA, is involved in branch migration of DNA crossovers. RuvA provides DNA binding specificity for RuvB by guiding it to Holliday junctions and is required for activation of RuvB helicase activity (26). Another example is the DnaB protein, the replicative helicase from E. coli. DnaB interacts with the γ subunit of the DNA polymerase III holoenzyme and coordinates the helicase activity of DnaB with other proteins engaged in replication fork movement (27, 28).

In previous studies, we described the isolation of two DNA helicases from human cells that were markedly stimulated by

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1 The abbreviations used are: ssDNA, single-stranded DNA; SSB, ssDNA-binding protein; RPA, replication protein A; SpRPA, S. pombe RPA; FPLC, fast performance liquid chromatography; ss, single-stranded circular; DTT, dithiothreitol; BSA, bovine serum albumin; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; ATP-S, adenosine 5’-3-O-thiotriphosphate; AppNp, 5’-adenylyl imidodiphosphate; AppCp, adenosine 5’-(β,γ-methylene)triphosphate; NEM, N-ethylmaleimide.
DNA Helicase Assay—The reaction mixture (20 μl) contained 25 mM Tris-HCl (pH 7.8), 2 mM MgCl₂, 2 mM DTT, 2 mM ATP, 0.25 mg/ml BSA, and the 3'-25P-labeled partial duplex DNA substrate (15 fmol). Reactions were incubated at 37 °C for 10 min throughout the purification procedure and 15 min in experiments concerned with the characterization of the DNA helicase activity. The reaction products were stoped with 4 μl of 6 x stop solution (60 mM EDTA (pH 8.0), 40% (w/v) sucrose, 0.6% SDS, 0.25% bromphenol blue, and 0.25% xylene cyanole). The reaction products were subjected to electrophoresis for 1.5 h at 150 V through 10% polyacrylamide gels containing 0.1% SDS in 0.5 x TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). The gel was dried on DEAE paper and radioographed. Labeled DNA products were quantitated with the use of a PhosphorImager. The background level detected in the absence of added DNA helicase was less than 2% of the input substrate, and this value was subtracted from the amount of displaced products formed in the presence of the DNA helicase.

Preparation of DNA Helicase—All purification steps were carried out at 0–4 °C. S. pombe strain 972 (150 liters) was grown to 30 °C until it reached the midexponential phase. The cells were harvested (1 kg of wet cells) and resuspended in 1 liter of suspension buffer containing 0.05 M Hepes-NaOH (pH 7.5), 0.1 mM 1 mM DTT, 0.1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1.5 μg/ml leupeptin, and 3 μg/ml peptatin A. The crude extract was prepared by processing the cell suspension with the Bead Beater-16. A large portion of RdRp and RdRp-like activities was removed. After centrifugation at 37,000 rpm in a Beckman 50.2TI rotor, the supernatant (crude extract; 16.5 mg/ml, 1.890 ml) was fractionated with ammonium sulfate. Purified ammonium sulfate (11.3 g/100 ml) was added slowly to the cleared supernatant while stirring to 20% saturation. The supernatant was collected by centrifugation at 12,000 rpm for 15 min in a Sorvall F-16/250 rotor. Ammonium sulfate (18.8 g/100 ml) was added to the supernatant to 50% saturation. The precipitate was collected by centrifugation at 14,000 rpm for 30 min, dissolved in 450 ml of buffer T (25 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM DTT, 100 μg/ml antipain, and 200 μg/ml leupeptin, dialyzed for 14 h against 5 liters of buffer T, and adjusted to 100 mM NaCl. The dialysates were centrifuged at 9,000 rpm in a Sorvall F-16/250 for 15 min to remove any insoluble material. The supernatant (24.1 mg/ml, 650 ml) was directly loaded onto a heparin-Sepharose (Pharmacia) column (2.5 x 27 cm, 133 ml) equilibrated with buffer T containing 100 mM NaCl, and the column was washed extensively with 10 column volumes of buffer T plus 100 mM NaCl. Proteins were eluted with a 1.2-linear gradient of 100–700 mM NaCl in buffer T. The major helicase activity, which eluted at 210 mM NaCl, was pooled, dialyzed against 3 liters of buffer T for 4 h, and adjusted to 50 mM NaCl.

The dialyzed fraction (2.1 mg/ml, 224 ml) was loaded onto a Q-Sepharose (Pharmacia) column (2.5 x 10.5 cm, 52 ml) column equilibrated with buffer T containing 50 mM NaCl. The column was washed with 70 ml of buffer T containing 50 mM NaCl and eluted with a 600-ml linear gradient of 50–500 mM NaCl in buffer T. The DNA helicase activity, which eluted at 200 mM NaCl, was pooled and dialyzed against 60 ml of buffer H (25 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM DTT, 100 μg/ml antipain, and 200 μg/ml leupeptin, plus an additional 20% glycerol (v/v)). The dialyzed fraction (2.7 mg/ml, 60 ml) was adjusted to 50 mM NaCl and loaded onto a Bio-Rex 70 (Bio-rad) (1.5 x 8.5 cm, 15 ml) column equilibrated with buffer H containing 50 mM NaCl. The column was washed with buffer H (80 ml) containing 50 mM NaCl and eluted with a 150 ml linear gradient of 50–1000 mM NaCl in buffer H. The peak helicase fractions (1.7 mg/ml, 35 ml), which eluted at 150 mM NaCl, were loaded directly without dialysis on to a ssDNA-cellulose (1 x 1.3 cm, 1 ml) column equilibrated with buffer T containing 150 mM NaCl. The column was washed successively with 5 ml of buffer T plus 250 mM NaCl and 15 ml of buffer T plus 700 mM NaCl. The latter fractions, which contained the majority of the DNA helicase activity, were concentrated 10-fold with the Ultrafree-15 centrifugal filter (Milipore Corp.) as recommended by the manufacturer and diluted to 70 mM NaCl prior to loading onto a Resource Q (Pharmacia) column equilibrated with buffer T containing 70 mM NaCl. The column was eluted with an 18-ml linear gradient of 70–750 mM NaCl in buffer T. The peak helicase fraction (25 ml) from the Resource Q column was purified by gel filtration gradient centrifugation (5 ml at 5–25% in buffer T plus 150 mM NaCl. After centrifugation at 30,000 rpm for 24 h a Beckman SW 55.1 rotor, fractions (200 ml) were collected from the top of the gradient and assayed for DNA helicase and ATPase activities. The active fractions (Figs. 2A; fractions 11–15) that contained >90% of total activity were pooled, supplemented with BSA (final concentration of 1 mg/ml) to increase their stability, and stored at −70 °C (glycerol gradient pool I fraction). The side fractions (Fig. 2A, 18-31).
Purification of DNA Helicase I—To detect DNA helicase activities in crude extracts prepared from S. pombe, we used a DNA helicase assay developed by Matson et al. (16) and Venkatesan et al. (17). This assay employs a partial duplex DNA as the substrate in which a short oligonucleotide radiolabeled at the 3'-end is annealed to bacteriophage φX174 sscDNA. DNA helicase activity is measured by displacement of the labeled oligonucleotide from the sscDNA.

The first step toward purification of the DNA helicase activity consisted of ammonium sulfate precipitation of proteins from whole cell extracts, and the subsequent steps are summarized in Table I. The DNA helicase activity could not be measured reliably in crude and ammonium sulfate fractions because of the presence of nucleases that degraded the DNA substrates; thus it was difficult to calculate both the -fold purification and yield of the enzyme achieved by these purification steps (Table I). The nuclease activity was progressively separated from the DNA helicase activity at each purification step. The final fraction (glycerol gradient centrifugation) had no detectable nuclease activity.

The majority of detectable helicase activity (>90%) was present in the 20–50% ammonium sulfate fraction (data not shown). Surprisingly, a single major DNA helicase activity that eluted from the first column chromatographic step (heparin-Sepharose, data not shown) was not further resolved into multiple helicase peaks throughout the entire purification procedure. However, a substantial amount of the total helicase activity (>60%) did not bind to the heparin-Sepharose column (data not shown). When the flow-through fraction was reloaded onto a freshly prepared heparin-Sepharose column, no helicase activity was retained (data not shown). It is not clear whether the helicase activity in the flow-through fraction was the same activity retained by the column.

Major purification was achieved during the ssDNA-cellulose chromatographic step, which removed more than 99% of the contaminating proteins (Table I). The DNA helicase activity is unstable at low protein concentrations, which may account for the low recovery (<20%) from the ssDNA-cellulose step (Table I). Analyses of fractions obtained from the Resource Q column chromatography in FPLC (Pharmacia) showed that the observed helicase activity was proportional to the intensity of a 95-kDa polypeptide band (Fig. 1), although several other polypeptides were detected in the same fractions. When DNA-dependent ATPase activity was examined with these fractions, the 95-kDa polypeptide also coincided with the ATPase activity (Fig. 1B).

To confirm that the 95-kDa polypeptide was responsible for DNA helicase activity, the peak fraction obtained from the Resource Q FPLC step (Fig. 1A, fraction 25) was further purified by glycerol gradient centrifugation. SDS-polyacrylamide gel electrophoresis (PAGE) of the resulting glycerol gradient fractions revealed the presence of a 95-kDa polypeptide that comigrated with both helicase and DNA-dependent ATPase activities (Fig. 2). We also detected a 95-kDa polypeptide using different purification procedures (data not shown).

The two major contaminating polypeptides (marked with asterisks in Fig. 2A) were 66 and 32 kDa in size and sedimented as a complex (Fig. 2A, fractions 12–16). These proteins were similar in size to the large and middle subunits of SpRPA. For this reason, we examined whether they were subunits of SpRPA. Immunoblot Western analysis with polyclonal antibodies raised against purified SpRPA demonstrated that the 66- and 32-kDa proteins were indeed SpRPA subunits (data not shown). It is not clear whether copurification of the SpRPA subunits and the DNA helicase activity was the result of protein-protein interactions or due to their similar behavior in column chromatographic steps.

In summary, the purification procedure for the isolation of DNA helicase I yielded a 390-fold increase in DNA helicase specific activity over that present in the heparin-Sepharose pooled fractions (Table I). Assuming that the glycerol gradient pool I fraction (Fig. 2A, fractions 11–15) was approximately 50% pure, the total amount of DNA helicase I obtained was 31 μg.

Hydrodynamic Properties of DNA Helicase I—The hydrodynamic properties of DNA helicase I were examined by determining the sedimentation coefficient and Stokes radius of the protein. Glycerol gradient and gel filtration analyses using Sephacryl S-200 yielded a sedimentation coefficient of 6.0 S (Fig. 2B) and a Stokes radius of 44.8 Å (data not shown), respectively. Assuming a partial specific volume of 0.725 ml/g, DNA helicase I was calculated to have a native molecular mass of 110 kDa and frictional ratio of 1.36 (44). The slight discrepancy in size determined by SDS-PAGE and the calculated value based on hydrodynamic properties may reflect the influence of SpRPA on the sedimentation or elution profile of DNA helicase.

### Table I

| Fraction       | Volume | Concentration | Protein | Total | Specific activity |
|----------------|--------|---------------|---------|-------|------------------|
| Crude extracts | 1,890  | 16.5          | 31,000  | 564   | 1.2              |
| 20–50% amm. sulfate | 650 | 24.1          | 15,700  | 564   | 1.2              |
| Heparin-Sepharose | 224 | 2.1           | 470     | 564   | 1.2              |
| Q-Sepharose     | 60    | 2.7           | 162     | 356   | 2.2              |
| Bio-Rex 70      | 35    | 1.7           | 59.5    | 286   | 4.8              |
| ssDNA-cellulose | 7    | 0.043         | 0.304   | 44    | 146              |
| Resource Q (FPLC) | 3.3 | 0.048         | 0.159   | 28    | 176              |
| Glycerol gradient* | 1.2 | 0.026*        | 0.031   | 15    | 470              |

*One unit of DNA helicase activity is defined as the amount of enzyme required to displace 1 pmol of the duplex DNA substrate after a 10-min incubation period at 37 °C in the standard assay mixture containing 15 fmol of partial duplex φX174 sscDNA with a 20-base oligonucleotide annealed to it.

*In this step, fraction 25 of Resource Q, which contained the peak of helicase activity, was used, and the results were calculated assuming that the entire Resource Q fraction was used in the glycerol gradient step.

*Protein concentration was estimated on the basis of silver staining using the Resource Q fraction as the protein standard.
Helicase activity was assayed in reaction mixtures (20 mM (Panel B) kDa). The load (min (45 kDa), carbonic anhydrase (31 kDa), and trypsin inhibitor (21.5 kDa) are marked by an asterisk). Desorption of the 95-kDa polypeptide is described (55), and the gel was silver-stained. The 95-kDa polypeptide is marked by an arrow. The protein molecular size markers (MW marker) used were: phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and trypsin inhibitor (21.5 kDa). The load (Load, the ssDNA cellulose fraction), flow-through (FT), and Resource Q fractions analyzed are indicated at the top of the figure. Panel B, DNA helicase activity (●) and DNA-dependent ATPase activity (○) were measured in fractions from the Resource Q FPLC column. Helicase activity was assayed in reaction mixtures (20 μl) that contained 0.5 μl of each fraction with 15 fmol of substrate that consisted of the 20-mer oligonucleotide annealed to 4X174 ssDNA under the conditions described under “Materials and Methods.” ATPase activity was measured in standard reaction mixtures (20 μl) that contained 0.5 μl of each fraction and 0.5 mM ATP as detailed under “Materials and Methods.”

I. These data suggest that DNA helicase I exists as an asymmetric, monomeric 95-kDa polypeptide.

**Enzyme Titration and Kinetics**—The characterization of DNA helicase I was carried out using a 20-mer oligonucleotide annealed to 4X174 ssDNA as substrate, unless otherwise indicated. Under the standard assay conditions as described under “Materials and Methods,” the oligonucleotide displacement reaction was proportional to the amount of helicase protein added. At saturating amounts of protein (>35 ng), more than 70% of the duplex fragments were displaced (Fig. 3, A and B). Displacement reactions carried out in the presence of less than 20 ng showed a sigmoidal curve. This suggests either a cooperative interaction between monomers or an induction of multimerization of DNA helicase I in the presence of ssDNA and/or ATP similar to that observed with the Rep helicase and SV40 T antigen (45, 46).

When kinetic analysis was carried out in the presence of 17 ng of DNA helicase I, the displacement reaction was rapid without an apparent lag period. The reaction proceeded linearly up to 5 min and then plateaued at 10 min after 40% of the DNA substrate had been unwound (Fig. 3, C and D).

**Directionality of Unwinding**—To determine the direction of DNA unwinding by DNA helicase I, a substrate was prepared that consisted of a linear 4X174 ssDNA containing a 23-nucleotide fragment at the 5′-end and a 29-nucleotide fragment at the 3′-end (Fig. 4A). Both fragments were labeled as described under “Materials and Methods.” If the helicase unwinds duplex DNA in a 5′ to 3′ direction with respect to single-stranded DNA (a 5′ → 3′ helicase), the 29-nucleotide fragment would be released. However, if the enzyme unwinds duplex DNA in a 3′ to 5′ direction (a 3′ → 5′ helicase), the 23-nucleotide fragment would be released. As shown in Fig. 4B, the
23-nucleotide fragment was preferentially displaced, whereas the 29-mer remained associated with the duplex substrate. The release of the fragment was dependent upon the presence of ATP (Fig. 4, lane 3). This demonstrates that DNA helicase I translocates along single-stranded DNA in a 3' to 5' direction.

Unwinding of Various Sizes of Partial Duplex DNA by DNA Helicase I—Because the helicase was capable of unwinding a 52-base pair (bp) DNA duplex as efficiently as a 20-bp DNA duplex (data not shown), we investigated the ability of DNA helicase I to unwind DNA fragments longer than 52 bp. Such DNA substrates were prepared by extending singly primed φX174 ssDNA with Klenow fragment as described under "Materials and Methods." The length of duplex fragments in the substrate ranged from 25 to 250 nucleotides (Fig. 5, lane 2). DNA helicase I displaced fragments up to 150 nucleotides in length, although the displacement reaction became less efficient as the length of the duplex increased (Fig. 5, lanes 5 and 6). This unwinding activity also depended on the presence of ATP (Fig. 5, lane 4).

Other Requirements for DNA Helicase Activity—As shown in Fig. 3 and Table II, the displacement of duplex DNA catalyzed by DNA helicase I required MgCl₂ and a hydrolyzable ATP derivative. Non-hydrolyzable ATP analogs such as ATPγS, AppNp, and AppCp did not substitute for ATP, which suggests that ATP hydrolysis is required for the unwinding activity of DNA helicase I. ADP did not substitute for ATP and did not inhibit unwinding when added at concentrations up to 1 mM (Table II). The divalent cation Mn⁺ completely substituted for Mg⁺, whereas Ca⁺ was only partially (27%) active (Table II). DNA helicase I activity was reduced 28% by 25 mM NaCl and inhibited completely (>95%) at 200 mM NaCl. The helicase activity was sensitive to NEM when incubated at 37 °C, suggesting that a sulfhydryl group(s) may be important in its function.

Nucleotide requirements for the helicase activity were also determined. Helicase activity was supported by dATP to the same extent as ATP at a concentration of 2 mM (Table II). Both GTP and dGTP substituted substantially for ATP (29 and 73%; 26 and 65%, respectively) at low (0.5 mM) and high (2 mM) concentrations, whereas other nucleotides were poorly active (<10%) (Table II). An exception was dCTP, which partially supported the unwinding activity only at high concentrations.

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**Fig. 3.** Influence of the addition of DNA helicase I on the oligonucleotide displacement reaction and kinetic analysis of the DNA helicase reaction. Panel A, the standard helicase assay was performed as described under "Materials and Methods" with the omissions indicated above the lanes (−ATP, −MgCl₂). The amount of DNA helicase I added (ng) is noted at the top of each lane. Panel B, quantitation of helicase activity in panel A. Panel C, helicase activity was measured as described under "Materials and Methods." The reaction mixture (180 μl) contained 153 ng of DNA helicase I; aliquots (20-μl) were withdrawn at the indicated times and subjected to nondenaturating polyacrylamide gel electrophoresis. The boiled enzyme control is indicated above the appropriate lane. Panel D, quantitation of results in panel C.

**Fig. 4.** Directionality of DNA helicase I unwinding of duplex DNA. Panel A, schematic structure of the linear partial duplex substrate as described under "Materials and Methods." Asterisks indicate radioisotopic labels at each 3'-end. Displacement of the 29-mer oligonucleotide from this substrate is indicative of 5' → 3' translocation, whereas the displacement of the 23-mer oligonucleotide is diagnostic of 3' → 5' translocation. Panel B, helicase activity was measured in the absence and presence of ATP with 11 ng of DNA helicase I and 15 fmol of the linear partial duplex substrate shown in panel A under standard reaction conditions described under "Materials and Methods." The reaction products were resolved by electrophoresis through a 15% nondenaturing polyacrylamide gel.

23-nucleotide fragment was preferentially displaced, whereas the 29-mer remained associated with the duplex substrate. The release of the fragment was dependent upon the presence of ATP (Fig. 4, lane 3). This demonstrates that DNA helicase I translocates along single-stranded DNA in a 3' to 5' direction.
Characterization of the DNA-dependent ATPase Activity—As shown in Figs. 1 and 2, both DNA helicase and DNA-dependent ATPase activities were detected coincidentally during chromatographic separation from the Resource Q column and on glycerol gradient centrifugation. The DNA-dependent ATPase activity was further characterized using the standard reaction conditions described under “Materials and Methods” (Table III). As observed in the DNA helicase assay, ATP hydrolysis required MgCl₂ and was completely inhibited by NEM (10 mM) (Fig. 6B). The complete reaction contained 15 fmol of partial duplex ssDNA substrate containing a 20-base oligonucleotide and 11 ng of DNA helicase I in the standard reaction mixture described under “Materials and Methods.”

The enzyme was first incubated for 10 min at 37 °C in the presence of the indicated amount of NEM.

**TABLE II**

| Requirements for DNA helicase I activity |
|----------------------------------------|
| Additions or omissions | Amount added | Relative activity% |
| Completeb | 100 |
| Add NaCl | 0.025, 0.05, 0.1, 0.2 mM | 72, 39, 18, 2 |
| Add ADP | 0.5 or 1 mM | 100 |
| Omit MgCl₂ | <1 |
| Omit ATP | 108 |
| Omit DTT + NEMc | 10 mM | <1 |
| Omit ADP | <1 |
| Omit GTP | 2 mM | <1 |
| Omit CTP | 2 mM | <1 |
| Omit UTP | 2 mM | <1 |
| Omit dGTP | 2 mM | <1 |
| Omit dATP | 2 mM | <1 |
| Omit dTTP | 2 mM | <1 |

**TABLE III**

| Properties of the NTPase activity of DNA helicase I |
|---------------------------------|
| Additions or omissions | Amount added | Relative activity% |
| Completeb | 100 |
| Add S. pombe RPA | 0.2, 0.4, 0.6 μg | 103.8, 107.4, 105.8 |
| Add E. coli SSB | 0.2, 0.4, 0.6 μg | 101.8, 102.5, 97.4 |
| Add NaCl | 0.05, 0.1, 0.2 mM | 104.3, 103.0, 96.0 |
| Omit MgCl₂ | <1 |
| Omit DTT + NEMc | 10 mM | <1 |
| Omit ATP | 2 mM | <1 |
| Add GTP or CTP or UTP | 250 μM each | 10.0, 14.9, 8.8 |
| Add dATP | 250 μM | 68.3 |
| Add dGTP or dCTP or dTTP | 250 μM each | 16.8, 10.1, 1 |

a The value of 100% in this experiment corresponded to 7.5 fmol of DNA substrate displaced.
b The complete reaction contained 15 fmol of partial duplex ssDNA substrate containing a 20-base oligonucleotide and 11 ng of DNA helicase I in the standard reaction mixture described under “Materials and Methods.”
c The enzyme was first incubated for 10 min at 37 °C in the presence of the indicated amount of NEM.

As shown in Fig. 6B, polynucleotide requirements for ATP hydrolysis were also examined in the standard reaction conditions containing 0.25 mM ATP (5000 pmol, total). Both M13 ssDNA and poly(dA)₄₀₀₀·oligo(dT)₁₂₋₁₈ stimulated the ATPase activity of DNA helicase I with equal efficiency. After a 10-min incubation period, about half of the added ATP was hydrolyzed. Double-stranded DNA (M13 replicative form I) also supported ATP hydrolysis but was 50% as effective as ssDNA. A synthetic RNA, poly(U), had little stimulatory effect on ATPase activity early in the reaction (<5 min) but was weakly stimulatory after prolonged incubation (Fig. 6B). However, a comparable level of ATP hydrolysis was observed after an extended incubation period even without added DNA. Thus, DNA helicase I appears to be devoid of an RNA helicase activity since it was unable to hydrolyze ATP in the presence of RNA.

Influence of S. pombe RPA on the DNA Helicase Activity—As...
discussed in the description of the purification of DNA helicase I (Figs. 1 and 2), its coelution with SpRPA prompted us to examine the effects of SpRPA on DNA helicase I activity. The reactions were carried out using standard conditions with either 0.2 (low level) or 2 mM (high level) ATP and 15 fmol of substrate (the 52-bp partial duplex). These two ATP concentrations were chosen specifically for examination of the effects of SpRPA on DNA helicase I. Because we failed to observe significant duplex unwinding by this helicase at levels below 0.2 mM ATP, the low level of ATP (0.2 mM) was expected to facilitate detection of an SpRPA stimulatory effect if any exists.

As shown in Fig. 7A, the displacement reaction that contained 2 mM ATP was stimulated marginally (1.4- and 2.1-fold) in the presence of 0.4 and 0.6 μg of SpRPA, respectively (Fig. 7A, lanes 6 and 7). Similar levels of E. coli SSB also stimulated helicase activity to similar extents (Fig. 7A, lanes 10 and 11). Addition of lower levels (0.2 μg) of SpRPA or E. coli SSB did not affect the displacement reaction (Fig. 7A, lanes 5 and 9).

At low ATP concentration (0.2 mM), helicase activity was hardly detectable (<0.2 fmol) in the absence of SpRPA (Fig. 7B, lane 4). Addition of SpRPA (0.2, 0.4, and 0.6 μg) stimulated helicase activity in proportion to the levels of SpRPA added (10-, 21-, and 38-fold, respectively) (Fig. 7B, lanes 5–7). In fact, the stimulatory effects of increasing levels of SpRPA were

FIG. 6. Determination of the \( K_m \) for ATP for the ATPase activity of DNA helicase I and the influence of different polynucleotides on the ATPase activity. Panel A, the \( K_m \) for ATP for the ATPase activity of DNA helicase I was determined. The double-reciprocal Lineweaver-Burk plot (1/reaction velocity, 1/v (1/pmol of ATP hydrolyzed/min)) versus 1/ATP) was based on results obtained in the standard ATPase assay containing 11 ng of DNA helicase I with varying concentrations of ATP after a 5-min incubation period. Panel B, kinetic analysis of polynucleotide-dependent ATPase activity. ATP hydrolysis was measured under the condition described under “Materials and Methods.” Reaction mixtures contained 11 ng of DNA helicase I and 50 ng of each designated polynucleotide and were incubated for the period of time indicated in the figure. RFI, replicative form I.

FIG. 7. Preferential stimulation of the oligonucleotide displacement reaction of DNA helicase I by SpRPA at low ATP concentrations (0.2 mM). The helicase assay was carried out in reaction mixtures containing either 0.2 mM ATP (panel A) or 2 mM ATP (panel B) with \( \lambda \)X174 ssDNA substrate consisting of a 52-bp partial duplex and 51 ng of DNA helicase I (glycerol gradient pool I fraction) as described under “Materials and Methods.” The amount of SpRPA or E. coli SSB added is noted at the top of each lane. The numbers at the bottom of the gel indicate the amount of oligonucleotide fragment displaced in fmol. The slower migrating product formed in the presence of E. coli SSB (lanes 9–11 in panels A and B) was identified as the displaced oligonucleotide complexed with E. coli SSB. Upon proteinase K treatment, this band was reduced markedly. Panel C, influence of low concentrations of SpRPA on DNA helicase I activity. The helicase activity was measured using 1 μl of glycerol gradient pool II fraction, which is relatively free of SpRPA contamination, in the presence of the indicated amounts of SpRPA (●) or E. coli SSB (○) at 0.2 mM ATP as described under “Materials and Methods.”
almost linear at the low ATP concentration. When E. coli SSB (0.2, 0.4, and 0.6 μg) was used in place of SpRPA, less stimulation was observed (5-, 7-, and 10-fold, respectively) (Fig. 7B, lanes 9–11). Neither SpRPA nor E. coli SSB had any detectable unwinding activity when tested in the absence of DNA helicase I (Fig. 7, A and B, lanes 8 and 12) regardless of the ATP concentration used. No stimulatory effect was observed upon addition of similar amounts of BSA carrier protein, eliminating the possibility that increased activity was a result of increased stability of the enzyme (data not shown). Based on these results, we considered two possibilities. (i) The weak helicase activity observed at a low ATP concentration might be attributed to either the depletion of ATP by a poten- tial ATPase activity of DNA helicase I or the inhibitory effects of ADP formed by the hydrolysis of ATP. (ii) SpRPA may block a reaction by which ATP hydrolysis is uncoupled from DNA unwinding.

To investigate the potential inhibitory effect of ADP, varying amounts of ADP were added to helicase reaction mixtures that contained either 0.2 or 2 mM ATP. There was no detectable inhibition of the unwinding activity (data not shown, Table I). Next, we supplemented the reaction mixture with an ATP-regenerating system to test whether inefficient displacement at low ATP concentrations was due to ATP depletion. The addition of creatine phosphate (10 mM) and creatine phosphokinase (CPK, 2.5 μg) to the reaction was performed under the standard assay condition with 52-mer oligonucleotide annealed dX174 ascDNA substrate (15 fmol) and 51 ng of DNA helicase I as described under “Materials and Methods.” Panel B, panel C, quantitation of results obtained in panel A. Refer to panel C for symbols in the graph. Panel C, quantitation of results from the same type of experiment as in panel A with a lower amount of DNA helicase I (17 ng). Symbols of each series of reactions are as indicated in the graph. CPK denotes additions of both creatine phosphate and creatine phosphokinase to the reaction as described in panel A.

In this report, we have described the isolation and biochemical properties of a DNA helicase, DNA helicase I, from S. pombe. This helicase activity, which was stimulated by the presence of SpRPA and an ATP-regenerating system, is the first DNA helicase to be purified from fission yeast.

We attempted to isolate a functional homolog from S. pombe of two human DNA helicases, helicase α and ε, previously isolated from HeLa cells (29, 30). Although it was stimulated by SpRPA, DNA helicase I presented in this report is not likely to be a functional homolog of the two human helicases described above. DNA helicase I was activated markedly by SpRPA only at limiting concentrations of ATP (<0.2 mM), whereas both helicase α and ε were stimulated by human RPA at high ATP concentrations (~1 mM). Unlike helicase α, DNA helicase I did not require a forked substrate (data not shown). Another distinct feature of DNA helicase I is that it is a catalytic DNA-dependent ATPase that hydrolyzes ATP at a high rate (30–50 ATPs/ enzyme) (Table III). In contrast, helicase α and ε were less active and had much lower ATP turnover rates (2.6 and 0.3 ATPs/ enzyme, respectively). Other DNA helicases from mammalian cells that translocate in the same direction as the DNA helicase I include DNA helicase A (47), nuclear DNA helicases I and II (10) from calf thymus, ATPase Q1 (48), and

FIG. 8. Effects of SpRPA, E. coli SSB, and the ATP-regenerating system on DNA helicase I activity at low ATP concentrations (25–200 μM).

Panel A, effects of varying concentrations of ATP (as indicated on the top of each lane) in the absence (−) or presence of SpRPA (0.4 μg) or E. coli SSB (0.4 μg). The same reactions were repeated with the addition of creatine phosphate (CP, 10 mM) and creatine phosphokinase (CPK, 2.5 μg). The helicase reaction was performed under the standard assay condition with 52-mer oligonucleotide annealed dX174 ascDNA substrate (15 fmol) and 51 ng of DNA helicase I as described under “Materials and Methods.” Panel B, quantitation of results described above. The presence of both SpRPA and the ATP-regenerating system had more than an additive effect and markedly increased the unwinding efficiency even at the lowest ATP concentration (25 μM) tested. These results are consistent with the idea that one function of SpRPA lowers the Km for ATP in the helicase reaction. The apparent Km of ATP in the presence of the ATP-regenerating system alone was 60 μM, and this value was reduced to 12.5 μM when both the ATP-regenerating system and SpRPA were added (Fig. 8B). Comparable changes in the apparent Km were obtained when the level of enzyme was reduced nearly 70% (17 ng) (Fig. 8C). Thus, under conditions where the ATP concentration was held constant, SpRPA stimulated helicase activity at least in part by substantially lowering the apparent Km of ATP for the unwinding reaction.

DISCUSSION

In contrast, helicase α and ε were less active and had much lower ATP turnover rates (2.6 and 0.3 ATPs/ enzyme, respectively). Other DNA helicases from mammalian cells that translocate in the same direction as the DNA helicase I include DNA helicase A (47), nuclear DNA helicases I and II (10) from calf thymus, ATPase Q1 (48), and
human DNA helicases I, II, III, V, and VI from HeLa cells (4). Among these DNA helicases, the calf DNA helicase A was the only one that was shown to be significantly stimulated by the heterotrimeric RPA. However, this enzyme was not stimulated by either heterologous human RPA or E. coli SSB. In addition, it was significantly smaller (47 kDa) in size than the S. pombe helicase. However, further experiments are necessary to distinguish whether they are related to each other since the calf helicase A was not examined at low ATP concentrations for RPA stimulation of unwinding. Comparison of other biochemical properties such as preference for nucleotides as energy source, sensitivity to salts, polynucleotide cofactors for ATPase activity, and molecular weight suggests that the DNA helicase I in this study is unlikely to be a functional homolog of any of these 3′→5′ mammalian helicases.

DNA helicase I is also not likely to be homologous to any known S. cerevisiae DNA helicase previously described (8, 49–53). The RAD3 protein (50), helicases B, C, and D (8), the HcsB protein (53), and DNA helicase III (51) translocate in the 5′→3′ direction, opposite that of the helicase activity characterized here. Although the Dna2 protein, a DNA helicase recently identified by genetic screening and biochemical studies, has a 3′→5′ directionality, its helicase activity requires a forked substrate (52) in contrast to the substrate specificity of S. pombe DNA helicase I. It remains to be shown whether ATPase III (49) is similar to DNA helicase I described in this study since comparable biochemical parameters of ATPase III have not been described.

We noted that the separation of SpRPA from DNA helicase I was difficult, and it was a major contaminant. Glycerol gradient sedimentation or gel filtration in the presence of varying levels of salt (0.15–0.5 M NaCl) failed to separate the two proteins or change the sedimentation or elution profile (data not shown). The presence of SpRPA in the helicase preparation (glycerol gradient pool I fraction) has made it difficult to evaluate the influence of low levels of SpRPA on DNA helicase I activity. To perform this experiment, we used the glycerol gradient pool II fraction that is relatively devoid of SpRPA (Fig. 7B, lane 4) may be partly due to SpRPA present in the helicase preparation.

Although several DNA helicases have been shown to be activated by RPA, the exact mechanism by which RPA stimulates unwinding is presently unknown. We postulate two possible mechanisms by which SpRPA stimulated DNA helicase I activity: 1) a direct protein-protein interaction between SpRPA and DNA helicase I induces a conformational change that allows the helicase to function efficiently at reduced concentrations of ATP (for example, lowering of the $K_m$ for ATP); or 2) SpRPA targets the helicase directly to the partial duplex junction, reducing the level of ATP required for unwinding possibly by avoiding a nonproductive ATP hydrolysis process (for example, translocation along ssDNA). These two possible roles of SpRPA in stimulating the DNA helicase I are not necessarily mutually exclusive. The precise mechanism by which SpRPA stimulates DNA helicase I remains to be elucidated. In addition to the key roles known to be played by RPA in major DNA metabolic processes, a recent study demonstrated that RPA from S. cerevisiae is phosphorylated specifically in vivo by MEC1 upon DNA damage (54). This finding suggests a novel function for RPA in cell cycle checkpoint signaling when DNA damage occurs. At present, the function of DNA helicase I is not known. Its interaction with RPA, a protein implicated in replication, repair, recombination, and perhaps in checkpoint signaling, suggests that DNA helicase I might be involved in any one if not all of these important reactions. Because DNA helicase I translocates in the 3′ to 5′ direction, it would move on the leading strand toward the replication fork if it were involved in DNA replication. Peptide sequencing of the 95-kDa polypeptide revealed that it is a novel helicase.2 The cloning of the full-length cDNA and genomic DNA for DNA helicase I is underway. The availability of cloned and overexpressed DNA helicase I should permit us to determine whether it is capable of interacting directly with SpRPA. Both biochemical and genetic studies will help clarify the biological function of DNA helicase I in S. pombe. In addition, we hope to identify structurally similar mammalian helicases and use S. pombe genetics to test whether they are functionally related to DNA helicase I.

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