Overexpression, Purification, DNA Binding, and Dimerization of the *Escherichia coli uvrD* Gene Product (Helicase II)†

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ABSTRACT: We have subcloned the *Escherichia coli uvrD* gene under control of the inducible phage λP_L promoter and report a procedure for the large-scale purification of helicase II protein. Yields of ~60 mg of >99% pure helicase II protein, free of detectable nuclease activity, are obtained starting from 250 g of induced *E. coli* cells containing the overexpression plasmid. Overproduction of helicase II protein at these levels is lethal in *E. coli*. The extinction coefficient of helicase II protein was determined to be $\epsilon_{280} = 1.06$ (± 0.05) $\times 10^5$ M^{-1} (monomer) cm^{-1} [20 mM Tris-HCl (pH 8.3 at 25 °C), 0.2 M NaCl, and 20% (v/v) glycerol, 25 °C]. We also present a preliminary characterization of the dimerization and DNA binding properties of helicase II and a systematic examination of its solubility properties. The apparent site size of a helicase II monomer on ss-DNA is 10 ± 2 nucleotides as determined by quenching of the intrinsic tryptophan fluorescence of the protein upon binding poly(dT). In the absence of DNA, helicase II protein can self-assemble to form at least a dimeric species at concentrations >0.25 μM (monomer) and exists in a monomer–dimer equilibrium under a variety of solution conditions. However, upon binding short oligodeoxynucleotides, the dimeric form of helicase II is stabilized, and dimerization stimulates the ss-DNA-dependent ATPase activity, suggesting that the dimer is functionally important. On the basis of these observations and similarities between helicase II and the *E. coli* Rep helicase, which appears to function as a dimer [Chao, K., & Lohman, T. (1991) *J. Mol. Biol. 221*, 1165–1181], we suggest that the active form of helicase II may also be a dimer or larger oligomer.

The *Escherichia coli* helicase II protein, encoded by the *uvrD* gene, functions in a number of DNA repair processes including methyl-directed mismatch repair (Modrich, 1991) and excision repair (Caron et al., 1985; Husain et al., 1985; Kumura et al., 1985). The protein is a helicase, i.e., a DNA-dependent ATPase that unwinds duplex DNA [for reviews, see Geider and Hoffmann-Berling (1981), Matson and Kaiser-Rogers (1990), Matson (1991), and Lohman (1992)]. At low protein concentrations, helicase II preferentially unwinds duplex DNA possessing a 3′-flanking ss-DNA (Matson, 1986; Runyon & Lohman, 1989), whereas at higher protein concentrations it can initiate unwinding at either a blunt-end or a nick (Runyon & Lohman, 1989; Runyon et al., 1990). This helicase is believed to unwind duplex DNA during these repair processes, possibly initiating at a nick (Runyon & Lohman, 1989; Runyon et al., 1990) or at the mismatch in mismatch repair (Langel-Rouault et al., 1987); however, its mechanism of unwinding is unknown.

Although the *uvrD* gene has been cloned (Oeda et al., 1981; Taucher-Scholtz & Hoffmann-Berling, 1983; Maples & Kushner, 1982), the protein has been overexpressed, and a number of purification protocols have been reported (Richet & Kohiyama, 1976; Abdel-Monem et al., 1977a; Hickson et al., 1983; Yamamoto et al., 1986; Kushner & Maples, 1988) since its discovery (Abdel-Monem et al., 1977a,b), it has been difficult to obtain the protein in a highly purified form in the large quantities and at the high concentrations necessary for many biochemical and biophysical studies. To address this problem, we have placed the *uvrD* gene under control of the strong inducible phage λP_L promoter and have developed a purification procedure that yields significantly larger quantities of highly purified protein than have previously been available. This has allowed us to examine more systematically many of the properties of this helicase, including its solubility under a variety of solution conditions, information that is important for biochemical and biophysical studies of this protein.

DNA helicases generally form oligomeric structures, usually dimers or hexamers, and the functionally active form of DNA helicases may be oligomeric in order to provide the helicase with multiple DNA binding sites, a feature that is essential for most proposed mechanisms of unwinding (Lohman, 1992). The availability of purified helicase II protein in large quantities has enabled us to examine its oligomerization properties, and although it has been reported that helicase II is monomeric (Richet & Kohiyama, 1976; Abdel-Monem et al., 1977a), we find that helicase II exists in a monomer–dimer equilibrium, which is influenced by solution conditions. Furthermore, the dimeric form is stabilized upon binding DNA, and the ATPase activity of helicase II is enhanced upon dimerization, suggesting that the helicase II dimer is functionally important. In this and other respects, helicase II is similar to the *E. coli* Rep helicase, which appears to function as a homodimer or higher oligomer to unwind duplex DNA (Chao & Lohman, 1991; Wong et al., 1992; Wong & Lohman, 1992), and therefore we suggest that the active form of helicase II may also be an oligomer.

MATERIALS AND METHODS

Buffers and Stock Solutions. Buffers were made with reagent-grade chemicals and distilled–deionized water that was passed through a Milli-Q system (Millipore, Bedford,

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MA). STE buffer is 10 mM tris(hydroxymethyl)aminomethane (Tris) base, pH 8.3 (titrated with HCl at 25 °C), 1 mM ethylenediaminetetraacetate trisodium salt (Na_3-EDTA), and 0.1 M NaCl. Lysis buffer is 50 mM Tris-HCl (pH 8.3 at 25 °C), 10% sucrose (w/v), 0.2 M NaCl, 5 mM Na_3EDTA, 0.5 mM disodium ethylene glycol bis(β-aminoethyl)ether)-*N,N,N',N'*-tetraacetic acid (Na_2EGTA), and 15 mM 2-mercaptoethanol. Buffer A is 20 mM Tris-HCl (pH 8.3 at 25 °C), 20% (v/v) glycerol, 1 mM Na_3EDTA, 0.5 mM Na_2EGTA, and 15 mM 2-mercaptoethanol. Buffer B is 20 mM Tris-HCl (pH 8.3 at 25 °C), 0.2 M NaCl, and 20% (v/v) glycerol. Buffer C is 20 mM Tris-HCl (pH 8.3 at 25 °C), 20% (v/v) glycerol, 1 mM Na_3EDTA, 0.5 mM Na_2EGTA, and 25 mM 2-mercaptoethanol. Buffer D is 25 mM Tris-HCl (pH 7.5 at 25 °C), 35 mM NaCl, 10% v/v glycerol, and 5 mM 2-mercaptoethanol. Buffer E is 25 mM Tris-HCl (pH 7.5 at 37 °C), 10 % v/v glycerol, 8 mM PEP, 0.9 mM NADH, and 5 mM 2-mercaptoethanol. Storage buffer is 20 mM Tris-HCl (pH 8.3 at 25 °C), 0.2 M NaCl, 50% (v/v) glycerol (spectral grade, Aldrich), 1 mM Na_3EDTA, 0.5 mM Na_2-EGTA, and 25 mM 2-mercaptoethanol. Stop buffer is 4% (w/v) sodium dodecyl sulfate (SDS), 0.05 M Na_3EDTA, and 20% (v/v) glycerol. SDS–polyacrylamide gel electrophoresis 2× sample buffer (made fresh before use) is 0.1 M Tris-HCl (pH 6.8 at 25 °C), 1.2 M 2-mercaptoethanol, 4.3% (w/v) SDS (Bio-Rad), 0.002% (w/v) bromophenol blue, and 10% (v/v) glycerol; 6 M guanidine hydrochloride (Schwarz/Mann) solutions were made in 20 mM potassium phosphate and adjusted to pH 6.8 with KOH at 25 °C. TE buffer is 10 mM Tris-HCl (pH 8.1 at 25 °C) and 1 mM Na_3EDTA; TAE buffer is 40 mM Tris–acetate, 5 mM sodium acetate, and 2 mM Na_3EDTA; and TBE buffer is 0.09 M Tris, 0.09 M boric acid, and 0.2 M Na_3EDTA.

A 10% (v/v) solution of Polymin P [50% (v/v) poly(ethylenimine) stock, M_r = 30K–40K; BRL], pH 6.9, was prepared as described (Jendrisak & Burgess, 1975). The following stock solutions were prepared directly before use: 0.05 M phenylmethanesulfonyl fluoride (PMSF) in 2-propanol; 4% (w/v) sodium deoxycholate (Sigma); lysozyme solutions (hen egg white, Boehringer Mannheim) in lysis buffer. Sodium dodecyl sulfate (SDS–polyacrylamide gel electrophoresis was performed as described (Lohman et al., 1986). $MgCl_2$ stock concentrations were determined by refractometry using an Abbe Model 1045 O refractometer. ATP (disodium salt) was from Calbiochem, and stocks were neutralized to pH 7 with NaOH to a final ratio of 3.8 mol of Na^+/mol of ATP. ATP solutions contained <2% ADP; ATPγS and ADP were from Boehringer Mannheim.

Column Materials. Heparin–agarose (0.8 ± 0.2 mg of heparin/mL) was from Bio-Rad. Single-stranded DNA–cellulose (~5.8 mg of ss-DNA/g of cellulose) and double-stranded DNA–cellulose (1 mg of ds-DNA/g of cellulose) (CF11 from Whatman) were prepared by the method of Littman (1968). Calf thymus DNA (Worthington) was extracted with phenol before use. The ss-DNA was made by denaturation under alkaline conditions. DEAE-cellulose (DE-52) was from Whatman (Hillsboro, OR).

Nucleic Acids. Poly(dT) (~1400 ± 200 nucleotides) (Midland Certified Reagent Co., Midland, TX) was resuspended in TE buffer + 0.2 M NaCl, extracted with phenol, precipitated in the presence of 0.1 M NaCl and ethanol (70% by volume), resuspended in TE buffer, and dialyzed extensively vs the appropriate buffer. The oligodeoxynucleotides $(dT)_8$, $(dT)_{10}$, $(dT)_{16}$, and the 16 base pair duplex hairpin, HP (5'-GACTCGTTACCTGAGT-TTTT-ACTCAGGTAAC-GAGTC-3'), were synthesized and purified as described (Chao & Lohman, 1991). Concentrations of $(dT)_8$, $(dT)_{10}$, $(dT)_{16}$, and poly(dT) were determined spectrophotometrically (TE buffer + 0.2 M NaCl) using $\epsilon_{260} = 8.1 \times 10^3$ M^{-1} (nucleotide) cm^{-1}.

Bacterial Strains and Plasmid DNA. E. coli N4830 (N^+, λcI857) (Gottesman et al., 1980) was provided by Dr. K. Ippen-Ihler (Medical Microbiology, Texas A&M University). Plasmid pGT26 (Taucher-Scholz & Hoffman-Berling, 1983) was provided by Dr. H. Hoffmann-Berling (Max Planck Institute, Heidelberg, Germany).

Growth of E. coli N4830/pTL51 for Helicase II Protein Overproduction. N4830/pTL51 was grown in LB broth with ampicillin (50 μg/mL) and its viability tested as described (Lohman et al., 1989). N4830/pTL51 for use in protein purification was grown in a 250-L fermenter (New Brunswick Scientific Co.) as described (Lohman et al., 1989); 188 L of LB broth containing thiamin (100 μg/mL) and ampicillin (50 μg/mL) at 30 °C was inoculated with 18 L of N4830/pTL51, started from a 200-mL inoculum, grown for 12 h in a 28-L fermenter (New Brunswick Scientific Co.) at 30 °C in LB broth plus ampicillin (50 μg/mL), resulting in an initial $OD_{600} \leq 0.2$. The pH of the media was kept constant at 7.0 by the sterile addition of 7 N NH_4OH. The temperature of the culture was increased to 42 °C at $OD_{600} = 0.6$ (~1.5 h after inoculation), maintained at 42 °C for 45 min, and then lowered to 37 °C for an additional 3 h. The cells were harvested by continuous-flow centrifugation at 8 °C (Cepa Z61), washed by resuspension in 50 L of STE buffer (4 °C), recentrifuged, and stored at −70 °C. The average yield of cell paste ranged from 1 to 1.2 kg.

ATPase Assays. Single-stranded DNA-dependent ATPase activity was measured in buffer containing 25 mM Tris-HCl (pH 7.8 at 37 °C), 3 mM $MgCl_2$, 1.5 mM ATP (pH 7, 5.7 mM Na^+ contribution), 10 mM 2-mercaptoethanol, 7.6 μM (nucleotides) poly(dT), and 1.2–4.1 nM helicase II by two methods. A spectrophotometric assay (Panuska & Goldthwait, 1980; Kreuzer & Jongeneel, 1983) was used to measure the ATPase activity continuously. In this assay, solutions (1 mL) also contained 25 units/mL pyruvate kinase (type II from rabbit muscle, Sigma), 25 units/mL lactic dehydrogenase (type II from rabbit muscle, Sigma), 4 mM phosphoenolpyruvate (PEP) (pH 7, 10.7 mM Na^+ contribution, Boehringer Mannheim), and 0.3 mM NADH (grade I, disodium salt, Boehringer Mannheim). Samples were preequilibrated for ~5 min at 37 °C, and the reaction was initiated by adding helicase II (5 μL). Hydrolysis of ATP was monitored by the decrease in absorbance at 340 nm. The initial rate of ATP hydrolysis was calculated usine ϵ_{340} = 6250 M^{-1} cm^{-1} for NADH (Kreuzer & Jongeneel, 1983). No component of the coupled enzyme reaction mixture was inhibitory or rate-limiting in the experiments reported here. Reactions were typically started by addition of either helicase II or DNA, although the ATPase activity was not dependent on the order of addition for experiments at low protein concentration.

ATPase activity was also measured using a discontinuous assay in which the rate of disappearance of [^{3}H]ATP was monitored by thin-layer chromatography. These reactions were carried out in 25 μL (or a multiple thereof) in the same buffer described above, containing phosphoenolpyruvate, but in the absence of pyruvate kinase, lactic dehydrogenase, and NADH unless stated otherwise. Reactions were preincubated for 5 min at 37 °C prior to initiating the reaction with helicase II and were stopped by addition of 5 μL of 250 mM Na_3-

EDTA. Samples (5 μL) were applied to poly(ethylenimine)–cellulose sheets (containing UV_{254} indicator, Schwarz/Mann) and developed with 1 M formic acid/0.5 M LiCl (Matson & Richardson, 1983). The spots containing ATP and ADP were cut out, and their radioactivity was determined by scintillation counting using Scintiverse II cocktail (American Scientific).

Helicase II Protein Concentration Determination. The extinction coefficient of native helicase II protein was determined by comparing the absorption spectra of the native protein in buffer B and the denatured protein in 6 M guanidine hydrochloride (Gdn-HCl) + 20 mM potassium phosphate (pH 6.8) as described (Lohman et al., 1989). Low concentrations of helicase II [1–30 μg/mL (12–360 nM)] were determined from measurements of ATPase activity in the presence of excess poly(dT) (ratios of ≥1000 nucleotides per helicase II monomer) or by the micro-Bradford assay (Bio-Rad) using purified helicase II as the standard. Use of BSA as the standard *overestimates* the helicase II concentration by nearly a factor of 2.

Nuclease Assays. Helicase II preparations were assayed for single- and double-stranded endonuclease by mixing different concentrations of helicase II [6–24 μM (monomer)] with supercoiled pUC8 or M13mp11 RF DNA [61 pmol of nucleotide (200 ng)] or circular ss M13mp11 DNA in 25 mM Tris-HCl (pH 7.5 at 37 °C), 10% (v/v) glycerol, 2.5 mM $MgCl_2$, 35 mM NaCl, and 1.5 mM ATP (pH 7) (30 μL) and incubating the mixture for 1–5 h at 37 °C as described (Runyon, 1991).

Glutaraldehyde Cross-Linking. Glutaraldehyde (pentane-1,5-dial) (grade I, Sigma) was obtained as an 8% aqueous solution and stored in 0.5-mL aliquots (–70 °C). Cross-linking of helicase II (2 μM monomer) was carried out at room temperature (~22 °C) in 20 mM Tricine, pH 8.3, 50 mM NaCl, 20% glycerol, 5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. When present, either $(dT)_{16}$ or a 16 base pair hairpin duplex oligodeoxynucleotide, HP, was at 2 μM, and AMPP(NH)P or ADP was at 2 mM. Cross-linking was initiated by the addition of glutaraldehyde to 0.1% and quenched after 30 min by the addition of lysine acetate to 10 mM. Products were analyzed by SDS–PAGE on a 10% acrylamide gel.

Helicase II Protein Solubility. Protein solubility was determined either by equilibrium dialysis or by filtering solutions through a 0.22-μm filter (GV_4, Millipore) as described (Lohman et al., 1989; Runyon, 1991). Seventy-five microliters of concentrated helicase II protein (>12 μM in storage buffer) was dialyzed for at least 10 h to reach equilibrium at 4 °C. In experiments examining the effects of ATP, ADP, and ATPγS, these ribonucleotides were added initially to both the protein sample and dialysate, at the concentration indicated, since these ribonucleotides pass through the dialysis membrane very slowly in solutions containing ≥10% (v/v) glycerol. After dialysis, the protein solution was transferred to a microfuge tube and allowed to equilibrate at the final temperature (4, 25, or 37 °C) for 30 min with occasional gentle mixing. Insoluble protein was pelleted by centrifugation in a microfuge for 5 min at the appropriate temperature. The temperature shift of protein solutions from 4 °C (dialysis conditions) to 25 or 37 °C resulted in a change of pH. The pH at the final temperature was calculated from the pK_a/°C value for each buffer (Good et al., 1966; Hirschman et al., 1967). Due to dilution effects, we could only measure solubilities that were lower than ~8.5 μM (monomer) in buffers containing 20% (v/v) glycerol, and ~6.3 μM (monomer) for buffers containing 10% (v/v) glycerol.

Gel Permeation Chromatography. The assembly state of helicase II was examined by small-zone gel permeation chromatography using HPLC (Waters W600 gradient module) and a Protein-Pak 300SW column (Waters) under isocratic conditions (flow rates of 0.1 or 0.25 mL/min). Column temperature was maintained using a Waters TCM column heater, and elution profiles were monitored by absorbance at 280 nm. The retention times of the following proteins displayed a linear dependence on the log of their molecular mass (in 100 mM NaCl/25% glycerol, pH 7.5, 37 °C) and thus were used as standards: horse spleen apoferritin (440 000) and rabbit muscle aldolase (158 000) (Pharmacia); sweet potato β-amylase (200 000), hen ovalbumin (43 000), and soybean trypsin inhibitor (21 500) (Sigma); human transferrin (80 000) (Calbiochem). In buffer D at low salt (25 mM NaCl), ovalbumin eluted anomalously on the column and thus was not used as a standard under these conditions. The retention times of the other protein standards were identical in solutions containing different [Tris], 25 mM HEPES–NaOH (pH 7.5), and between 10 and 25% (v/v) glycerol, ±5 mM $MgCl_2$ or 1.5 mM ATP (data not shown).

Helicase II samples were preincubated in column buffer for at least 15 min at 37 °C prior to injection onto the column, using a Hamilton glass syringe (10 or 25 μL) preequilibrated at 37 °C. The partition coefficient, K_D, is defined as $(V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume for the protein, V_0 is the excluded volume, and V_t is the total column volume. Blue Dextran and guanosine were used to determine V_0 and V_t, respectively. V_e was determined by the position of the peak for each sample. In buffers containing ATP, helicase II was detected by ss-DNA-dependent ATPase activity.

Fluorescence Measurements. Titrations of helicase II with poly(dT) were performed in buffer D (pH 7.5) + 0.1 mM Na_3EDTA at 25.0 ± 0.2 °C by monitoring the quenching of intrinsic tryptophan fluorescence (λ_{ex} = 296 nm, 1-mm slit; λ_{em} = 335 nm, 8-mm slit) with an SLM 8000C spectrofluorometer (Lohman & Overman, 1985).

RESULTS

Expression of the uvrD Gene under λP_L Control. The 2.9 kilobase pair (kpb) *Pvu*II fragment from plasmid pGT26 (Taucher-Scholz & Hoffmann-Berling, 1983), containing the complete *uvrD* gene (encoding helicase II) and its regulatory region, was cloned into the *Hpa*I site of pKC30 (Shimatake & Rosenberg, 1981), placing it under control of the inducible phage λP_L promoter. After transformation, two plasmids were isolated, each containing the 2.9 kbp insert but in opposite orientations. The plasmid pTL51 contains the *uvrD* gene in the proper orientation for transcriptional initiation from the λP_L promoter; the start of the *uvrD* structural gene in pTL51 is approximately 400 bp from the λP_L promoter. The plasmid pTL52 contains the *uvrD* gene inserted in the opposite orientation. *E. coli* strain N4830 (N^+, cI857) (Gottesman et al., 1980) was tranformed with these plasmids. Following a temperature shift (from 30 to 42 °C) of N4830/pTL51, helicase II protein (M_r = 82 116) (Yamamoto et al., 1986; Finch & Emmerson, 1984) is expressed to ~1–2% of the total cellular protein as estimated from the total cellular ATPase activity and polyacrylamide gel electrophoresis, whereas no overexpression was observed with N4830/pTL52. Helicase II expression in N4830/pTL51 increased during the first 2 h after temperature induction, after which no further increase was observed.

Figure 1 indicates that N4830/pTL51 viability decreases significantly after a temperature shift from 30 to 42 °C whereas

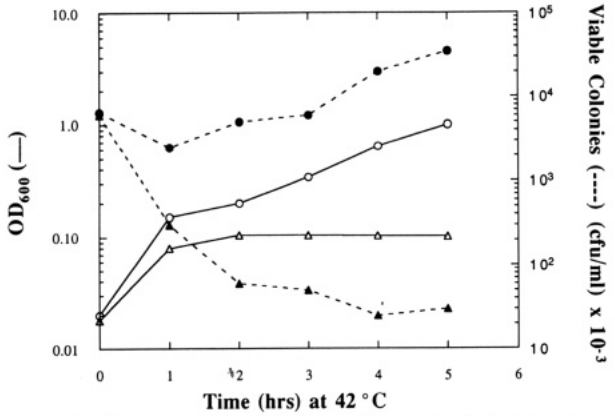

FIGURE 1: Overexpression of helicase II protein is lethal. *E. coli* N4830 containing plasmids pTL51 (triangles) or pTL52 (circles) were grown at 30 °C in LB broth and induced by a shift in temperature to 42 °C as described under Materials and Methods. The OD_{600} (open symbols) and the number of colony forming units (cfu) (filled symbols) in the cultures were determined as a function of time after induction.

Table I: Purification of Helicase II Protein from N4830/pTL51[a]

purification step	vol (mL)	total[b] protein (mg)	total[c] ATPase act. (units)	sp ATPase act. (units/mg)	purification (*x*-fold)
cell lysate	1250	15200	67300	4.43	1.0
Polymin P supernatant	1160	12400	59000	4.75	1.1
ammonium sulfate	750	780	60500	77.6	17.5
heparin–agarose	488	136	38700	285	64.3
ss-DNA–cellulose	412	77.2	27800	360	81.3
ds-DNA–cellulose	2500	60.8	22100	364	82.2

[a] Starting from 250 g (wet weight) of N4830/pTL51 cells. [b] Measured using the Bradford (Bio-Rad) protein assay with purified helicase II protein as a standard. [c] ATPase activity was determined in the presence of excess poly(dT) (≥2500 nucleotides/helicase II monomer).

N4830/pTL52 remains viable after this same treatment, indicating that overexpression of the *uvrD* gene product results in cell death. N4830/pTL51 also forms filaments after temperature induction as observed by light microscopy. These properties of N4830/pTL51 are qualitatively similar to those observed for MZ-1/pRepO, which overexpresses the *E. coli rep* gene product (Lohman et al., 1989).

Purification of Helicase II Protein from N4830/pTL51. The following procedure is for 250 g of induced N4830/pTL51; however, the procedure can be scaled down in direct proportion to the quantity of starting cells with similar relative yields. All steps were carried out at 4 °C or on ice unless stated otherwise. Table I and Figure 2 summarize the steps in the purification.

Cell Lysis. Prewashed, frozen N4830/pTL51 cells (250 g) are resuspended in 1 L of lysis buffer in a Waring blender at low speed, and phenylmethanesulfonyl fluoride (PMSF) is added to 0.1 mM. The cell suspension is adjusted to pH 8–8.5 using 2 M Tris. Lysozyme, from a freshly made stock, is added to a final concentration of 200 μg/mL, and the suspension is incubated for 30 min at 4 °C with frequent stirring. Sodium deoxycholate [freshly made 4% (w/v) stock] is added dropwise to the suspension to a final concentration of 0.05% (w/v) and incubated for 30 min at 15 °C with intermittent mixing. The lysate is placed at 4 °C, brought to 0.48 M NaCl by addition of cold 5 M NaCl (0.059 mL/mL of lysate), and stirred continuously for 15 min. This [NaCl] increases the helicase II protein solubility and may facilitate separation of helicase II protein from the cell debris. (N.B.: If the [NaCl] exceeds 0.48 M, some RNA polymerase will copurify with helicase II through the subsequent steps.) The lysate is sonicated on ice for 3 min (50% duty cycle, power setting 8; Heat systems W-225) and centrifuged at 26000*g* (13 000 rpm in a Beckman JA-14 motor) for 90 min at 4 °C. The supernatant is fraction I (~1250 mL) (Figure 2, lane C).

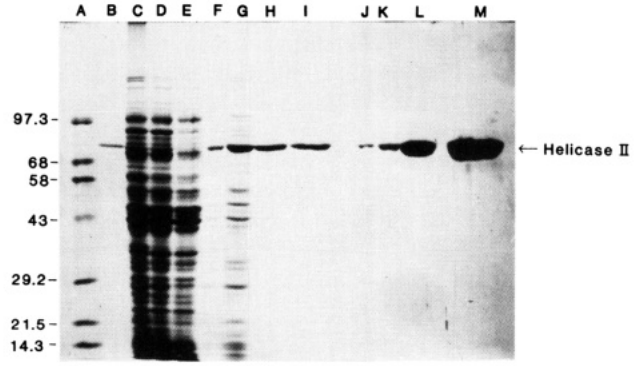

FIGURE 2: Helicase II protein purification. The various fractions obtained during the purification of helicase II were examined by SDS–polyacrylamide gel electrophoresis (6–14% gradient gel) followed by staining with Coomassie Blue. Lanes: (A) molecular weight standards; (B) purified helicase II (400 ng); (C) fraction I; (D) fraction II; (E) supernatant from the ammonium sulfate precipitation; (F) fraction III; (G) fraction III at 6 times the amount of protein as in lane F; (H) fraction IV; (I) fraction V; (J) fraction VI (200 ng of purified helicase II); (K) fraction VI (1 μg); (L) fraction VI (10 μg); (M) fraction VI (30 μg). Equivalent amounts of total protein were loaded in lanes C–F. Equivalent amounts of total protein were loaded in lanes G–I, although these levels were 6-fold higher than the levels in lanes C–F.

Polymin-P Precipitation. Ten percent (v/v) Polymin-P (pH 6.9) is added slowly to fraction I *with constant stirring* to a final concentration of 0.3% (v/v), and stirring is continued for an additional 15 min. Helicase II protein *remains in the supernatant* while nucleic acids and most acidic proteins are precipitated. The sample is centrifuged at 13 000 rpm in a JA-14 rotor for 30 min at 4 °C. The supernatant is fraction II (~1150 mL) (Figure 2, lane D).

Ammonium Sulfate Fractionation. Helicase II is precipitated by adding solid ammonium sulfate (176 g/L) to fraction II (~30% of saturation) and is collected by centrifugation (13 000 rpm in a JA-14 rotor) for 30 min at 4 °C. The ammonium sulfate pellets are drained, and each pellet is resuspended in 125 mL of buffer A + 0.3 M NaCl. The samples are cleared by centrifugation for 10 min at 4 °C (10 000 rpm, JA-14 rotor). The supernatant is fraction III (~750 mL) (Figure 2, lane F).

Heparin–Agarose Chromatography. Fraction III is loaded onto a 250-mL heparin–agarose column (12.7 cm × 5 cm i.d.), preequilibrated in buffer A + 0.1 M NaCl, by mixing 1:2 (v/v) with buffer A (1500 mL). [N.B.: It is important that fraction III is diluted with buffer A *directly as it is loaded onto the column*, using four-way tubing and a peristaltic pump to suction from three beakers of identical volumes (750 mL each), one containing the protein sample and two containing buffer A (without NaCl). If fraction III is mixed with buffer A in one dilution step before loading, significant losses result from the formation of fibrous helicase II aggregates, which can only be resolubilized using 6 M guanidine hydrochloride.] The column is loaded at 16 mL cm^{-2} h^{-1} (315 mL/h) and washed to base line with buffer A + 0.1 M NaCl (~4–5 column volumes).

The heparin–agarose column is eluted with a 1400-mL linear NaCl gradient from 0.1 to 0.45 M in buffer A, after which the column is washed to base line with buffer A + 0.45 M NaCl. Fractions (12 mL) are collected throughout the

gradient and wash steps and screened for helicase II by SDS–PAGE. Helicase II is contained in column fractions ~35–140 (corresponding to ~0.15–0.44 M NaCl), with the peak occurring near 0.42 M NaCl (fraction 100). Two protein contaminants (~22 and 29 kDa) elute near the end of the gradient [starting near ~0.41 M NaCl (fraction 114)] and should be avoided since they are not separated in the following steps. The pooled fractions containing helicase II have a conductivity equivalent to 0.25–0.3 M NaCl. This is fraction IV (~490 mL) (Fraction 2, lane H).

Single-Stranded DNA–Cellulose. Fraction IV is diluted *slowly* with buffer A to a final conductivity equivalent to 0.2 M NaCl in buffer A and loaded onto a 100-mL ss-DNA–cellulose column (5 cm × 5 cm i.d.) (~5.8 mg of ss-DNA/g of cellulose) preequilibrated in buffer A + 0.2 M NaCl (14 mL cm^{-2} h^{-1}, 275 mL/h). While fractions are collected (12 mL), the column is washed to base line with 3–4 column volumes of buffer A + 0.25 M NaCl and then washed with 1 column volume of buffer A + 0.325 M NaCl. Helicase II is eluted with buffer A + 1.0 M NaCl in ~15 fractions which are screened by SDS–PAGE (12.5%). The early fractions from the 0.325 M NaCl elution (~fractions 5–10) often contain a ~26-kDa polypeptide contaminant and should not be pooled, since the presence of this contaminant seems to reduce the recovery of helicase II from the double-stranded DNA column. (N.B.: As much as 15–20% of the total helicase II protein that is eluted from the ss-DNA column is discarded in this step.) The pooled fractions from the 1 M NaCl elution are fraction V (~410 mL) (Figure 2, lane I) and contain helicase II that is 98–99% pure. However, fraction V contains a 3′–5′ ss-DNA-dependent exonuclease contaminant that can be removed in the following step.

Double-Stranded DNA–Cellulose. Fraction V (~410 mL) is slowly diluted with buffer A + 0.15 M NaCl to a protein concentration of ~100 μg/mL [estimated by the Bradford assay or ss-DNA-dependent ATPase activity (see Materials and Methods)]. The final volume after dilution is ~800 mL. This sample is dialyzed vs 2 × 8 L of buffer A + 0.15 M NaCl in two steps (8 L for 6 h each). If the final conductivity of the protein solution is not equivalent to 0.15 M NaCl in buffer A, then buffer A should be added slowly to achieve this conductivity. A ds-DNA–cellulose column (~75 mL of 1 mg of ds-DNA/g of cellulose), preequilibrated in buffer A + 0.075 M NaCl, is prepared (5-cm i.d. column) (~1 mL of ds-DNA column material is required per milligram of helicase II). Fraction V is then mixed 1:1 with buffer A + no salt, *directly as it is loaded onto the ds-DNA column*, as described for the heparin–agarose column. (*A one-step dilution of fraction V will result in precipitation of the protein.*) A maximum flow rate of 14 mL cm^{-2} h^{-1} (275 mL/h with a 5.0-cm i.d. column) is used. The helicase II (>90%) *flows through* the column at this [NaCl] (0.075 M NaCl) and is collected. The protein that binds to the column is eluted upon continual washing with buffer A + 0.075 M NaCl (~6 column volumes is usually sufficient). The contaminating nuclease binds to the ds-DNA column under these conditions. It is essential that the buffers used in this step be maintained at a conductivity equivalent to buffer A + 0.075 (±0.005) M NaCl, since the nuclease elutes from the column at a conductivity equivalent to buffer A + 0.1 M NaCl, whereas helicase II will bind to the ds-DNA column if the [NaCl] is <0.075 M NaCl. The helicase II in the flow-through and in the wash is pooled [fraction VI (~2500 mL) (Figure 2, lanes J–M)]. It is better to test for nuclease activity after fraction VI is concentrated. If the 3′–5′ exonuclease activity is still present at a significant level, then the helicase II protein should be rediluted to 100 μg/mL in buffer A + 0.15 M NaCl and reapplied to the ds-DNA column by 1:1 dilution with buffer A as described above.

Concentration and Storage of Helicase II. Fraction VI is brought to 0.2 M NaCl by adding buffer A + 5 M NaCl, and helicase II is precipitated by adding ammonium sulfate to ~55% of saturation (350 g/L), over a period of 1 h, with constant *very* slow stirring to avoid formation of filamentous aggregates. The protein solution is stirred for an additional 2 h after all of the ammonium sulfate has dissolved. Using this procedure, approximately 90% of the protein is precipitated. Addition of the 0.2 M NaCl inhibits irreversible formation of fibrous helicase II aggregates. The ammonium sulfate pellet is collected by centrifugation for 30 min at 4 °C (13 000 rpm, JA-14 rotor), drained, and resuspended gently in storage buffer [0.7 mL of storage buffer per milligram of helicase II protein (~42 mL)]. The amount of helicase II in fraction VI should be estimated before ammonium sulfate precipitation in order to determine the volume of storage buffer used to resuspend the pellets. The concentrated protein is dialyzed vs 2 L of storage buffer (4 × 500 mL for ~8 h each), followed by centrifugation at 4 °C to remove any insoluble material [60 min at 40K rpm in 10-mL polycarbonate Oak Ridge tubes (Beckman Ti70.1 rotor)]. The final helicase II concentration in storage buffer should be ~1–2 mg/mL (estimated by the UV absorbance as described below). Protein is stored at −70 °C in 0.5–1-mL aliquots.

Helicase II protein purified by this procedure has no detectable 5′–3′ ss-DNA exonuclease activity, ds-DNA exonuclease activity of either polarity, or ss-DNA- or ds-DNA-dependent endonuclease activity. The 3′–5′ ss-DNA exonuclease activity is very low (less than 3% of the label on ss-DNA ends is digested after 1 h). The specific ss-DNA-dependent ATPase activity of helicase II is 2.9 (±0.2) × 10^4 mol of ATP hydrolyzed (mol of helicase II monomer)$^{-1}$ min^{-1} in our standard conditions (see Materials and Methods). The helicase activity of helicase II has been characterized previously (Runyon & Lohman, 1989; Runyon et al., 1990). When stored at −70 °C, helicase II protein retains full ss-DNA-dependent ATPase and helicase activities for at least 3 years. These preparations also retain full ss-DNA-dependent ATPase and helicase activities for at least 6 months at −20 °C and 1 month at 4 °C (the longest periods tested). At helicase II concentrations >0.1 μM monomer, a DNA-independent ATPase activity is detectable in our preparations. For different protein preparations, this activity ranges between 10 and 40 mol of ATP hydrolyzed (mol of helicase II protein)$^{-1}$ min^{-1} (i.e., ~0.03–0.13% of the ATPase activity observed in the presence of ss-DNA).

Extinction Coefficient of Helicase II Protein. The extinction coefficient of native helicase II protein was determined by comparison of the UV absorption spectra of native and denatured (in 6 M guanidine hydrochloride) protein with the spectra for model compounds of tryptophan and tyrosine as described (Lohman et al., 1989) (see Materials and Methods). Helicase II has 13 tryptophans and 23 tyrosines per monomer (Yamamoto et al., 1986); hence, for the *denatured protein*, we calculate ϵ_{280} (6 M Gdn-HCl) = 1.02 × 10^5 M^{-1} cm^{-1}. An average of 11 independent determinations yielded a value for the native protein of ϵ_{280} = 1.06 (±0.05) × 10^5 M^{-1} (monomer) cm^{-1} [1.29 (±0.06) mL mg^{-1} cm^{-1})] in buffer B (25 °C). For the native protein, A_{280}/A_{260} = 1.65 ± 0.11.

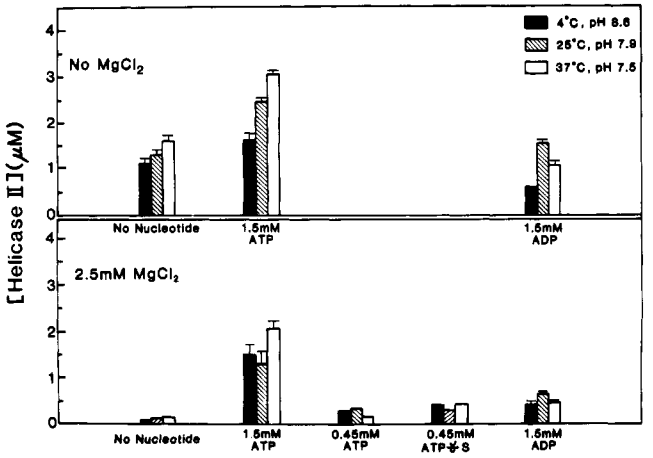

FIGURE 3: Helicase II solubility in buffers used to study ATPase and helicase activities. Helicase II protein solubility (micromolar monomer) was measured by equilibrium dialysis in buffer D in the presence of the indicated concentrations of ATP, ADP, and ATPγS at 4, 25, and 37 °C.

Table II: Effects of Monovalent Salt and ATP Concentration on Helicase II Protein Solubility

[NaX] (mM)	helicase II protein solubility (μM monomer)[a] NaCl	$NaCH_3COO$	NaCl + 1.5 mM ATP
50	1.5 ± 0.1	0.73 ± 0.05	nd[b]
75	1.4 ± 0.1	nd	4.6 ± 0.3
100	1.4 ± 0.1	0.39 ± 0.3	nd
150	2.3 ± 0.2	0.49 ± 0.04	3.9 ± 0.3
200	5.7 ± 0.2	0.91 ± 0.07	nd
250	8.3 ± 0.2	1.2 ± 0.01	8.3 ± 0.6
300	≥8.2	1.7 ± 0.1	nd

[a] Measured by equilibrium dialysis vs buffer C (pH 8.9), 20% (v/v) glycerol, 4 °C. [b] nd, not determined.

Table III: Effects of Temperature, pH, and [NaCl] on Helicase II Protein Solubility[a]

[NaCl] (mM)	helicase II protein solubility (μM monomer)[a] 25 °C pH 7.9	25 °C pH 8.3	37 °C pH 7.5	37 °C pH 7.9
0	2.5 ± 0.2	2.2 ± 0.2	2.4 ± 0.2	2.4 ± 0.2
25	1.5 ± 0.1	2.0 ± 0.2	1.2 ± 0.1	1.9 ± 0.2
50	1.1 ± 0.1	1.3 ± 0.2	0.89 ± 0.07	1.3 ± 0.1
75	1.2 ± 0.1	1.3 ± 0.2	0.99 ± 0.07	1.2 ± 0.1
100	1.3 ± 0.1	2.0 ± 0.2	1.3 ± 0.1	1.4 ± 0.1
150	2.7 ± 0.2	2.1 ± 0.2	2.3 ± 0.2	2.9 ± 0.2
200	2.6 ± 0.2	2.6 ± 0.2	3.1 ± 0.2	3.4 ± 0.2

[a] Measured by equilibrium dialysis vs buffer D, 10% (v/v) glycerol.

Solubility Properties of Helicase II Protein. During our attempts to develop an efficient purification protocol for the helicase II protein, it became apparent that the low solubility of this protein presents a major limitation. Since this also is a limitation for biochemical and physical characterizations of the protein, we undertook a systematic examination of the solubility properties of helicase II protein in a range of solution conditions that are commonly used to study this protein. The results of these studies are presented in Figure 3 and in Tables II–VI. In general, helicase II solubility is quite sensitive to solution conditions, increasing with increasing [glycerol], [NaCl], pH, temperature, and [ATP], but decreasing dramatically in the presence of magnesium.

Helicase II Protein Exists in a Monomer–Dimer Equilibrium. We examined the assembly state of helicase II protein as a function of solution conditions using small-zone gel permeation chromatography by HPLC as described under Materials and Methods, with particular emphasis on solution conditions used in studies of the ATPase and helicase properties of the protein. The average retention time of helicase II protein was examined as a function of protein concentration up to 1.5 μM (monomer), in buffer D (pH 7.5) at 37 °C in the presence and absence of 1.5 mM ATP and/or 2.5 mM $MgCl_2$. A single peak of helicase II protein was observed under all conditions; however, the retention time of this peak was affected by protein concentration, indicating that helicase II undergoes self-assembly and that the oligomeric forms are in rapid equilibrium on the time scale of the chromatographic experiment. On the basis of our solubility studies, all of the helicase II protein was soluble at the concentrations and conditions used in these studies.

Figure 4 shows the protein concentration dependence of the apparent molecular mass of helicase II under several solution conditions. In the absence of Mg^{2+} (±ATP), the apparent molecular mass increases with increasing protein concentration until it reaches a plateau value equivalent to that of a dimer at concentrations near 0.5–1 μM (monomer). In the presence of 2.5 mM Mg^{2+} (no ATP), the apparent molecular mass in this same protein concentration range was lower than in the absence of Mg^{2+}, but still exhibited concentration-dependent behavior consistent with self-assembly; however, a plateau was not reached at these protein concentrations. In the presence of both 2.5 mM Mg^{2+} and 1.5 mM ATP, helicase II eluted at a position equivalent to a monomer at concentrations up to 1.5 μM (monomer). We have also observed that self-assembly of helicase II is inhibited upon raising the [NaCl] (data not shown). Therefore, at these protein concentrations, helicase II exists in a monomer–dimer equilibrium that is sensitive to solution conditions.

Helicase II Dimers Are Stabilized upon Binding DNA. We examined helicase II dimer formation in the presence and absence of ss and ds oligodeoxynucleotides in order to determine if DNA binding affects the monomer–dimer equilibrium. This was examined by treating helicase II with 0.1% glutaraldehyde and monitoring the formation of cross-linked protein dimers by polyacrylamide gel electrophoresis as shown in Figure 5. Cross-linking was carried out in the presence and absence of the single-stranded (ss) oligodeoxynucleotide $d(pT)_{16}$ as well as a 16 bp hairpin duplex HP (see Materials and Methods). On the basis of the estimated helicase II site size on poly(dT) of 10 ± 2 nucleotides (see below), both of these oligodeoxynucleotides are short enough to preclude binding of multiple helicase II monomers in tandem to each oligonucleotide. Cross-linking was performed in the presence of Mg^{2+}, ADP–Mg^{2+}, or the nonhydrolyzable ATP analogue adenosine 5′-(β,γ-imidotriphosphate) [AMP-P(NH)P–Mg^{2+}]. Under the conditions of the experiment, a small amount of cross-linked dimer is observed in the absence of DNA as shown in Figure 5 (lanes B–D). However, in the presence of $d(pT)_{16}$ (lanes E–G) as well as the hairpin duplex (lanes H–J), the extent of dimer formation increased significantly, indicating that helicase II dimerization is facilitated by binding either ss- or ds-DNA. This behavior is similar to that observed for the *E. coli* Rep protein, although a major difference is that Rep dimers are induced only upon binding DNA but do not form detectably in the absence of DNA (at least below 8 μM monomer) (Chao & Lohman, 1991).

We also examined the assembly state of helicase II in the presence of the ss oligonucleotide $d(pT)_8$ by small-zone gel chromatography on Sephacryl S-300HR. In these experiments, $d(pT)_8$ was included in the elution buffer to ensure

Table IV: Effects of pH on Helicase II Protein Solubility[a,b]

buffer	4 °C pH	4 °C solubility (μM monomer)	25 °C pH	25 °C solubility (μM monomer)	37 °C pH	37 °C solubility (μM monomer)
cacodylate–NaOH	6.6	0.027 ± 0.002	6.6	0.14 ± 0.01	6.6	0.035 ± 0.002
cacodylate–NaOH	7.0	0.045 ± 0.003	7.0	0.14 ± 0.01	7.0	0.097 ± 0.007
cacodylate–NaOH	7.4	0.13 ± 0.01	7.4	0.45 ± 0.03	7.4	0.43 ± 0.03
HEPES–NaOH	7.5	0.31 ± 0.02	7.3	0.85 ± 0.06	7.1	0.77 ± 0.06
HEPES–NaOH	7.8	0.58 ± 0.04	7.5	1.6 ± 0.1	7.3	2.2 ± 0.2
HEPES–NaOH	8.1	1.4 ± 0.1	7.8	2.8 ± 0.2	7.6	3.1 ± 0.2
Tris-HCl	7.8	0.23 ± 0.02	7.1	0.31 ± 0.02	6.7	0.20 ± 0.02
Tris-HCl	8.2	0.60 ± 0.05	7.5	1.0 ± 0.1	7.1	0.95 ± 0.07
Tris-HCl	8.3	0.66 ± 0.05	7.6	1.0 ± 0.1	7.2	1.0 ± 0.1
Tris-HCl	8.8	2.5 ± 0.2	8.1	3.1 ± 0.2	7.7	2.1 ± 0.2
Tris-HCl	8.9	2.3 ± 0.2	8.3	2.4 ± 0.2	7.9	1.8 ± 0.2
CHES–NaOH	8.7	2.6 ± 0.2	8.5	2.6 ± 0.2	8.4	2.2 ± 0.2
CHES–NaOH	8.9	2.3 ± 0.2	8.7	2.3 ± 0.2	8.6	2.3 ± 0.2
CHES–NaOH	9.2	1.8 ± 0.2	9.0	2.2 ± 0.2	8.9	1.8 ± 0.2

[a] Measured by equilibrium dialysis. [b] The total concentration of buffer is 25 mM in each experiment. Solutions also contain 10% (v/v) glycerol, 35 mM NaCl, and 5 mM 2-mercaptoethanol.

Table V: Effects of [$MgCl_2$] on Helicase II Protein Solubility

[$MgCl_2$] (mM)	helicase II protein solubility (μM monomer)[a] 5% (v/v) glycerol	10% (v/v) glycerol
0.5	0.66 ± 0.05	1.1 ± 0.1
1	0.38 ± 0.03	0.64 ± 0.05
2	0.13 ± 0.01	0.30 ± 0.02
2.5	0.090 ± 0.007	0.17 ± 0.01
3	0.066 ± 0.005	0.11 ± 0.01
4	0.039 ± 0.003	0.11 ± 0.01
5	0.039 ± 0.003	0.075 ± 0.006
10	0.043 ± 0.003	0.060 ± 0.004

[a] Measured using the filtration assay in buffer D (pH 7.5), 35 mM NaCl, 37 °C.

Table VI: Effects of [Glycerol] and [$MgCl_2$] on Helicase II Protein Solubility

[glycerol] (v/v)	helicase II protein solubility (μM monomer)[a] at [$MgCl_2$] of none	2.5 mM	10 mM
5	0.71 ± 0.05	0.098 ± 0.007	0.024 ± 0.002
6.5	0.79 ± 0.06	0.16 ± 0.01	0.049 ± 0.004
10	1.0 ± 0.1	0.22 ± 0.02	0.11 ± 0.01
15	1.5 ± 0.1	0.34 ± 0.03	0.24 ± 0.02
20	≥1.5	0.59 ± 0.04	0.49 ± 0.04
25	≥1.5	1.0 ± 0.1	1.0 ± 0.1

[a] Measured using the filtration assay in buffer D (pH 7.5), 35 mM NaCl, 37 °C.

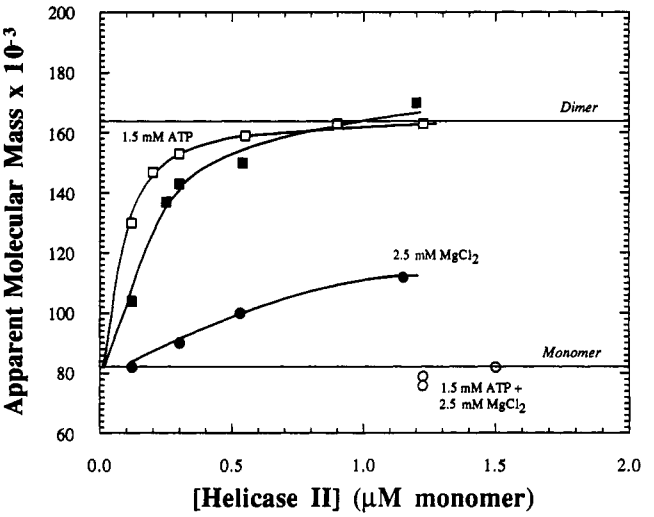

FIGURE 4: Effects of ATP and $MgCl_2$ on the assembly state of helicase II protein in enzymatic reaction conditions. The apparent molecular mass of helicase II protein was estimated by small-zone gel permeation chromatography as a function of helicase II concentration as described under Materials and Methods. The elution buffer was 25 mM Tris-HCl, 10% (v/v) glycerol, 35 mM NaCl, and 4 mM 2-mercaptoethanol, pH 7.5 at 37 °C, and (■) no ATP or $MgCl_2$, (□) 1.5 mM ATP, (●) 2.5 mM $MgCl_2$, or (○) 1.5 mM ATP + 2.5 mM $MgCl_2$.

that helicase II was saturated with oligodeoxynucleotide. Upon application of 16 μL of 2.5 μM helicase II in the presence of 5 μM $d(pT)_8$, helicase II eluted in a symmetrical peak at the same elution volume as alcohol dehydrogenase (M_r = 150 000), indicating that it is dimeric under these conditions (data not shown). As a control, we also performed the same experiment with Rep protein in the presence of saturating $d(pT)_8$ and observed that Rep also eluted at a position consistent with a dimer as observed previously (Chao & Lohman, 1991).

Dimerization of Helicase II Stimulates Its ss-DNA-Dependent ATPase Activity. In order to determine whether dimerization influences the enzymatic properties of helicase II, we examined its ss-DNA-dependent ATPase activity as a function of helicase II concentration in the presence of excess (5 μM) $d(pT)_{10}$. This oligodeoxynucleotide is short enough to preclude the binding of multiple helicase II monomers to an individual oligodeoxynucleotide. Figure 6 indicates that in the presence of 5 μM $d(pT)_{10}$, the specific ATPase activity of helicase II is constant [~2.1 (±0.2) × 10^3 mol of ATP hydrolyzed (mol of helicase II monomer)$^{-1}$ min^{-1}] at monomer concentrations below ~1 nM; however, it increases upon increasing the [helicase II] above 1 nM, until a second plateau is reached above ~10 nM monomer [~5.2 (±0.2) × 10^3 mol of ATP hydrolyzed (mol of helicase II monomer)$^{-1}$ min^{-1}]. The midpoint of the transition occurs at ~5 nM under these conditions. This behavior indicates a concentration-dependent assembly of helicase II into an oligomeric form, presumably a dimer on the basis of the gel filtration and cross-linking data. Furthermore, the data in Figure 6 indicate that dimerization stimulates the ss-DNA-dependent ATPase activity of helicase II, suggesting that dimerization is functionally important.

Apparent Site Size for Helicase II Binding to ss-DNA. Helicase II protein has 13 Trp and 23 Tyr per monomer (Yamamoto et al., 1986; Finch & Emmerson, 1984); however, the fluorescence emission spectrum of helicase II reflects only emission from Trp ($\lambda_{em,max}$ = 336 nm, independent of excitation wavelength). The intrinsic Trp fluorescence is quenched upon binding ss-DNA, and we have used this signal to estimate the average number of nucleotides occluded per helicase II

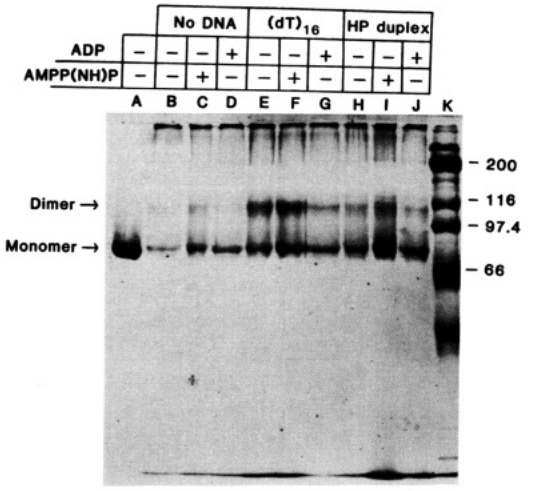

FIGURE 5: Glutaraldehyde cross-linking of helicase II dimers. Helicase II dimers were covalently cross-linked using 0.1% glutaraldehyde under a variety of solution conditions as shown by analysis on a 10% polyacrylamide gel. All cross-linking reactions contained 2 μM helicase II in 20 mM Tricine, pH 8.3, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 20% (v/v) glycerol. Lanes C, F, and I contained 2 mM AMPP(NH)P, and lanes D, G, and J contained 2 mM ADP. Lanes B–D contained no DNA, lanes E–G contained 2 μM $(dT)_{16}$, and lanes H–J contained 2 μM HP.

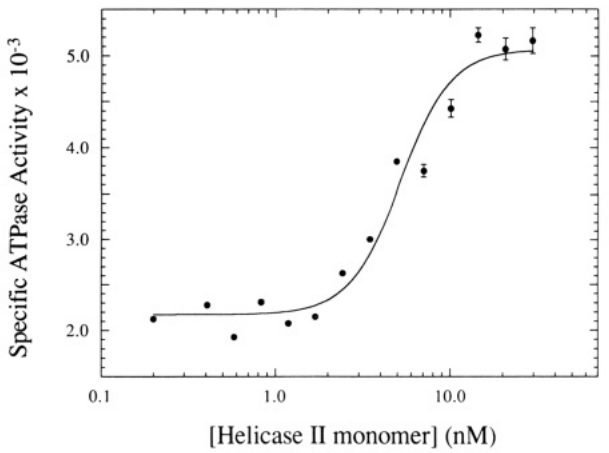

FIGURE 6: Dimerization of helicase II stimulates its ss-DNA-dependent ATPase activity. The ss-DNA-dependent ATPase activity of helicase II was examined as a function of monomer concentration in the presence of excess $(dT)_{10}$ (5 μM) at 37 °C. All ATPase activities were determined using the spectrophotometric assay described under Materials and Methods. Specific Activities (moles of ATP hydrolyzed per minute per mole of helicase II monomer) were obtained by division of the measured activity by the total enzyme concentration.

monomer when bound to ss-DNA (i.e., the apparent site size). Titrations of helicase II with poly(dT) were performed as described under Materials and Methods (Lohman & Overman, 1985). Results of a study performed in buffer D (pH 7.5), 25 °C, at two helicase II concentrations (0.15 and 0.38 μM helicase II monomer) are shown in Figure 7. Under these conditions, the helicase II tryptophan fluorescence is quenched in direct proportion to the amount of poly(dT) added until an initial plateau is reached at 22–24% quenching. The apparent site size, estimated from the intersection of the linear change in quenching and the plateau (see Figure 7), is $\sim 10 \pm 2$ nucleotides per helicase II monomer. Studies at pH 8.3, in the presence of either 100 mM NaCl or 100 mM KCl, gave identical results (L. B. Overman, unpublished observations).

However, upon further addition of poly(dT), the apparent quenching of the protein fluorescence continues to increase without reaching a plateau, even at very high poly(dT) concentrations (see Figure 7). Furthermore, upon addition

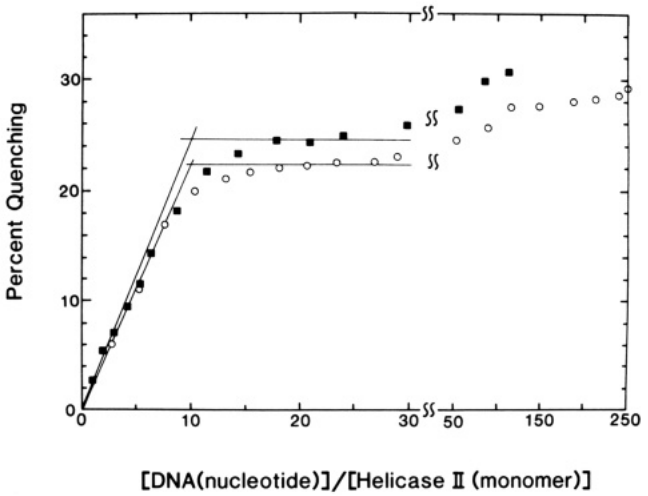

FIGURE 7: Apparent site size of helicase II monomer on poly(dT). The percent quenching of the helicase II tryptophan fluorescence upon binding poly(dT) is plotted as a function of the ratio of total poly(dT) (nucleotide) to the total helicase II monomer concentration in solution. Titrations of helicase II [(○) 0.15 μM and (■) 0.38 μM monomer] with poly(dT) were performed in 25 mM Tris-HCl (pH 7.5), 35 mM NaCl, and 10% (v/v) glycerol at 25 °C. The apparent site sizes (nucleotides occluded per helicase II monomer) were estimated from the point of intersection of the initial linear portion of the titrations and the initial plateau region.

of NaCl in order to dissociate the complex, only a fraction of the initial fluorescence was recovered. The amount of fluorescence recoverable by addition of NaCl was equal to the amount of quenching that occurred up until the first plateau. The additional apparent fluorescence quenching observed at higher poly(dT) concentrations was not due to inner filter or photobleaching effects since corrections for these effects were applied as previously described (Lohman & Overman, 1985; Loman & Mascotti, 1992). Since we were not able to determine the basis for these effects, we did not investigate helicase II–ss-DNA interactions further using fluorescence quenching methods. Although we feel that the estimate of an apparent site size of 10 ± 2 nucleotides per helicase II monomer is not likely to have been influenced by these problems, the resulting site size estimate must be viewed as preliminary at this time. Furthermore, we emphasize that the molecular interpretation of this apparent site size is not necessarily simple, especially since helicase II assembles to a dimer upon binding ss-DNA. If both subunits of the helicase II dimer bind poly(dT), then 10 ± 2 nucleotides represents the true site size per monomer. However, if only 1 of the 2 subunits binds poly(dT) under these conditions, then the true site size is ~ 20 nucleotides per monomer. Therefore, the value of 10 ± 2 should be considered a lower limit at this time.

Effects of ATP Regeneration on ss-DNA-Dependent ATPase Activity of Helicase II Protein. Helicase II protein solubility is low in the presence of Mg^{2+} (no ATP), and ADP does not increase its solubility. As a result, problems due to protein insolubility can arise during ATPase and helicase reactions performed at high helicase II concentrations if ATP is depleted during the reaction. This potential problem is of special concern for DNA unwinding studies that require high helicase II concentrations (Runyon & Lohman, 1989; Runyon et al., 1990); however, this effect can be eliminated if an ATP-regenerating system is used. In light of this, we examined the effect of an ATP-regenerating system on the ss-DNA-dependent ATPase activity of helicase II at low protein concentrations such that the protein remains soluble. ATP hydrolysis was compared using the spectrophotometric (ATP regeneration) and the thin-layer chromatography (TLC)

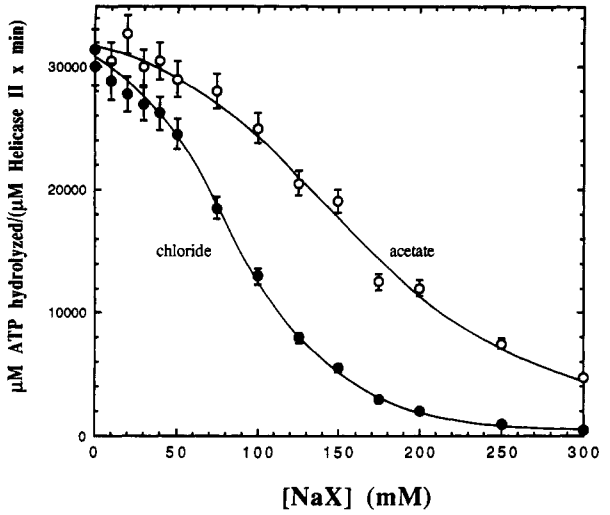

FIGURE 8: Dependence of single-stranded DNA-dependent ATPase activity of helicase II protein on monovalent salt concentration. The steady-state rate of ATP hydrolysis by helicase II (4.1 nM monomer) in the presence of poly(dT) (8.5 μM nucleotide) was measured as a function of the concentration of (●) NaCl and (○) $NaCH_3CO_2$ using the spectrophotometric assay as described under Materials and Methods, except that the concentration of coupling enzymes (pyruvate kinase and lactic dehydrogenase) was increased to 60 units/mL. The ATPase activity is plotted as a function of [NaX] added to the standard reaction mixture which also contains 17 mM Na^+ that is contributed from the ATP and PEP stocks.

assays (no ATP regeneration) under identical reaction conditions [3.7 nM helicase II (monomer) and 8 μM poly(dT) (nucleotide)]. All of the components for the ATP-regenerating system, except pyruvate kinase, were included in the TLC assay in order to maintain equivalent conditions in the absence of ATP regeneration. In one experiment, lactic dehydrogenase (70 μg/mL) was also included as a carrier protein in the TLC assay.

A significantly higher rate of ATP hydrolysis is obtained in the presence of the ATP-regenerating system. The initial velocities, v_0, were 82.5 μM ATP hydrolyzed/min in the TLC assay vs 110 μM ATP/min for the spectrophotometric assay. The ATPase activity measured by TLC was also lower (v_0 = 74.5 μM ATP hydrolyzed/min) in the absence of lactate dehydrogenase (70 μg/mL), but the activity did not increase upon further addition of this carrier protein. Furthermore, the time course of the spectrophotometric assay remained linear up to 10 min, whereas the TLC assay was nonlinear over this time period, presumably due to product inhibition. This result suggests that the helicase II ATPase activity is sensitive to product inhibition and that ATP regeneration eliminates this inhibition.

Effects of Solution Conditions on ss-DNA-Dependent ATPase Activity. The affinity of most proteins for DNA decreases upon raising the monovalent salt concentration (Lohman & Mascotti, 1992; Record et al., 1976, 1978). As a result, the ATPase activity of DNA-dependent ATPases, such as helicases, is also generally inhibited upon increasing the salt concentration. Although the dominant effect of salt on protein–DNA interactions is generally due to the displacement of cations from the DNA upon protein binding, effects of anions have also been observed indicating anion binding to the protein (Lohman et al., 1989; Lohman & Mascotti, 1992; Overman et al., 1988; Leirmo et al., 1987). We have previously shown that the DNA helicase activities of both helicase II (Runyon & Lohman, 1989) and *E. coli* Rep helicase (Lohman et al., 1989) decrease with increasing monovalent salt concentration, with clear differences due to anion type (Runyon & Lohman, 1989). Therefore, we compared the effects of NaCl vs $NaCH_3CO_2$ on the poly(dT)-dependent ATPase activity of helicase II, and the results are shown in Figure 8. Increasing salt concentration inhibits the ss-DNA-dependent ATPase activity over the range from 0 to 300 mM Na^+. However, above 10 mM Na^+, higher ATPase activity was observed when acetate replaces chloride.

Experiments at high helicase II concentrations [>0.25 μM (monomer)] are typically performed in buffers containing 10–20% (v/v) glycerol to increase helicase II solubility. Therefore, we examined the effects of glycerol concentration on helicase II ATPase activity over the range from 0 to 25% (v/v) glycerol. ATPase activity decreases linearly with increasing glycerol concentration as described by eq 1 (specific

$$\text{specific ATPase activity} = (3 \times 10^4) - 573[\%\ \text{glycerol (v/v)}] \quad (1)$$

ATPase activity has units of moles of ATP hydrolyzed per mole of helicase II monomer per minute). This is qualitatively similar to the behavior observed for the *E. coli* Rep protein (Lohman et al., 1989), although the relative inhibition of helicase II ATPase activity is less than observed for Rep protein.

The molecular interpretations of the effects of salt concentration and glycerol or any solution variable (e.g., pH, temperature) on the ATPase activity of helicase II are complicated since ATPase activity is clearly dependent upon helicase II DNA binding affinity as well as its monomer–dimer equilibrium. Therefore, such interpretations must await direct quantitative studies of the effects of these solution variables on DNA binding and dimerization.

DISCUSSION

We describe a procedure for the large-scale purification of *E. coli* helicase II protein, starting with cells in which helicase II protein accumulates to approximately 2% of the total cellular protein after temperature induction. The procedure yields ~60 mg of >99% pure helicase II protein from 250 g of induced N4830/pTL51 cells, which is a substantial improvement over previous procedures (Richet & Kohiyama, 1976; Abdel-Monem et al., 1977a; Hickson et al., 1983; Yamamoto et al., 1986; Kushner & Maples, 1988). Development of this procedure was aided by systematic studies of helicase II protein solubility as a function of solution conditions, which also enabled us to obtain concentrated protein stocks (1–2 mg/mL). Knowledge of the limits of helicase II protein solubility has also facilitated biochemical and biophysical characterization of the protein, in particular the unwinding of blunt-ended and nicked-circular DNA which requires high helicase II concentrations (Runyon & Lohman, 1989; Runyon et al., 1990).

Helicase II solubility is sensitive to glycerol, monovalent salt, pH, temperature, Mg^{2+}, and nucleotide cofactors. Solubility is dramatically reduced in all conditions that are generally used to measure enzyme activity when Mg^{2+} is present in the absence of ATP. However, inclusion of ATP (>1 mM) increases helicase II solubility in solutions containing Mg^{2+}. We estimate the solubility of helicase II to be >4.5 μM (monomer) in buffer D in the presence of ATP and Mg^{2+}. The protein will remain soluble at this concentration throughout an enzymatic reaction in the presence of an ATP-regenerating system, whereas in the absence of ATP regeneration helicase II will precipitate as ATP is depleted. Our studies also show that in solutions which contain Mg^{2+},

insoluble helicase II protein can be rendered soluble by addition of ATP or poly(dT).

Helicase II Protein Undergoes a Monomer–Dimer Equilibrium, and Dimerization Stimulates Its ATPase Activity. Knowledge of the assembly state of enzymes that bind DNA, such as helicases, and the factors that influence the assembly state (Mg^{2+}, ATP, ADP, salt, pH, temperature, DNA) is required to obtain a molecular understanding of how such enzymes function. In general, the enzymatic properties of most enzymes are influenced by their state of assembly. Furthermore, if DNA binding is linked to assembly, then this linkage must be understood in order to interpret DNA binding studies (Wong et al., 1992; Wong & Lohman, 1992). Previous studies have reported that helicase II remains monomeric (Richet & Kohiyama, 1976; Abdel-Monem et al., 1977a); however, we have shown that helicase II can self-assemble to form at least dimers under a variety of solution conditions in the absence of DNA. Furthermore, dimers of helicase II are stabilized upon binding short oligodeoxynucleotides, and dimerization stimulates the ss-DNA-dependent ATPase activity of helicase II. Under conditions that are typically used to investigate its ATPase and helicase activities (1.5 mM ATP and 2.5 mM $MgCl_2$), helicase II remains monomeric up to concentrations of at least 1.5 μM in the absence of DNA; however, dimerization is facilitated upon binding oligodeoxynucleotides. Therefore, under such conditions, helicase II dimerization is linked to oligodeoxynucleotide binding and thus is similar to the DNA-induced dimerization of the *E. coli* Rep protein. However, Rep protein remains monomeric up to concentrations of at least 8 μM and is induced to dimerize only upon binding DNA under all conditions tested (Chao & Lohman, 1991; Wong et al., 1992; Wong & Lohman, 1992). On the other hand, helicase II dimers can form even in the absence of DNA under some conditions, particularly in the absence of Mg^{2+}. Although we have shown that helicase II dimers are formed upon binding short oligodeoxynucleotides, 16 nucleotides or base pairs long, we have not determined whether larger helicase II oligomers can form upon binding longer stetches of DNA or at ss/ds-DNA junctions.

The assembly states of all helicases that have been examined carefully have been shown to be oligomeric, usually dimeric or hexameric (Lohman, 1992), although the *E. coli* Rep helicase dimerizes only upon binding DNA (Chao & Lohman, 1991). There is also good evidence that the active forms of at least three helicases, *E. coli* Rep, *E. coli* RecBCD, and SV40 T antigen, are oligomeric (Lohman, 1992). Most proposed mechanisms of DNA unwinding require the functional helicase to possess at least two DNA binding sites whose affinity for DNA can be modulated by binding and hydrolysis of nucleotide cofactors (Lohman, 1992; Yarranton & Gefter, 1979). In fact, a relatively simple solution to the problem of obtaining multiple DNA binding sites is for an active helicase to be oligomeric (Lohman, 1992). It has been proposed that a Rep homodimer is the active form of the Rep helicase on the basis of the fact that a cross-linked Rep dimer retains its ability to unwind duplex DNA in vitro (Chao & Lohman, 1991), and a model has been proposed in which the Rep dimer unwinds DNA by forming an intermediate complex in which the dimer is bound simultaneously to both ss-DNA and duplex DNA at an unwinding fork (Lohman, 1992; Wong & Lohman, 1992). A conformational change that is coupled to ATP hydrolysis is proposed to destabilize a region of the duplex at the fork, and translocation of the dimer then occurs by a "rolling" mechanism (Lohman, 1992; Wong & Lohman, 1992). Such a mechanism is formally possible for any oligomeric helicase and hence also helicase II if its active form is dimeric.

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