Yeast DNA Helicase A: Cloning, Expression, Purification, and Enzymatic Characterization†

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ABSTRACT: We have cloned and expressed the yeast DNA helicase A in *Escherichia coli* at a high level (∼30 mg/L of culture) in soluble form. We describe here a simple two-step purification protocol that produces reasonable quantities of homogeneous enzyme. In denaturing gel electrophoresis the enzyme behaved as a ∼90 kDa protein. The native structure, determined by gel-filtration studies, appeared to be hexameric and its quaternary structure was salt (NaCl) dependent. In low-salt buffers (containing 50 mM NaCl), the enzyme eluted in a single activity peak at an elution volume that appeared to correlate with a possible hexameric structure. In higher salt buffer (containing greater than 150 mM NaCl), the enzyme eluted as smaller assemblies (monomer/dimer). The recombinant helicase A was able to hydrolyze ATP or dATP with equal efficiency. The ATPase activity of the enzyme was absolutely DNA-dependent. The nucleotidase activities were comparable to those of the native enzyme. Kinetic analysis of the ATPase activity demonstrated that the K_m of the enzyme was ∼90 μM and the rate of ATP hydrolysis was ∼20 ATP s^{-1} $molecule^{-1}$. DNA sequences containing pyrimidine stretches were more effective activators than those containing purine stretches. However, poly(dC) appeared to be the most effective activator of the ATPase activity. The ATPase activity was inhibited by salt (NaCl) above 50 mM with a half-maximal inhibition at ∼110 mM. It is likely that the active state of helicase A is hexameric. The helicase activity of the recombinant enzyme was stimulated significantly by the yeast replication protein A (RPA) and to a lower extent by the single-stranded DNA binding protein of *E. coli* (SSB). The DNA helicase migrated on a DNA template in a 5′ → 3′ direction. Helicase A appeared to share a number of enzymatic characteristics including directionality, stimulation by RPA/SSB, and quaternary structure (monomer−hexamer) dynamics that are common to known replicative helicases such as DnaB helicase and the SV40 T-antigen.

The two strands of DNA in a double helix structure are held together in a very stable form by a large number of hydrogen bonds (H-bond)[1] or Watson−Crick base pairs (Watson & Crick, 1953; Wilkins et al., 1953; Arnott et al., 1965). The cumulative effect of a large number of H-bonds and other interactions such as stacking between pairs leads to a significant amount of energy that is required to unwind even a short stretch of double helix. Consequently, most enzymes such as the various polymerases in the cell are incapable of unwinding the duplex DNA during cellular processes. DNA helicases are highly specialized energy-transducing enzymes that utilize the energy of nucleotide triphosphate hydrolysis to unwind the duplex DNA and carry out this role.

Many helicases have been described over the last few decades from *Escherichia coli* to human (Kornberg & Baker, 1992; DePamphilis, 1993a,b; Tuteja et al., 1995; Tuteja & Tuteja, 1996). It is now evident that there are a number of DNA helicases in the cell and the functions of many of these helicases remain unknown. In *E. coli*, out of a large number of helicases, only DnaB helicase has been shown to be definitely involved in chromosomal and λ phage DNA replication (Lebowitz & McMacken, 1986). Several bacteriophages code for their own replicative helicases, for example, gene 4 helicase/primase of T7 bacteriophage and gp41 of T4 bacteriophage (Notarnicola et al., 1995; Dong et al., 1995; Dong & von Hippel, 1996). Among yeast DNA helicases, only RAD3 helicase has been shown to be involved in nucleotide excision repair (Prakash et al., 1993; Sung et al., 1994). Replicative helicases of eukaryotes remain quite unclear at the present time. T-antigen, the helicase derived from the mammalian virus SV40, is definitely involved in viral DNA replication (Goetz et al., 1988; Scheffner et al., 1989). The lack of an *in vitro* DNA replication system in eukaryotic organisms makes it especially difficult to correctly evaluate the role of putative replicative helicases. Almost all of the current studies involving identification of a eukaryotic replicative helicase suffer from this limitation. Nevertheless, it is still possible to develop a general profile of a replicative helicase and then determine how well a putative helicase fits this profile.

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[1] Abbreviations: HcsA, helicase A; H-bond, hydrogen bond; pol α, DNA polymerase α; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RPA, replication protein A; SSB, single-stranded DNA binding protein of *Escherichia coli*; PCNA, proliferating cell nuclear antigen.

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The most well studied of all replicative helicases is the DnaB helicase (Lebowitz & McMacken, 1986). Interestingly, DnaB helicase displays certain unique features that are not generally common to most helicases, such as interaction with other DNA replication proteins, formation of an oligomeric structure, stimulation by single-stranded DNA binding proteins (SSB), and a 5′ → 3′ directionality (Kornberg & Baker, 1992; Lebowitz & McMacken, 1986). Other bacteriophage-derived replicative helicases from T4 and T7 fit these characteristics very well. The SV40 T-antigen helicase also fits this profile except that it has a 3′ → 5′ directionality (although T-antigen is a multifunctional protein that is known to have origin binding and transcriptional regulatory activity in addition to its ATPase and helicase activities). Consequently, replicative DNA helicases appear to have a common profile of structure and function.

We have reported earlier the identification and purification of a 90 kDa helicase by immunoaffinity chromatography using immobilized monoclonal antibody against DNA polymerase α (Biswas et al., 1993). Due to the low yields of enzyme obtained using this method, only a limited amount of characterizations have been carried out, although initial studies suggest that it fits this profile. Two other yeast DNA helicases and their respective genes, 127 kDa DNA helicase B (ORF61 gene) and 170 kDa DNA2 helicase, have recently been described (Biswas et al., 1995a; Budd et al., 1995). At the present time only a limited amount of information on their physical properties is available. Consequently it is not possible to ascertain the roles these helicases play. However, eukaryotic DNA replication is a complex process. Three different DNA polymerases appear to function at the replication fork and there are multiple origins of replication in the chromosome. Therefore, the possibility that more than one DNA helicase could participate in DNA replication definitely exists.

In the accompanying paper (Biswas et al., 1997), we reported a detailed purification and sequence analysis of yeast DNA helicase A. Interestingly, the DNA helicase A appears to have a limited but striking sequence homolgy to *E. coli* DnaB helicase despite the fact that these two organisms are vastly seperated by evolution. At the present time, we do not fully understand its implications. We describe here the cloning and large-scale expression of yeast helicase A in *E. coli*. With the availability of large amounts of highly purified helicase A, it was possible to carry out important mechanistic studies in order to assess its structure and possible cellular function.

MATERIALS AND METHODS

Nucleic Acids, Enzymes, and Other Reagents. Synthetic homopolymers were from Pharmacia-LKB (Piscataway, NJ), and oligonucleotides were synthesized by Oligos Etc. (Portland, OR). Ultrapure deoxy- and ribonucleotides were obtained from Pharmacia (Piscataway, NJ) and were used without further purification. [α-^{32}P]ATP, [α-^{32}P]dATP, and [γ-^{32}P]ATP were obtained from Dupont/NEN (Boston, MA). RPA used in this study was purified to homogeneity from wild-type yeast as described by Brill and Stillman (1989). *E. coli* SSB and terminal deoxynucleotidyltransferase was purchased from U.S. Biochemical Corp. (Cleveland, OH). T4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). High-fidelity thermostable Pfu polymerase was from Stratagene (La Jolla, CA). All chemicals used to prepare buffers and solutions were reagent grade and were purchased from Fisher Scientific (Pittsburgh, PA). Protease inhibitors were from Bachem (Los Angeles, CA). Polyethylenimine−cellulose TLC strips were from J. T. Baker. The T7 expression system vector pET29b was from Novagen (Madison, WI). Transformation-competent *E. coli* strain DH5αF′ was from Life Technologies (Bethesda, MD) and strains BL21(DE3) and BL21(DE3)pLysS were from Novagen (Madison, WI).

Buffers. Buffer A contained 25 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.01% (v/v) NP40, 1 mM EDTA, and NaCl as indicated. Buffer B was analogous to buffer A, except that it contained 25 mM Hepes (pH 7.5) instead of Tris-HCl. Buffer C contained 25 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mg/mL BSA, and 5 mM DTT. 1× TBE buffer was 89 mM Tris−borate and 2.5 mM EDTA (pH 8.3).

ATPase Assays. The ATPase assays were carried out as previously described (Biswas et al., 1993). For enzyme characterizations, the standard assay contained 7.5 ng of helicase A Fr III.

Helicase Assays. The helicase assays were based on the methods described by Matson et al. (1983) and Biswas et al. (1993).

(A) Preparation of Helicase Substrates. A synthetic 45-mer oligonucleotide, complementary to a 35 bp sequence between nucleotides 6268 and 6302 of M13mp19 ssDNA, contained a 10 nucleotide tail on its 3′ terminus. The oligonucleotide was labeled at its 5′ end using T4 polynucleotide kinase. The oligonucleotide was hybridized to M13mp19 as previously described (Biswas et al., 1993). Excess unhybridized labeled oligomer was removed by spin column purification (Promega Biotech, Madison, WI). The purified substrate was diluted to 17 fmol/μL with 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Directionality substrate: A synthetic 60-mer oligonucleotide, complementary to a 50 bp sequence between nucleotides 6268 and 6317 of M13mp19 ssDNA was hybridized to M13mp19, following labeling at its 5′ and 3′ ends as described (Biswas et al., 1993). Excess unhybridized labeled 60-mer was removed by spin column purification. The directionality substrate was prepared by digesting the aforementioned substrate with *Sac*I restriction enzyme, generating a linear substrate with duplex ends consisting of a 43-mer at the 5′ terminus and a 17-mer at the 3′ terminus.

(B) Assay Conditions. Reaction mixtures were set up on ice as follows: a standard 20 μL reaction volume contained, in buffer C, 10 mM $MgCl_2$, 3.4 mM ATP, helicase substrate, and the indicated amount of DNA helicase. The mixtures were incubated at 30 °C for the times indicated and the reactions were terminated by the addition of 4 μL of 1% SDS, 60 mM EDTA, and 1% bromophenol blue. A fraction (25%) of each reaction mixture was analyzed on 8% polyacrylamide gels in 1× TBE and 0.1% SDS. The electrophoresis was carried out in 1× TBE and 0.1% SDS for 1 h at 160 V. Following electrophoresis, the gels were dried and exposed to Fuji XAR-5 film for 12 h at −80 °C.

PCR Amplification, Cloning, and Expression of the HcsA Gene. Amplification of the open reading frame of the *HcsA* gene was carried out using high-fidelity Pfu DNA polymerase and primers specific to the 5′- (starting at the ATG codon initiator codon) and 3′-ends of helicase A, based on the nucleotide sequence as obtained from the search of GenBank (Biswas et al., 1997). The primers were designed such that

the amplified DNA would contain the restriction endonuclease sites for *Bgl*II and *Eco*RI at its 5′ and 3′ ends, respectively, in order to facilitate subsequent subcloning. The primer sequences were as follows: left, 5′-T CTC TCA GAT CTA ATG AAC AAA GAA TTG GCT TC-3′; right, 5′-TCT GAA TTC ACA CCA TTC TGA CCA ACT-3′. These primers were used to amplify the entire coding region of the HcsA gene from the genomic DNA. PCR was carried out for 25 cycles with Pfu DNA polymerase (Stratagene Inc., La Jolla, CA) according to manufacturer's instructions, and using high-fidelity conditions, such as low dNTP concentration and a limited number of cycles.

Following amplification from *Saccharomyces cerevisiae* genomic DNA, the PCR DNA was digested using the appropriate restriction endonucleases, purified, and cloned into a T7 expression vector (Novagen Inc., Madison, WI) following the manufacturer's recommendations. Plasmid DNA was prepared following transformation of DH5α F′ cells. The authenticity of the clones was verified by restriction endonuclease mapping based on sites predicted to be present within the sequence. *E. coli* strain BL21(DE3) was then transformed with pET-HcsA plasmid for subsequent expression of the protein. The expression plasmids were created such that the expressed proteins would have a purification tag such as $(His)_6$-tag or S-tag, etc. The purification scheme described here was for helicase A containing a S-tag however, this purification scheme also applies for helicase A with or without an affinity tag.

Photo-Cross-Linking of the ATP Binding Domain of the Helicase Protein. Photo-cross-linking was carried out essentially as described earlier (Biswas & Kornberg, 1984; Biswas et al., 1993).

Other Methods. Protein concentrations were estimated according to the method of Bradford (1976), using bovine serum albumin as a standard. SDS−PAGE was carried out as described by Laemmli (1970).

RESULTS

Cloning and Expression of the Yeast Helicase A. The *HcsA* gene was amplified using polymerase chain reaction (PCR) methodologies along with a high fidelity thermostable DNA polymerase, Pfu DNA polymerase, and reaction conditions that favor high fidelity, such as a low concentration of deoxynucleoside triphosphates and a lower number of PCR cycles. The PCR product was cloned into a T7 expression vector in which expression is under control of the IPTG-inducible *lacUV5* promoter, as described earlier for the PCNA gene (Biswas et al., 1995b). Helicase A was obtained in a soluble form through controlled expression at the T7 promoter; this was accomplished by lowering the concentration of the inducer to 100 μM and the temperature to 12 °C over a period of 24−48 h. Under these conditions it was estimated that the *HcsA* gene product contributed to approximately 30% of the total cellular protein (Figure 1). Expression carried out at higher IPTG concentrations or temperatures resulted in the expression of the protein in an insoluble form, and all attempts at solubilization and purification of the protein were unsuccessful in restoring the enzymatic activities. We have also expressed helicase A in a baculovirus system; however, the protein was insoluble (data not shown). Consequently, the expression system described here is perhaps the best method of expressing helicase A.

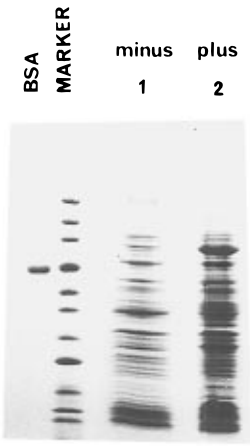

FIGURE 1: SDS−PAGE analysis of expression of helicase A in *E. coli*. Induction of expression was carried out as described in Results in the presence of 0.1 mM IPTG. Equal amounts of cells before and after induction were analyzed. Lane 2: BL21(DE3)/pET29bHcsA cells after IPTG induction. The protein standards were broad range markers from New England Biolabs (Beverly, MA), and the sizes are, from top to bottom, 156, 116, 97, 66, 55, 42, 36, 27, 20, 14, and 6.5 kDa.

Purification of Recombinant Helicase A. The extraction and all purification steps were carried out at 4 °C. In all purification steps, 0.1 mM ATP and 5 mM $MgCl_2$ were added in order to aid in the stability of the recombinant enzyme.

The relatively high levels of expression aided immensely in the purification of the recombinant protein. The purification of the recombinant protein was designed following a modified protocol of purification of the native protein from yeast [Biswas et al., 1997 (preceding paper in this issue)]. A flowchart of the purification is shown below (Scheme 1).

Scheme 1

E. coli extract (fraction I)

↓

POROS-HQ/H HPLC (fraction II)

↓

POROS-HS/H HPLC (fraction III)

An extract was prepared from the induced *E. coli* [BL21-(DE3)/pET29b-HcsA] by sonication in buffer A-500. The lysate was spun at 100000*g* for 30 min (fraction I). The supernatant was dialyzed to adjust the conductivity to that of A-50 and then loaded on a 10 mL HQ/H column equilibrated with buffer A-50. Helicase A was eluted with a gradient from buffer A-50 to A-500. The fractions were analyzed for ATPase activity and by SDS−PAGE, and the results are presented in Figure 2A,B. The ATPase activity eluted as a single major peak at a conductivity of approximately 300 mM (Figure 2B). This activity comigrated with the recombinant helicase A polypeptide; its size was approximately 90 kDa on SDS−PAGE (Figure 2A). The HQ/H pool (fraction II) was dialyzed against buffer H-0 until the conductivity had reached that of buffer H-100 and then loaded on a 2 mL HS/H column equilibrated with H 100. The column was eluted with a gradient of buffer H-100→H-1000, following which the fractions were analyzed for helicase and ATPase activities and by SDS−PAGE. The helicase and ATPase activities eluted as a single peak (Figure

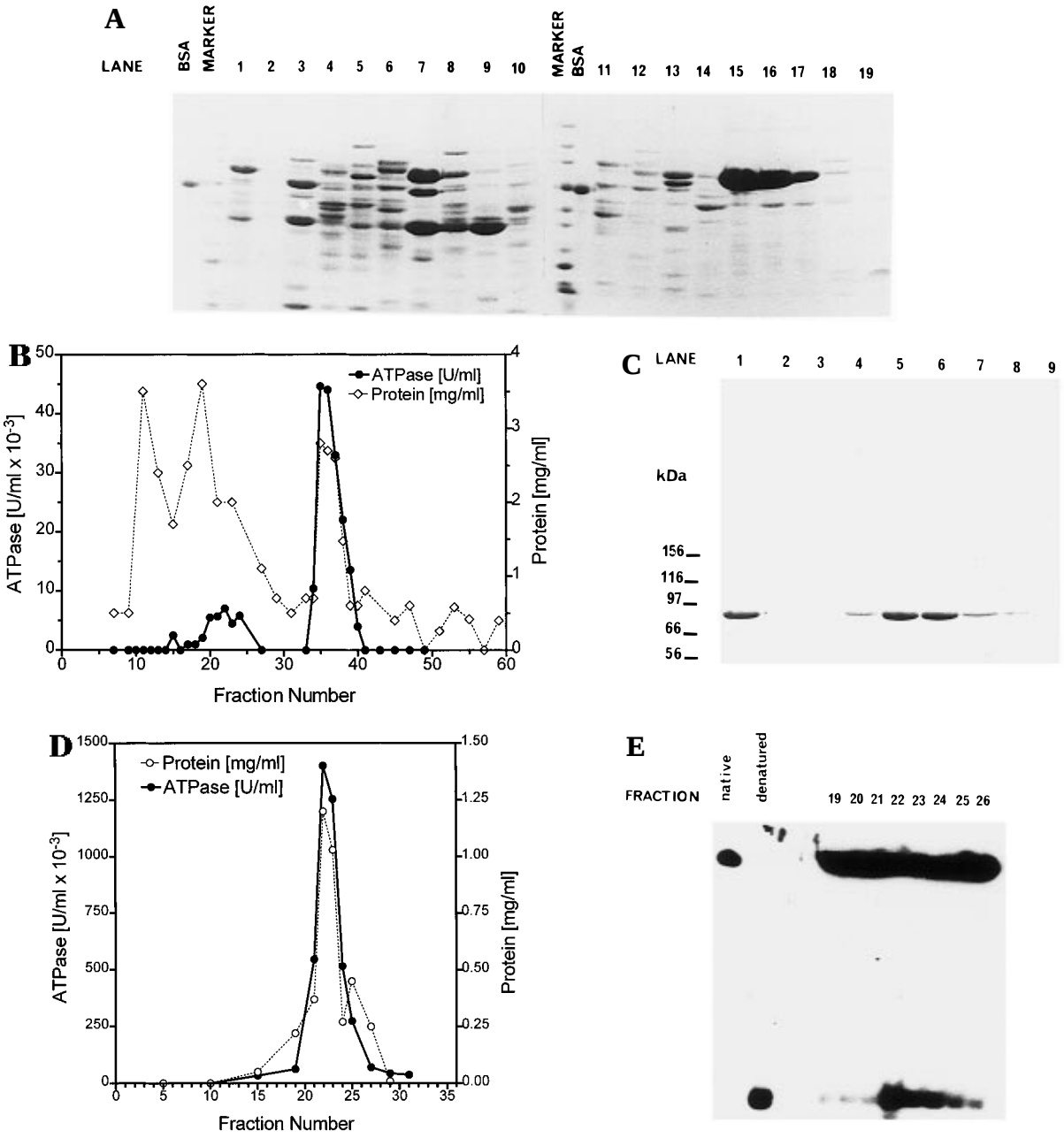

FIGURE 2: Chromatographic purification of recombinant helicase A. (A) SDS−PAGE analysis of POROS HQ/H anion-exchange fractionation Aliquots (25 μL) of the indicated fractions were resolved on an SDS polyacrylamide gel (5 → 18%) followed by Coomassie staining of the gel. Protein standards were as in Figure 1. Lane 1, load; 2, flow through; lane 3, A-50 wash; lanes 4−19, fractions 13−43, alternate odd-numbered fractions. (B) Chromatogram demonstrating protein and ATPase activity profile of HQ/H fractions. A standard ATPase assay was carried out using 0.05 μL of the indicated fractions. The percent hydrolysis represents that obtained during a 10 min assay. (C) SDS−PAGE analysis of POROS HS/H cation-exchange fractionation. Aliquots (25 μL) of the indicated fractions were resolved on an SDS polyacrylamide gel (5 → 18%) followed by Coomassie staining of the gel. Lane 1, load; lane 2, flowthrough; lane 3, Fr 19; lane 4, Fr 21; lane 5, Fr 22; lane 6, Fr 23; lane 7, Fr 24; lane 8, Fr 25; lane 9, Fr 27. (D) Chromatogram demonstrating protein and ATPase activity profile of HS/H fractions. Assays were carried out across the fractions using 0.05 μL of the indicated fractions. (E) Analysis of the helicase activity across the HS/H fractionation. A standard helicase assay was carried out using 0.5 μL of the indicated fractions, and the corresponding autoradiogram is shown.

2D,E). The helicase protein eluting from the HS/H column appeared to be 90−95% pure as determined by SDS−PAGE (Figure 2C). Fractions 22 and 23 constituted the purified helicase A pool (fraction III). The helicase eluted from the column at approximately 300 mM NaCl. The half-life of the purified protein appeared to depend on the ionic strength of the storage buffer; at high salt the protein was stable for at least 4 months, while at low salt (50 mM NaCl) the enzyme more readily lost activity. As a consequence, the enzyme was stored in 300 mM NaCl buffer, in aliquots, at −80 °C until further use. The final yield of purified recombinant helicase A was ∼10 mg/L of induced cell culture, indicating a 33% recovery of the enzyme.

Oligomeric Structure of Helicase A. The oligomeric structure of a DNA helicase is an important physical parameter and could play a significant role in its mechanism of action. The native helicase A purified from yeast cells was obtained in very small quantity; as a result, we could not carry out many important physical studies. The high levels of helicase A expression in *E. coli* made estimation

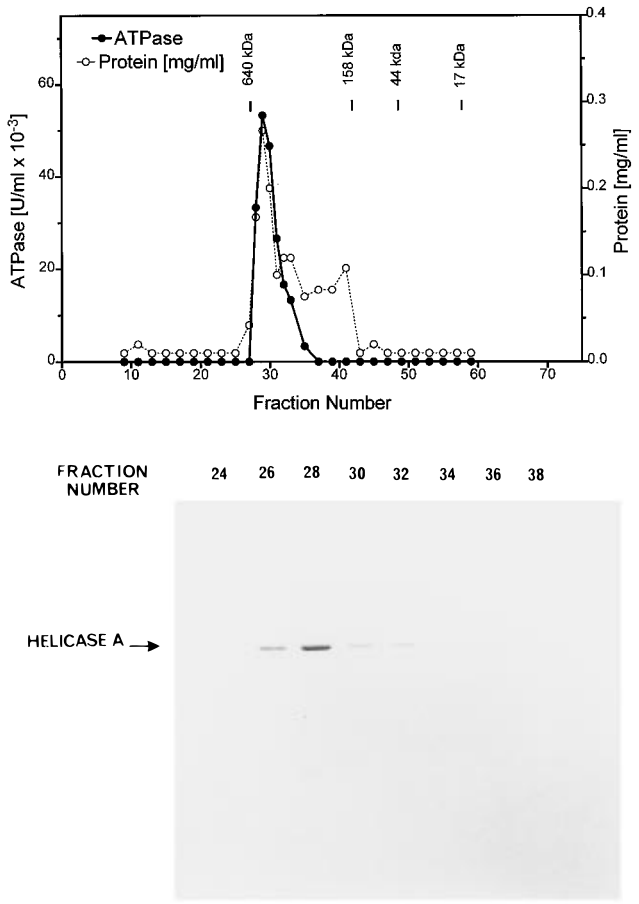

FIGURE 3: Size-exclusion HPLC of helicase A. Fractionation of recombinant helicase on a Sephacryl S-300 HR column. (A, top panel) Analysis of protein and ATPase activities of the fractions. Native molecular weight markers were gel-filtration standards from Bio-Rad Laboratories (Richmond, CA) and the proteins were as follows: thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), and equine myoglobin (17 kDa). (B, bottom panel) Western immunoblot analysis. SDS polyacrylamide gel (5 → 18%) electrophoresis was carried out using 25 mL aliquots of the indicated fractions. The blot was probed with antibody against the S-tag.

of native molecular weight using size-exclusion HPLC (SE-HPLC) relatively simple. Helicase A (fraction I) was dialyzed extensively against buffer B-50 containing 0.1 mM ATP and 5 mM $MgCl_2$ and fractionated on a Sephacryl S-300 (1.2 × 60 cm) gel-filtration column equilibrated with the same buffer. The column fractions were analyzed for protein and ATPase and by Western blot using antibody directed against the affinity tag of the recombinant protein. The results of the SE-HPLC are shown in Figure 3. The ATPase and Western blot clearly indicated elution at a volume corresponding to a molecular weight of ~500 kDa, as determined by a least-squares analysis of elution of molecular weight standards. This is consistent with a hexameric native structure of helicase A, given the observed denatured molecular weight. Oligomerization was found to be salt-sensitive, as oligomeric structure was not observed with salt concentrations greater than 150 mM NaCl (data not shown). It should be noted that we have reported earlier the salt-dependent elution of immunoaffinity-purified helicase A in SE-HPLC (Biswas et al., 1993). Because of the lack of understanding of the oligomeric structure of helicase A, we were not able interpret the SE-HPLC data at that time. Taken together, these data suggest a possible salt-dependent dynamic monomer−hexamer equilibrium of this helicase.

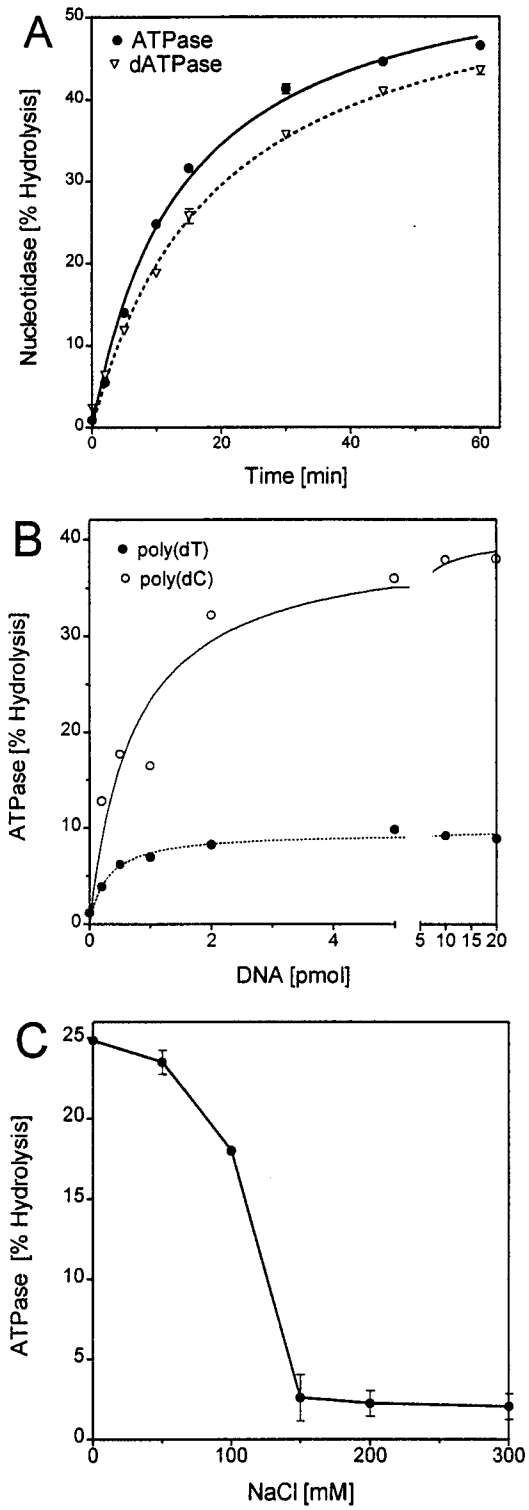

FIGURE 4: Characterization of the ATPase Activity of Helicase A. (A) Time course analysis. Time course analysis of helicase A in an ATPase assay was performed in the presence of 200 pmol of M13mp19 ssDNA. (B) Comparison of poly(dC) and poly(dT) DNA as effectors of the ATPase. (C) Influence of salt (NaCl) on the ATPase activity. Standard assays were carried out in the presence of increasing concentrations of NaCl.

Characterization of ATPase Activity. Analysis of the ATPase and dATPase activities showed that helicase A was able to hydrolyze either nucleotide with comparable efficiency (Figure 4A). The ATPase activity was strictly DNA-dependent, and half-maximal stimulation was observed at 1 nmol/mL (as nucleotide) of ssM13mp19 DNA (data not shown). DNA substrate specificity was examined and the results are presented in Figure 4C and Table 1. Helicase A

Table 1: Influence of Various DNA Effectors on the ATPase Activity of Recombinant Helicase A[a]

Polynucleotides [100 pmol]	% Activity
M13mp18 ssDNA	100
Poly(dA)	10
Poly(rA)	5
Poly(dT)	112
Poly(dC)	380
60-Mer Oligonucleotide	28
31-Mer Oligonucleotide	22
$(dA)_{20}$	5
$(dT)_{20}$	49
$(dA)_{10}$	≤1

[a] The ATPase assay was carried out as described in Materials and Methods using 7.5 ng of recombinant helicase A and the reaction was carried out for 5 min at 37 °C. The 100% activity refers to 18% hydrolysis (180 pmol) of the input ATP.

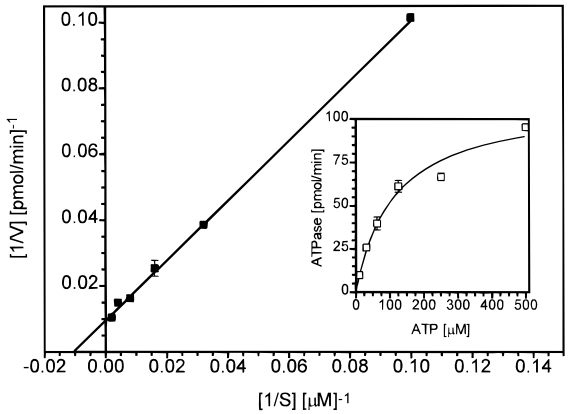

FIGURE 5: Kinetic analysis of the ATPase activity of Helicase A. Lineweaver−Burke plot. Double-reciprocal ($1/V$ vs $1/[S]$) plot of the ATPase activity demonstrating the influence of substrate concentration on the initial rate of ATP hydrolysis. The ATPase (V) vs ATP concentration ([S]) plot is shown in the inset and the curve was generated using nonlinear regression analysis of the data. The double-reciprocal plot was generated from the data in the inset using a linear least-squares regression analysis.

was found to exhibit a unique and specific preference for poly(dC) as the polynucleotide cofactor. ATP hydrolysis was 3-fold higher in the presence of poly(dC) than poly(dT) or native DNA templates. In general, polynucleotides were preferred over oligonucleotides and oligonucleotides smaller than 20 bp did not support ATP hydrolysis. The ATPase activity of helicase A was significantly modulated by salt (NaCl) concentration in the reaction and it was found that this influence was comparable to that previously observed for maintenance of oligomeric structure (Figure 4C). Thus, these data perhaps suggest a relationship between the oligomeric structure and the ATPase activity. Kinetic analysis of the ATPase activity showed that the K_m was 90 μM and the V_{max} was $\sim 1.4 \times 10^7$ pmol min^{-1} mg^{-1} (Figure 5). The value for K_m was comparable to that of the purified native enzyme, but the value of the V_{max} was significantly higher than the native enzyme [Biswas et al., 1997 (preceding paper in this issue)]. This difference could well be due to partial inactivation of the enzyme during purification and may also be due to partial protease degradation during purification. Photo-cross-linking of the recombinant helicase A is shown in Figure 6. The polypeptide was found to cross-link with reasonable efficiency in a dose-dependent manner.

Characterization of Helicase Activity. The recombinant helicase A was able to carry out DNA unwinding in a standard helicase assay as shown in Figure 7. In a titration

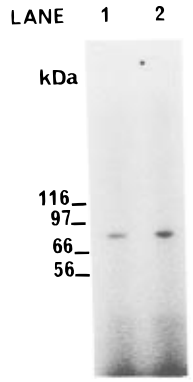

FIGURE 6: ATP cross-linking by helicase A. Cross-linking to [α-^{32}P]-ATP was carried out as described in Materials and Methods using purified recombinant helicase A (Fr III). Lane 1, 500 ng; lane 2, 1 μg of helicase A.

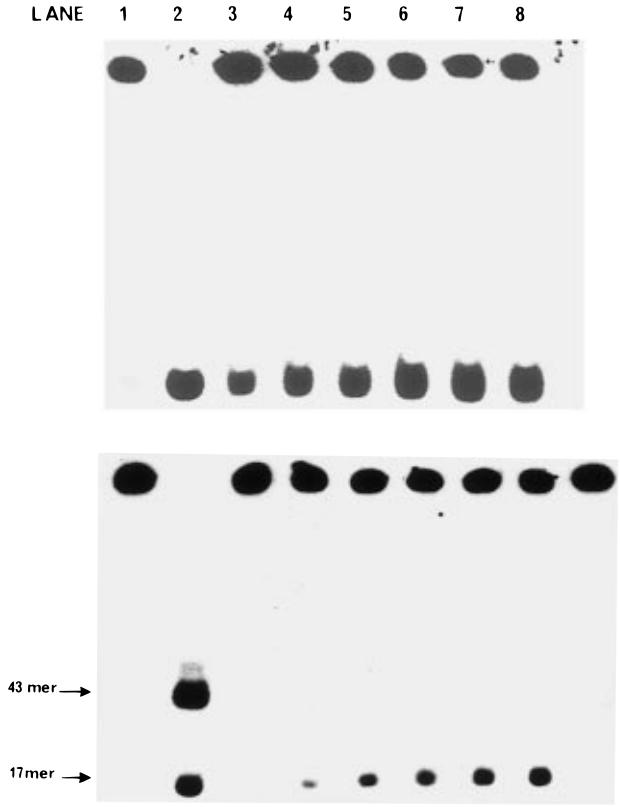

FIGURE 7: Characterization of the helicase activity of helicase A. (A, top panel) Protein titration of the helicase activity. A standard helicase assay was carried out using increasing amounts of helicase A (Fr III), in the presence of 250 ng of yRPA. Lane 1, native substrate; lane 2, heat-denatured substrate; lane 3, 200 ng; lane 4, 400 ng; lane 5, 600 ng; lane 6, 800 ng; lane 7, 1 μg; lane 8, 2 μg of recombinant helicase A. (B, bottom panel) Analysis of the polarity of translocation. A time course analysis of helicase activity (100 ng of HcsA Fr III) using the directionality substrate, as described in Materials and Methods, was carried out in the presence of 250 ng of yRPA. Lane 1, native substrate; lane 2, heat-denatured substrate; lane 3, 0 min; lane 4, 5 min; lane 5, 10 min; lane 6, 15 min; lane 7, 30 min; lane 8, 45 min; lane 9, 45 min, minus helicase A, 250 ng of yRPA.

of protein it was found that ~200 ng of helicase protein was required to unwind 30% of the input substrate at 30 °C in 30 min (Figure 7A) and saturated at 800 ng of the enzyme. A helicase directionality substrate as described in Materials and Methods was used to determine the polarity of translocation of helicase A on a template DNA. Helicase A was found, unambiguously, to have a 5′ → 3′ polarity of movement (Figure 7B). This result is consistent with that

Table 2: Properties of Several Known DNA Helicases

DNA helicase	polarity of movement	structural/ functional interaction	subunit mass (kDa)	effects of RPA/SSB	subunit structure
DnaB Helicase[a]	5′ → 3′	(a) pol III holo (b) primase	52	stimulation	hexameric
T4 gene 41 DNA helicase[b]	5′ → 3′	part of T4 helicase/primase	53	stimulation	hexameric
T7 gene 4 DNA helicase[c]	5′ → 3′	T7 primase	63, 56	stimulation	hexameric
SV40 T antigen[d]	3′ → 5′	DNA polymerase α−primase	92	stimulation	hexameric
DNA helicase A[e]	5′ → 3′	DNA polymerase α−primase	78	stimulation	hexameric

[a] Arai & Kornberg, 1981; Lebowitz & McMacken, 1986; Kornberg & Baker, 1992; Mok & Marians, 1987; Kim et al., 1996. [b] Venkatesan et al. 1982; Cha & Alberts, 1989; Kornberg & Baker, 1992; Dong et al., 1995; Dong & von Hippel, 1996. [c] Matson et al., 1983; Nakai & Richardson, 1988; Kornberg & Baker, 1992; Notarnicola et al., 1995. [d] Goetz et al., 1988; Dean et al., 1992; Kornberg & Baker, 1992. [e] Biswas et al., 1993; this paper.

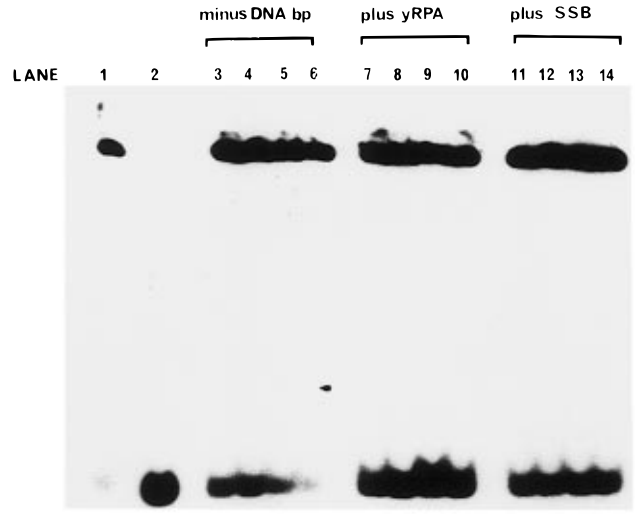

FIGURE 8: Influence of single-stranded DNA binding proteins (DNA bp) on DNA unwinding. A protein titration of the helicase activity of helicase A (Fr III) in the absence and in the presence of either yeast RPA (250 ng) or *E. coli* SSB (500 ng). The corresponding autoradiogram is shown. Lane 1: native substrate; lane 2, heat-denatured substrate. Lanes 3−6, no DNA binding protein: 3, 50 ng; 4, 100 ng; 5, 200 ng; and 6, 300 ng of helicase A. Lanes 7−10, reaction in the presence of yRPA: 7, 50 ng; 8, 100 ng; 9, 200 ng; and 10, 300 ng of helicase A. Lanes 11−14, reaction in the presence of *E. coli* SSB: 11, 50 ng; 12, 100 ng; 13, 200 ng; and 14, 300 ng of helicase A.

previously shown for helicase A isolated by immunoaffinity chromatography (Biswas et al., 1993). In this regard, helicase A appeared to share the same polarity with other common replicative DNA helicases such as DnaB helicase and T4 and T7 phage-encoded DNA helicases (Table 2).

Influence of Single-Stranded DNA Binding Proteins. Previously we have shown that the helicase activity of HcsA, isolated in a complex with polymerase α, is stimulated by the presence of yeast replication protein A (yRPA). We examined both yRPA and *E. coli* SSB for their ability to stimulate the helicase activity of recombinant helicase A, and the results are presented in Figure 8. An approximate 4-fold stimulation of unwinding was observed in the presence of yRPA. *E. coli* SSB was also able to stimulate the activity, although to a lesser degree.

DISCUSSION

DNA helicases play pivotal roles in a number of important cellular processes including DNA replication. These enzymes are normally present in the cell at a very low level (20 copies of DnaB helicase/cell in *E. coli*), making purification and enzymatic characterization of the helicase difficult. In the past, cloning and expression of the helicases greatly facilitated structural and functional characterizations of these enzymes. In the case of DnaB helicase, the availability of large quantities of the enzyme has allowed extensive elucidation of its mechanism of action. Consequently, we have expressed the *HcsA* gene product in a number of different expression systems including baculovirus. We were most successful in expressing the helicase A at a high level, in active and soluble form, with an *E. coli* T7 expression system (Rosenberg et al., 1987; Studier et al., 1990; Biswas et al., 1995b) as demonstrated in Figure 1. The level of expression simplified purification of the protein from *E. coli* extract, which consisted of two-step ion-exchange chromatography (Figure 2). Not surprisingly, the recombinant helicase A behaved in a similar manner as the native enzyme, in both anion- and cation-exchange chromatographies.

With the availability of large quantities of recombinant protein, it was possible to address important questions regarding the physical properties of helicase A that we were previously unable to answer due to scarcity of purified native protein. One such question was regarding the quaternary structure of helicase A. Various enzymes that are involved in DNA replication, recombination, and repair appear to form unique multimeric structures in order to process multiple strands of DNA structures such as replication fork, Holliday junction, etc. In addition to helicases, it has been shown recently that the Mu transposes of bacteriophage Mu and Flp site-specific recombinase of *S. cerevisiae* create ordered tetrameric structures during strand exchange (Lee et al., 1996; Voziyanov et al., 1996; Yang et al., 1996). In the case of helicases, hexamerization appears to be an important characteristic of all replicative DNA helicases including DnaB helicase and the SV40 T-antigen (Table 2). In most cases, hexameric structure is in a dynamic equilibrium with smaller subassemblies, such as monomer, dimer, etc. Certain conditions appear to favor the hexameric state; for example, Mg^{2+}, ATP, and NaCl. DnaB helicase forms a stable hexamer in the presence of ATP and Mg^{2+}. On the other hand, SV40 T-antigen is a stable monomer that appears to form a hexamer in the presence of ATP preceding its helicase action (Dean et al., 1992). Our present studies demonstrated that helicase A shared similar structural features with these two enzymes. It is stable in high salt as a monomer and long-term storage was possible in this state. Its enzymatic activities absolutely required low ionic strength (<150 mM NaCl), even for the ATPase activity. Gel-filtration studies indicated that Helicase A likely exists in a monomer−hexamer dynamic equilibrium and monomer is favored at higher salt, and above 150 mM NaCl it appeared to be monomeric. Taken together, it appears that the hexameric form is the active form. Interestingly, our studies also indicated that in a low-salt state (∼50

mM NaCl), helicase A lost its activity at a slow rate, approximately 50% in 6 h at 0 °C (data not shown). This property appears to resemble closely the structural dynamics of SV40 T-antigen.

The ATPase activity of the recombinant helicase A was similar in its properties to the native enzyme. Kinetic analysis of the ATPase indicated that the K_m was 90 μM and the rate of ATP hydrolysis was 20 ATP s^{-1} (enzyme monomer)$^{-1}$ (Figure 5). The recombinant enzyme was strictly DNA-dependent and no nucleotide hydrolysis was observed in the absence of a DNA cofactor. In general, pyrimidine-rich DNA was preferred, and small oligonucleotide served as poor effectors (Figure 4A−C). Poly(dC) was uniquely effective as a cofactor, and the rate of hydrolysis was 3-fold higher than that of the native template.

The recombinant protein was fully active in a standard helicase assay. Under our assay conditions the unwinding increased linearly with enzyme concentration (Figure 7A). Polarity of translocation is an important identifying characteristic of a DNA helicase. Previous studies with polarity of helicase A demonstrated that the direction of translocation was 5′ → 3′, which is also the direction of movement of the lagging strand of the replication fork. The present studies with recombinant helicase A indicated a 5′ → 3′ polarity of movement (Figure 7B). The ability of single-stranded DNA binding proteins to stimulate unwinding of DNA helicases has been demonstrated in several laboratories. Previously we have shown that the yeast replication protein A, and to a lesser extent *E. coli* SSB, was capable of stimulating the activity of native helicase A (Biswas, et al., 1993). The helicase activity was stimulated severalfold by yeast RPA and to a lesser extent by *E. coli* SSB.

The results of our present and previous studies (Biswas et al., 1993), taken together, demonstrated the following important characteristics of yeast helicase A: (i) helicase A is a hexameric helicase with monomer−hexamer dynamics similar to those observed with SV40 T-antigen; (ii) it interacts with pol α-primase similar to the interactions observed with *E. coli* DnaB helicase, SV40 T-antigen, and T4 and T7 replicative DNA helicases; (iii) the helicase activity is stimulated by single-stranded DNA binding proteins; (iv) it has 5′ → 3′ polarity of movement, a common polarity of replicative DNA helicases (except for SV40 T-antigen, which is known to have additional roles other than its helicase action in viral DNA replication); and finally (v) its subunit mass of 78.3 kDa is in the normal range of 50−92 kDa of replicative DNA helicases. In the absence of an *in vitro* DNA replication system for the yeast, *S. cerevisiae*, or other eukaryotic organisms, it is difficult to assess the exact role, if any, of an identified helicase in DNA replication. At the present time, helicase A appears to be a good candidate for the cellular replicative DNA helicase. Further studies are required for determining its cellular function.

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