The *Escherichia coli* RecQ Helicase Functions as a Monomer*

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The RecQ helicases belong to an important family of highly conserved DNA helicases that play key roles in chromosomal maintenance, and their defects have been shown to lead to several disorders and cancer in humans. In this work, the conformational and functional properties of the *Escherichia coli* RecQ helicase have been determined using a wide array of biochemical and biophysical techniques. The results obtained clearly indicate that *E. coli* RecQ helicase is monomeric in solution up to a concentration of 20 μM and in a temperature range between 4 and 37 °C. Furthermore, these properties are not affected by the presence of ATP, which is strictly required for the unwinding and translocating activity of the protein, or by its nonhydrolyzable analogue 5′-adenyl-β,γ-imidodiphosphate. Consistent with the structural properties, functional analysis shows that both DNA unwinding activity and single-stranded DNA-stimulated ATPase specific activity were independent of RecQ concentration. The monomeric state was further confirmed by the ATPase-deficient mutants of RecQ protein. The rate of unwinding was unchanged when the wild type RecQ helicase was mixed with the ATPase-deficient mutants, indicating that non-protein-protein interactions were involved in the unwinding processes. Taken together, these results indicate that RecQ helicase functions as a monomer and provide new data on the structural and functional properties of RecQ helicase that may help elucidate its mechanism in solution.

Genetic information is stored into the double-stranded DNA molecule that is stabilized through specific hydrogen-bonded base pairs. However, replication and repair as well as recombination of the DNA molecule require a single-stranded DNA to be available at least transiently. In cells, unwinding and separation of the complementary strands of duplex DNAs (dsDNA) into single-stranded DNAs (ssDNA) are catalyzed by a class of enzymes known as helicases. DNA helicases destabilize and unwind the duplex DNA through a series of energetic states, driven by the binding and hydrolysis of ATP, and subsequent release of NDP and inorganic phosphate. Thus, DNA helicases convert the chemical energy into mechanical energy for DNA unwinding and translocation along the nucleic acid lattice (for reviews, see Refs. 1–3).

During unwinding of dsDNA, and to translocate processively without dissociation from DNA, the helicase must use at least two DNA-binding sites to keep contact with the DNA lattice; one binds to ssDNA for translocating, whereas the other binds to dsDNA for DNA unwinding. These two DNA binding sites may be located at different domains within a single polypeptide of an oligomer or be held by two different polypeptides within a dimer or an oligomer for providing multiple DNA-binding sites. Corresponding to the first possibility, the “inchworm” model was proposed, in which the helicase is assumed to possess two nonidentical DNA binding sites; the “leading” site binds both ssDNA and dsDNA and interacts with the duplex to be unwound during successive unwinding cycles, whereas the “lagging” site interacts only with ssDNA. The disruption of the dsDNA at the leading site and the translocation of the enzyme are the result of conformational change of the enzyme modulated by binding and hydrolysis of ATP. Recent crystal structures of complexes of PcrA helicase with a partial dsDNA duplex substrate show that the ssDNA and dsDNA bind, respectively, on two domains of this monomeric helicase (5). These data provided direct proof to support an inchworm mechanism (6).

Corresponding to the second possibility, the active “rolling” model requires that the enzyme be oligomeric and at least dimeric. Each protomer possesses an identical DNA binding site. Both sites could bind either ssDNA or dsDNA, and binding of ssDNA and dsDNA cannot occur simultaneously in the same subunit. Binding of ATP leads to the enzyme interacting alternatively with the ssDNA and dsDNA at the junction region. Furthermore, hydrolysis of ATP destabilizes hydrogen bonds between the base pairs of the duplex. This model was originally based on the observed allosteric effects of ATP and ADP on the ssDNA and dsDNA binding properties of the *Escherichia coli* Rep dimer (7). However, the crystal structures of Rep helicase bound to ssDNA alone or bound to both ssDNA and ADP have revealed that the protein remained monomeric; no protein-protein interactions were observed (8).

An essential difference between the “inchworm” and “rolling” model is that an oligomer, at least a dimer, is absolutely required for translocation and unwinding in the case of the rolling model, whereas a monomeric form or any oligomeric form could function in the inchworm model. Thus, the knowledge of the oligomeric structure of a helicase is of fundamental concern in understanding the mechanism by which the protein unwinds DNA.

The objective of this work is to investigate the structural state of the *E. coli* RecQ helicase in solution, a protein of 610
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EXPERIMENTAL PROCEDURES

Reagents—Chemicals were reagent grade, and all solutions were prepared using ELGA pure water. ATP and AMPNP were purchased from Sigma. Buffer A is 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 3 mM MgCl₂, 0.1 mM dithiothreitol.

Nucleic Acid Substrates—Oligonucleotides were purchased from Genset and further purified by polyacrylamide gel electrophoresis. The sequences of the oligonucleotides used in this study are listed in Table I. A 100 μM working stock of double-stranded DNA was prepared by mixing equal concentrations of each oligonucleotide in 10 mM Tris-HCl (pH 7.5 at 25 °C), 100 mM NaCl, followed by heating the mix for 5 min at 85 °C and then cooling it slowly to room temperature.

Protein Expression—A full coding region of RecQ DNA was prepared by PCR using E. coli chromosome DNA. A 2.2-Kb PCR product was cloned into pGEM-T Easy vector, and the sequence of this insert was shown to be identical to the published RecQ gene sequence (20). The DNA corresponding to the coding sequence of the RecQ helicase gene was amplified again by PCR-mediated amplification, using a set of oligonucleotides corresponding to the 5′- and 3′-ends of the gene. These primers had additional bases at each end for creation of restriction sites: NdeI at the 5′-end (5′-GGATCCTTCGAGATTTAGGTTAAGTTAGGTTAACGCTGGC-3′) and XhoI at the 3′-end of the gene (5′-GGATCCTTCGAGATTTAGGTTAAGTTAGGTTAACGCTGGC-3′). The amplified DNA was subcloned into pGEM-T Easy vector and transformed into E. coli. The recombinant plasmid was purified and subcloned, respectively, into NdeI/XhoI sites of the vector PET-15b and pNB5 (21). The PET-15b vector encodes a hexahistidine tag at the amino terminus that allows purification of the expressed protein on a nickel-chelating column. A thrombin cleavage site was adjacent to the histidine tag. The constructed plasmid was transformed into E. coli strain BL21 (DE3). The bacteria were grown at 37 °C in terrific broth supplemented with 50 μg/ml ampicillin. The expression of the protein was induced by adding isopropylthio-β-D-galactoside to 0.5 mM at low log phase (A₆₀₀ = 0.6). The culture was then incubated for 3 h at 37 °C. Cells were harvested by centrifugation at 3,000 × g for 15 min at 4 °C. For expression of the RecQ helicase bearing a c-myc tag at the carboxyl terminus, but without the histidine tag at the amino terminus, the pNB5 vector containing the RecQ gene was transformed into E. coli BL21(DE3). The protein expression and purification were essentially as described by Benaroudj et al. (21).

Protein Purification—His-tagged RecQ helicase was overexpressed in E. coli BL21 (DE3) and purified under native conditions. Briefly, harvested cells were suspended in 30 ml of suspension buffer (20 mM Tris-HCl, pH 7.9, 0.5 mM imidazole, 500 mM NaCl) and were lysed by using a French pressure cell. The lysate was then sonicated in order to shear DNA into small fragments. The lysate was cleared by centrifugation at 70,000 × g for 30 min at 4 °C. The supernatant was applied to the column charged with histidine binding resin (Novagen). The column was washed with 20 mM Tris-HCl (pH 7.9) buffer containing 500 mM NaCl, 80 mM imidazole. The proteins bound to the column were eluted stepwise using 20 mM Tris-HCl (pH 7.9) buffer containing 100, 200, 300, 400, or 500 mM imidazole. RecQ helicase-containing fractions, identified by both DNA-dependent ATP hydrolysis and helicase activity assays, were pooled. The histidine tag was cleaved using biotinylated thrombin during a dialysis step. The removal of biotinylated thrombin was accomplished using streptavidin-agarose magnetic beads (Novagen, Madison, WI). RecQ helicase was further purified by FPLC size exclusion chromatography (Superdex 200; Amersham Biosciences). Finally, ion exchange chromatography (DEAE Sephadex A-50) was used to remove the contaminating DNA. The active fractions were pooled, dialyzed against storage buffer (25 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 500 mM NaCl, 2 mM dithiothreitol), and stored at −80 °C. The protein was pure as judged by Coomassie staining and electrophoresis mass spectrometry.

Protein concentration was determined spectrophotometrically using an extinction coefficient at 280 nm of 1.54 × 10⁴ M⁻¹ cm⁻¹.

Mutagenesis—Site-directed mutagenesis was performed using the QuikChange™ kit (Stratagene) according to the manufacturer’s instructions. Amino acid substitutions were made in conserved motif I (K55A) and in conserved motif II (D148A) using oligonucleotides C and D (Table I). The mutations were confirmed by DNA sequencing (MWG Biotech). The mutant helicases were expressed in E. coli BL21 (DE3) cells and purified as described above for wild type enzyme.

Immunoprecipitation—Anti-His tag monoclonal antibodies were immobilized onto CNBr-activated Sepharose 4B at 2.5 mg of protein/ml of packed beads (22, 23). For homodimerization studies, 0.2 ml of the mix of 5 or 10 μM His-tagged and c-myc-tagged tagged helicase was incubated at 4 °C in buffer A for 1 h. The samples were mixed with 150 μl of anti-His tag antibody beads. The beads were then washed twice with 5 ml of 10 mM Heps, pH 7.4. The bound proteins were eluted from the beads by the addition of 0.1 M NaCl, 20 mM Heps, pH 7.4, 1 M acetic acid containing 0.2% Triton X-100.

SDS-PAGE and Western Blotting—SDS-polyacrylamide gel electrophoresis was carried out on 10% polyacrylamide gels. For Western blotting analysis, 25 μl of the samples from immunoprecipitation experiments were analyzed by SDS-PAGE. The gels were electroblotted onto Immobilon membranes. For immunoblotting analysis, the blots were blocked in phosphate-buffered saline, containing 0.9% Tween 20 and 5% nonfat milk, and incubated with anti-c-myc monoclonal antibodies (dilution 1:1000) for 45 min. The blots were then washed in phosphate buffer (0.05% Tween 20) and further incubated in blocking buffer containing 1:1000 anti-mouse immunoglobulin peroxidase for 30 min. The blots were washed again in phosphate buffer (0.05% Tween 20) and visualized using the Western blotting detection kit (KPL Inc., Gaithersburg, MD). The blots were subsequently exposed to Kodak X-OMAT film.

ATPase and Helicase assays—The ATPase activity was measured according to the literature (24). One unit of ATPase activity is defined as the amount of RecQ protein required to hydrolyze 1 nmol of ATP/min at 37 °C (16). Helicase activity was determined by Xu et al. (25) using DNA substrate B (Table I).

| Substrate | Length | Structure and sequence |
|-----------|--------|------------------------|
| A         | 21     | 5′-GGT TGG AAA ATC TCT AGC AGT-3′ |
| B         | 63/45  | 5′-GGAGATACAGTGGCGTTCGACAGCGC-3′ |
| C         | 33     | 5′-GGGACATACAGTGGCGTTCGACAGCGC-3′ |

F represents the fluorescein chemical group.
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Helicase-DNA Binding Activity Assay—Binding of RecQ helicase to DNA was analyzed by fluorescence polarization using a BioTek fluorescence polarization spectrophotometer (PanVera). RecQ helicase (200 nM) was added to a 150-μl aliquot of buffer A containing 1 mM of 21-mer fluorescein-labeled DNA (substrate A). Each sample was incubated for 5 min at 25 °C, after which fluorescence polarization was measured. In order to ensure that the mixture had reached equilibrium, the sample was further incubated for 30 min, and a second reading then was taken. No significant change was observed between the two measurements, indicating that equilibrium was reached.

Size Exclusion Chromatography—Size exclusion chromatography was done at 25 °C, using an FPLC system (Amersham Biosciences), on a Superdex 200 (analytical grade) column equilibrated with buffer A. Elution was performed using the same buffer. The RecQ helicase was quantified by monitoring the absorbance at 280 nm. Fractions of 0.5 ml were collected at a flow rate of 0.4 ml/min, and absorbance was measured at 280 and 260 nm. For ATP and AMP-PNP experiments, 1 mM ATP or AMP-PNP was present in all buffers. It is well known that proteins migrate through a gel filtration column as a function of both the molecular weight and the molecular shape. The Rs values (Rg designates the Stoke radius of the protein) of the RecQ molecule in different conditions were determined from the plot of log Rs versus Vm using the different Stokes radii of the standards. The partition coefficient Kst was calculated using the formula Kst = (Vc − Vo)/Vm, where Vc is the elution volume of the sample, Vo is the excluded volume of the column, and Vm is the total volume of the column. The excluded volume, Ve (7.52 ml), and the total volume, Vt (23.5 ml) were measured by calibration with dextran blue and thymidine. The calibration graph of log Rs versus Vm was constructed using a high and low molecular weight calibration kit from Sigma (26): cytochrome c (12.4 kDa; Rs = 12 Å), carbonic anhydrase (29 kDa; Rs = 22.5 Å), albumin (67 kDa; Rs = 35.5 Å), phosphorylase b (97.4 kDa; Rs = 38.75 Å), and thyroglobulin (669 kDa; Rs = 85 Å). Assuming similar shape factors, the plot calibration of log Mw versus Rs allowed the determination in a first approximation of the molecular weight of helicase.

Sedimentation Velocity—Sedimentation velocity experiments were performed at 4 °C with a Beckman Optima XL-A analytical ultracentrifuge equipped with an An T6 60 titanium four-hole rotor with two-channel 12-mm path length centripetals. Sample volumes of 400 μl in the buffer A were centrifuged at 55,000 rpm, and radial scans of absorbance (λ = 280 nm) were taken at 10-min intervals. The experimental base lines were measured for each sample at the end of the run. RecQ protein remained stable over the course of the run as shown by both the ATPase and the helicase activity assays. Data analysis and determination of the hydrodynamic parameters (the diffusion constant D and the sedimentation coefficient s) were performed using the computer programs supplied by Beckman, SEDTHERP and SVEDBERG supplied by John Philo. The partial specific volume, Vm, 0.7286 ml/g, was calculated from the amino acid composition. Molecular mass is related to D and s by the equation,

\[ \text{Mass} = \frac{RTS(1 - \nu)}{D} \]  

where \( \nu \) is the solvent density, and R and T are the molar gas constant and the absolute temperature, respectively.

Sedimentation Equilibrium—Sedimentation equilibrium experiments were carried out as described by Bailey et al. (27) at 4 °C using different rotor speeds (from 4,500 to 15,000 rpm). Enzyme samples were dialyzed extensively against buffer A. During the dialysis, the buffer was changed three times. The reference cells were filled by the buffer used for the last dialysis. Radial scans of the absorbance at 280 nm were taken at 2-h intervals, and samples were judged to be at equilibrium by the absence of systematic deviation in overlaid successive scans and when a constant average Mr was obtained in the plot of Mr versus centrifugation time. RecQ protein remained stable over the course of the run as judged by both the ATPase and the helicase activity assays. The experimental data were fitted to a model for a single homogeneous species following the equation,

\[ A(r) = [A(r_\infty) \exp \sigma (r^2 - r_\infty^2)] + \delta \]  

where \( \sigma = Wa_0^2RTW / MW(1 - \nu) \), \( A(r_\infty) \) is the absorbance of the solute at the meniscus, \( A(r) \) is the absorbance at a radial distance \( r \) from the center of rotation, and W is the buoyant molecular weight. Mr and r designate molecular weight and partial specific volume of solute, respectively, \( \rho \) is the density of the solvent, \( \omega \) is the angular velocity, R and T are the molar gas constant and the absolute temperature, respectively, and \( \delta \) is the base line offset. The partial specific volume for the protein was calculated from the amino acid composition of the protein sample, and the densities of the different buffers were determined from published tables (28, 29).

Time-resolved Fluorescence Measurements—The time-resolved fluorescence anisotropy was obtained from the two polarized emission decays Ivt(t) and Ivh(t), using the time-correlated single-photon counting technique. Ivt corresponds to the emission intensity (I) when both excitation and emission polarizers are vertical (v). Ivh corresponds to the emission intensity (I) when excitation polarizer is vertical (v) and emission polarizer is horizontal (h). The excitation light pulse source was a Ti:sapphire subpicosecond laser (Tsunami, Spectra Physics) associated with a third harmonic generator tuned at 299 nm for tryptophan fluorescence. The repetition of the laser was set down to 4 MHz, and to ensure the single-photon counting condition, the counting rate never exceeded 40 kHz. The emission light pulse was triggered by a Hamamatsu photodiode (S4753). The fluorescence emission was detected through a monochromator (SpectraPro 150, ARC) set at the appropriate wavelength (Δλ = 15 nm) (emission wavelength was 350 nm). A time-correlated single photon-counting SPC-430 card (Becker-Hick GmbH) was used for the acquisition. The function of the instrumental response of the laser pulse (100 ps) was recorded by detecting the light scattered by a water solution. The time scaling was 11 ps/channel, and 4096 channels were used. The two polarized components of the emission decay were collected until the total count of the Ivt component reached 15–20 million for tryptophan fluorescence or 25–35 million for fluorescein fluorescence. The microcoul (volume 50 μl) was thermostated with a Haake type-F9 circulating bath. Unless otherwise specified, the Trp fluorescence measurement mixture included 20 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 0.1 mM dithiothreitol, and helicase RecQ at the appropriate concentration in the presence of either 75 or 500 mM NaCl. The data were analyzed by the quantified maximum entropy method (30, 31), which displays the lifetime (τ) and the correlation time (θ) distributions according to Equations 3 and 4,

\[ Ivt(t) = \frac{1}{3} \sum_{i=1}^{n} a_i \exp \left( -\frac{1}{2} \sum_{j=1}^{m} \rho_{ij} e^{\Delta \nu_i} \right) \]  

and

\[ Ivh(t) = \frac{1}{3} \sum_{i=1}^{n} a_i \exp \left( -\frac{1}{2} \sum_{j=1}^{m} \rho_{ij} e^{-\Delta \nu_i} \right) \]  

where \( a_i \) represents the relative population of fluorophores with lifetime \( \tau_i \) and \( \rho_{ij} \) represents the initial anisotropy of molecules characterized by the rotational correlation time \( \theta_i \). The decay of the total fluorescence intensity, \( I(t) \), is calculated from both polarized components by the equation,

\[ I(t) = Ivt(t) + 2G \times Ivh(t) \]  

where G represents the correction factor for the difference in the monochromator transmission between parallel and perpendicular polarized components (G-factor = Ivt/Ivh). The anisotropy (r) is then defined by the following relation.

\[ r(t) = \frac{Ivt(t) - G \times Ivh(t)}{I(t)} \]  

For a spherical macromolecule, the hydrated molecular volume (V) is related to \( \theta \) by the Perrin equation,

\[ \theta = \frac{4V}{kT} \]
RESULTS

Purification and Characterization of the RecQ Helicase—The RecQ helicase was overexpressed in and purified from *E. coli*. Gel electrophoresis of the protein in denaturing conditions gave a single band corresponding to a molecular mass of about 70 kDa, consistent with the value determined from the amino acid sequence (68,290 Da). The purity and the molecular mass of the protein were further confirmed by electrospray mass spectrometry, which gives a mass of 69,000 ± 250, with purity exceeding 95%. The protein purified as above is active as an ATPase with a specific activity of 420 units/µg of protein, which is comparable with the previous determinations (16). The protein also displays DNA unwinding activity with an apparent unwinding rate of 2 bases/s, consistent with previous results (19).

Analysis of RecQ Helicase by Immunoprecipitation—RecQ helicase was first studied qualitatively by immunoprecipitation analysis. We reasoned that if RecQ helicase functions as an oligomer, the protein-protein interactions in the oligomer could be revealed by immunoprecipitation analysis. For this purpose, two different recombinant proteins were prepared; one was His-tagged at the NH₂-terminal end, and the other had a c-myc epitope at the COOH-terminal end. A complex between the purified His-tagged and c-myc-tagged helicases was allowed to form at different concentrations of salt or different temperatures (between 20 and 42 °C) and in the absence or in the presence of ATP. Anti-His tag monoclonal antibodies were used to immunoprecipitate RecQ helicase in the complexes. The immunoprecipitated samples were then analyzed by Western blotting with antibodies against c-myc epitope for detection of the c-myc-tagged helicase. The immunoprecipitation of His-tagged helicase by the anti-His tag monoclonal antibodies failed to co-precipitate the helicase having the c-myc epitope, even in the presence of ATP (results not shown). However, it is still possible that the purified helicases preformed a stable oligomer so tightly that no monomer exchange between the preformed oligomers can occur. We therefore co-expressed the two tagged helicases in the same *E. coli* cell, and then analyzed the cell extracts by immunoprecipitation analysis. No protein-protein interaction could be detected (results not shown). These results, taken together, suggest that RecQ helicase is mainly monomeric both in vitro and in vivo conditions.

Analysis of RecQ Helicase by Size Exclusion Chromatography—In order to further characterize the structure of the RecQ helicase, the protein free in solution was analyzed by size exclusion chromatography. These experiments were first performed in the presence of low concentrations of NaCl (50 mM), conditions in which both the optimum ATPase and DNA unwinding activities are observed. In these conditions, RecQ helicase elutes as a protein of about 57 kDa having a Stokes weight obtained was smaller than expected for a protein of 610 kDa and Stoke radius of 34 Å (Fig. 1, inset; Table II, line 3). This value is in accordance with the one obtained (33.6 Å) using the following empirical relation:

\[ \log(R_s) = 0.369 \log(M_r) - 0.254 \]

where

\[ M_r = \frac{2 \times 10^6 \log(R_s) + 5.42}{4.35} \]

Log(Rs) = log of the Stokes radius (Rs) and log(Mr) = log of the molecular weight (Mr). The data for the RecQ helicase fit well to a single species model with a calculated frictional ratio of 1.1 suggests that the RecQ helicase is mainly monomeric both in vitro and in vivo conditions.

Analysis of RecQ Helicase by Analytical Ultracentrifugation—To obtain further information about the quaternary structure of RecQ helicase in solution, analytical ultracentrifugation experiments were performed. First, sedimentation velocity confirmed the monomeric nature of RecQ helicase as indicated by the presence of a single sharp boundary. Moreover, from 3.6 to 10 μM, the sedimentation coefficient was rather constant (not shown), as expected for a nonassociative single particle. Extrapolating this data to infinite dilution gave a sedimentation coefficient, S20,\textsubscript{w}, of 3.42 S and, when corrected for temperature, an S20,\textsubscript{w}, of 5.28 S (see Table III). The diffusion coefficient was 6.89 cm²/s and allowed the calculation for both the Stokes radius (R = 32.11 Å) and a mass of 69,120 g/mol according to Equation 1 (see “Experimental Procedures”). Thus, the molecular weight obtained by sedimentation velocity was consistent with the one determined by size exclusion chromatography and corresponded to that of a monomer. The calculated frictional ratio of 1.1 suggests that the RecQ helicase is a monomer rather globular (Table III).

Second, sedimentation equilibrium of RecQ helicase, at different rotor speeds and protein concentration, was performed. The data for the RecQ helicase fit well to a single species model that yielded a molecular mass of 69,420 ± 380 Da (Table IV and Fig. 2), in good agreement with the molecular weight obtained by size exclusion chromatography and sedimentation velocity. This is further confirmed using the DISCREEQ program (35), which indicated that 100% of RecQ helicase was monomeric. These data are consistent with the above observations that RecQ helicase is monomeric.

Analysis of RecQ Helicase by Time-resolved Fluorescence Anisotropy—Time-resolved fluorescence anisotropy (TFA) experiments confirmed most of the results obtained by size exclusion chromatography and analytical ultracentrifugation. TFA is
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RecQ helicase was analyzed on a Superdex 200 column in buffer A containing 50 mM NaCl (see “Experimental Procedures”).

| Loaded sample     | Sample concentration | Kav a | R s | Apparent mass |
|-------------------|----------------------|-------|-----|--------------|
| 1                 | RecQ                 | 10    | 0.4 | 29           |
| 2                 | RecQ                 | 20    | 0.4 | 29           |
| 3                 | RecQ/ATP             | 10/1000 | 0.35 | 34           |
| 4                 | RecQ/AMPPNP          | 10/1000 | 0.4 | 29           |
| 5                 | RecQ/AMPPNP          | 10/1000 | 0.4 | 29           |

a The distribution coefficient Kav was measured from the retention time on the column and was used to determine both the apparent molecular weight and the Stokes radius from the calibration curves (see “Experimental Procedures”).

b The experiment was performed with elution buffer containing 300 mM NaCl.

### Table III

Hydrodynamic parameters of RecQ helicase

The molecular weight is obtained by the Svedberg equation. The hydrodynamic parameters were calculated using the partial specific volume value determined from the amino acid composition. s20,w is the corrected sedimentation coefficient at a given temperature in water. f and f° are the experimental and theoretical frictional coefficients, respectively. D20,° is the corrected diffusion coefficient at 20°C in water.

| Parameter          | Value          |
|--------------------|----------------|
| Molecular weight   | 69,120 ± 650   |
| s20,w (S)          | 3.42 ± 0.11    |
| R s (Å)            | 31.12 ± 0.13   |
| f/f°               | 1.1 ± 0.01     |
| D20,° (cm²/s)      | 6.89 ± 0.02    |

### Table IV

RecQ molecular weight determination by sedimentation equilibrium

Angular velocity | RecQ concentration | M_loop | r.m.s.a |
|-----------------|--------------------|--------|---------|
| rpm             | µM                 |        |
| 10,000          | 10                 | 69,420 ± 380 | 0.006   |
| 15              | 68,325 ± 1012      | 0.013  |
| 12,500          | 10                 | 67,453 ± 906 | 0.007   |
| 15              | 67,523 ± 1102      | 0.030  |
| 15,000          | 10                 | 68,638 ± 1001 | 0.023 |
| 15              | 69,258 ± 1005      | 0.024  |
| 17,000          | 10                 | 67,630 ± 960 | 0.006   |
| 15              | 77,215 ± 1203      | 0.014  |

a The r.m.s. is defined as the square root of the variance of the fit and is expressed in optical density units.

Based on the depolarization of light that occurs during the rotational diffusion of macromolecules or biological complexes. The extent of light depolarization between excitation and emission times is then related to the molecular size of the macromolecule. The analysis of the TFA data by the maximum entropy method displays the distribution of rotational correlation times (θ), which are related to the hydrodynamics volumes (see Equation 7). Such a distribution is shown in Fig. 3. RecQ helicase was studied using intrinsic tryptophan fluorescence.

RecQ helicase contains 4 tryptophan residues distributed along the sequence of the entire protein. The lifetime distribution as revealed by the maximum entropy method analysis (τ1 = 0.37 ± 0.04 ns (39%); τ2 = 1.6 ± 0.1 ns (33%); τ3 = 4.05 ± 0.2 ns (21%); τ4 = 7.35 ± 0.25 ns (7%)) leads to an average lifetime of 2.05 ns. As shown in Fig. 3, a 1 µM RecQ helicase preparation displayed a long correlation time of about 50 ns at 20°C, consistent with a low order of oligomerization, mainly a monomeric form (see “Discussion”). Another peak was detected at about 4.9 ns. This peak corresponds most likely to a domain motion that is independent of the global tumbling of the entire protein. The flexibility motions of Trp were characterized by a correlation time distribution below 1 ns (not shown). Between 0.5 and 20 µM, the RecQ helicase displayed a similar long rotational correlation time, indicating that its oligomeric state is not strongly dependent upon the protein concentration in this range (Table V). Since most of the ultracentrifugation data were obtained at 4°C, the TFA experiments were repeated at different temperatures (Table VI). From 4 to 37°C, the long correlation time decreased from 90 ± 13 to 38 ± 5 ns. To take into account the modulation of correlation times by external factors such as the temperature and viscosity, all of the data were normalized at 20°C according to the Perrin equation (Equation 7). As indicated in Table VI, the normalized rotational correlation times did not change significantly as a function of temperature.

![Fig. 2. Analysis of RecQ helicase by sedimentation equilibrium.](image1)

![Fig. 3. Rotational correlation time distribution of DNA-free RecQ helicases.](image2)
RecQ ATPase and Helicase Activities

In order to further confirm that RecQ helicases function as a monomer and no cooperative interactions between the subunits existed, two ATPase-deficient RecQ helicases were prepared for this purpose. It is well established that specific amino acids in the helicase conserved motifs I and II were involved in the ATPase activity of the enzyme (36, 37). With the intention of preparing ATPase-deficient mutants, the amino acid lysine 55 in the helicase conserved motif I and asparagine 147 in motif II were mutated separately. If only one amino acid that is highly conserved in the nucleotide-binding loop is mutated in each modified RecQ protein, the modification should not change the three-dimensional structure of the protein. Such ATPase-deficient mutant should be still able to bind to DNA and involved in the protein–protein interactions but unable to unwind duplex DNA substrates. We reasoned that if RecQ helicases function as a monomer, then the ATPase and helicase specific activities should not be influenced by the increasing concentration of protein. The ssDNA-stimulated ATPase activity of RecQ helicase has been studied as a function of RecQ protein concentration. As shown in Fig. 4, the $k_{\text{cat}}$ value is not affected by protein concentration up to 80 nM. Similar results were observed in the presence of ssDNA and dsDNA of different sequences or different lengths or at a higher temperature (42 °C), suggesting that RecQ helicase monomers are an active form of the enzyme, at least as an ATPase. We next characterized the DNA unwinding activity of RecQ helicase at different protein concentrations. The time courses of the helicase reactions are shown in Fig. 5A. The unwinding rate was obtained from the fit of the unwinding kinetics curve by using a single exponential equation. Consistent with the ATPase assay, the DNA unwinding rate was unchanged as RecQ protein concentration increased (Fig. 5B). All of these experiments indicate that the ATPase and helicase specific activities do not increase by increasing the protein concentration.

Effect of the ATPase-deficient RecQ Helicase on the Wild Type RecQ ATPase and Helicase Activities

In order to further confirm that RecQ helicase functions as a monomer and no cooperative interactions between the subunits existed, two ATPase-deficient RecQ helicases were prepared for this purpose. It is well established that specific amino acids in the helicase conserved motifs I and II were involved in the ATPase activity of the enzyme (36, 37). With the intention of preparing ATPase-deficient mutants, the amino acid lysine 55 in the helicase conserved motif I and asparagine 147 in motif II were mutated separately. If only one amino acid that is highly conserved in the nucleotide-binding loop is mutated in each modified RecQ protein, the modification should not change the three-dimensional structure of the protein. Such ATPase-deficient mutant should be still able to bind to DNA and involved in the protein–protein interactions but unable to unwind duplex DNA substrates. We reasoned that if RecQ helicases function as a monomer, then the ATPase and helicase specific activities should not be influenced by the increasing concentration of protein. The ssDNA-stimulated ATPase activity of RecQ helicase has been studied as a function of RecQ protein concentration. As shown in Fig. 4, the $k_{\text{cat}}$ value is not affected by protein concentration up to 80 nM. Similar results were observed in the presence of ssDNA and dsDNA of different sequences or different lengths or at a higher temperature (42 °C), suggesting that RecQ helicase monomers are an active form of the enzyme, at least as an ATPase. We next characterized the DNA unwinding activity of RecQ helicase at different protein concentrations. The time courses of the helicase reactions are shown in Fig. 5A. The unwinding rate was obtained from the fit of the unwinding kinetics curve by using a single exponential equation. Consistent with the ATPase assay, the DNA unwinding rate was unchanged as RecQ protein concentration increased (Fig. 5B). All of these experiments indicate that the ATPase and helicase specific activities do not increase by increasing the protein concentration.

Therefore, this result shows that RecQ helicase is mainly monomeric in the 4–37 °C temperature range.

**ATPase Activity and DNA Unwinding Activities Assay Versus RecQ Helicase Concentration**—If RecQ helicase functions as a monomer, then the ATPase and helicase specific activities of RecQ helicase should not be influenced by the increasing concentration of protein. The ssDNA-stimulated ATPase activity of RecQ helicase has been studied as a function of RecQ protein concentration. As shown in Fig. 4, the $k_{\text{cat}}$ value is not affected by protein concentration up to 80 nM. Similar results were observed in the presence of ssDNA and dsDNA of different sequences or different lengths or at a higher temperature (42 °C), suggesting that RecQ helicase monomers are an active form of the enzyme, at least as an ATPase. We next characterized the DNA unwinding activity of RecQ helicase at different protein concentrations. The time courses of the helicase reactions are shown in Fig. 5A. The unwinding rate was obtained from the fit of the unwinding kinetics curve by using a single exponential equation. Consistent with the ATPase assay, the DNA unwinding rate was unchanged as RecQ protein concentration increased (Fig. 5B). All of these experiments indicate that the ATPase and helicase specific activities do not increase by increasing the protein concentration.

![Fig. 4. DNA-stimulated ATPase activity of RecQ at varying enzyme concentration.](image)

The ATPase activity was measured for different concentrations of RecQ protein in the presence of 1.0 mM ATP at 25 °C. The DNA unwinding rates, $k_{\text{obs}}$, are plotted as a function of increasing concentration of RecQ helicase. Data in Fig. 5A from the time courses were fitted to the exponential equation: $A_t = A_{\text{exp}} \exp(-k_{\text{obs}} t)$, where $A_t$ is the anisotropy amplitude at time $t$, and $k_{\text{obs}}$ is the observed rate constant.

![Fig. 5. DNA unwinding activity of RecQ helicase at varying enzyme concentrations.](image)

A, DNA unwinding RecQ helicase activity was determined by the fluorescence polarization method (25). 5 nM 63/45-mer duplex DNA (substrate B) was incubated with different concentrations of RecQ helicase. Open circles, 5 nM; closed circles, 10 nM; closed squares, 20 nM; closed triangles, 30 nM; open squares, 40 nM; open triangles, 50 nM. DNA unwinding was initiated upon the addition of 1 mM ATP at 25 °C. B, DNA unwinding rates, $k_{\text{obs}}$, are plotted as a function of increasing concentration of RecQ helicase. Data in Fig. 5A from the time courses were fitted to the exponential equation: $A_t = A_{\text{exp}} \exp(-k_{\text{obs}} t)$, where $A_t$ is the anisotropy amplitude at time $t$, and $k_{\text{obs}}$ is the observed rate constant.

### Table V

| [Helicase] | Long rotational correlation time |
|-----------|--------------------------------|
| $\mu M$   | ns                             |
| 0.5       | 44 ± 9                         |
| 1         | 50 ± 7                         |
| 1.8       | 56 ± 8                         |
| 5         | 56 ± 6                         |
| 10        | 57 ± 3                         |
| 20        | 51 ± 3                         |

### Table VI

| Temperature | Long rotational correlation time | Normalized long rotational correlation time$^a$ |
|-------------|---------------------------------|---------------------------------------------|
| °C          | ns                              | ns                                         |
| 4           | 90 ± 13                         | 56                                         |
| 20          | 56 ± 8                          | 56                                         |
| 25          | 49 ± 6                          | 54                                         |
| 37          | 38 ± 5                          | 54                                         |

$^a$ The normalized values (reference temperature = 20°C) were obtained using the Perrin equation (see “Experimental Procedures”).
omer, the catalytic efficiencies for ATPase and DNA unwinding activities should not be influenced in the presence of increasing concentrations of ATPase-deficient helicase.

As expected, both mutants show no detectable ATPase and helicase unwinding activity (Table VII). The circular dichroism spectrum of both wild type and mutant proteins displayed no difference (results not shown), ruling out the possibility of structural change as the result of the mutation. In order to confirm that the ability of the mutants to bind to DNA was not impaired by a single amino acid substitution, the binding of the mutated RecQ helicases to DNA was studied under the equilibrium binding conditions (Fig. 6). The data obtained from wild type helicase fit well to a hyperbolic equation, whereas the binding isotherms determined from both modified RecQ helicases are sigmoidal. To determine the apparent Kd values, the data were fitted to the Hill equation. The apparent dissociation constants are 25, 29, and 56 nM for wild type, K55A, and D148A, respectively (Table VII). These results indicated that the DNA binding activity of the ATPase-deficient mutants were essentially unchanged compared with that of the wild type helicase.

The ATPase activity of wild type RecQ helicase was determined in the presence of increasing amounts of either the K55A or D148A mutant protein. We observed that the rate of ATP hydrolysis by wild type RecQ helicase did not change as the mutant protein concentration was increased (results not shown), suggesting that there were nonprotein-protein interactions involved in ATPase activity. We next performed a series of unwinding experiments to determine the effect of the mutant protein on the activity of wild type RecQ helicase. In order to give to the DNA substrate an equal opportunity to bind to the protein, all of the experiments described below were performed by premixing the wild type RecQ helicase and mutant helicase, and then the premixed solution was added to the reaction solution containing DNA. First, the unwinding activity of wild type enzyme was measured under the condition in which the different concentrations of wild type RecQ and mutant RecQ were mixed at a 1:1 ratio. Each kinetic curve was fit to a single exponential equation; the apparent unwinding rates were then plotted against the mutant helicase concentrations. As shown in Fig. 7A, the apparent unwinding rate of wild type RecQ helicase remains unchanged, whatever the mutant concentration. In a second experiment, the wild type RecQ helicase was held constant at 30 nM, and the unwinding activity was measured in the presence of increasing amounts of mutant helicase. As shown in Fig. 7B, a mild decrease of the apparent unwinding rate was detected as mutant K55A RecQ concentration increases. A similar phenomenon was observed with D148A RecQ mutant. The observed decrease of the apparent rate can be attributed either to interacting subunits or to simple competition between wild type and mutant protein for binding the DNA substrates. As will be discussed under “Discussion,” these decreases arise from the competition between the two proteins for DNA binding rather than protein-protein interactions. Finally, we performed single-turnover kinetics using the premixed proteins to determine the unwinding rate constants. In this experiment, a large excess of nonspecific “trapping” DNA was added with ATP solution to prevent helicase from reassociating with the DNA substrates, thus ensuring that only the first cycle of unwinding activity is observed. Under these conditions, the unwinding rate constants of wild type helicase are not significantly influenced by the increasing concentration of mutant helicase (Fig. 7B, square). Taken together, these results provide strong evidence that no protein-protein interactions were involved in the DNA unwinding process.

**DISCUSSION**

We have investigated in this work the structural and functional properties of the E. coli RecQ helicase in solution by immunoprecipitation, size exclusion chromatography, analytical ultracentrifugation, and time-resolved fluorescence anisotropy as well as enzyme kinetics. The results presented shed some light on the hydrodynamic and conformational properties of this protein.

**Conformational Properties of RecQ Helicase Free in Solution**—First, all of the data of this study show that RecQ helicase is monomeric in solution. Immunoprecipitation analysis showed that no helicase-helicase interactions occur even in the presence of ATP or AMPPNP. Size exclusion chromatography and sedimentation velocity confirmed this result, since the Rₛ values are consistent with a monomeric 68,290 Da protein. Sedimentation equilibrium experiments further confirmed these observations by a direct determination of the Mₛ of the enzyme. Furthermore, the long correlation time as determined by TFA (about 55 ns at 20 °C) is compatible with the monomeric state, since, for instance, serum albumin, which has similar mass (66 kDa), has a correlation time in the same range (46 ns), and the γ-interferon (34 kDa), half of the mass of RecQ helicase, has a correlation time divided by 2 (27 ns). TFA experiments established that RecQ helicase exists as a monomer up to a concentration of at least 20 μM and that this monomer is stable from 4 to 37 °C.

Size exclusion chromatography and sedimentation velocity experiments allowed the determination of the RecQ helicase Stokes radius. The values found for RecQ helicase are comparable with the above methods and gave Rₛ equal to 34 and 31.1 Å, respectively. This Rₛ knowledge allows deeper insight into the molecular shape of the protein. In fact, the expected theoretical Rₛ for an unhydrated spherical protein characterized by

| RecQ protein | ATPase rate | Helicase rate | Apparent Kd | Hill coefficient |
|--------------|-------------|---------------|-------------|-----------------|
| Wild type    | 35 ± 1.25 s⁻¹ | 0.035 ± 0.012 s⁻¹ | 25 ± 1.01 nM | 1.05 ± 0.17  |
| K55A         | ND*         | ND            | 29 ± 1.12 nM | 1.63 ± 0.28    |
| D148A        | ND          | ND            | 56 ± 2.31 nM | 4.99 ± 0.32    |

* ND, not detectable.
Structural Properties of the RecQ Helicase

TABLE VIII

| Helicase | Amino acids | Identity residues in seven motifs | Oligomerization state | Reference |
|----------|-------------|----------------------------------|-----------------------|-----------|
| RecQ     | 610         | 100                              | Monomer               | This work |
| RecQ1    | 649         | 42.3                             | Dimer                 | 51        |
| WRN      | 1432        | 43                               | Trimer                | 52        |
| BLM      | 1417        | 45                               | Hexamer               | 40        |

*Helicase sequences are from GenBank™. The identity was determined using the Alignp program (infoBiogen), and the sequence of E. coli RecQ helicase was taken as the reference. WRN, Werner syndrome protein; BLM, Bloom syndrome protein.

The three-dimensional structure of RecQ helicase is not yet available. However, the crystallized helicases PcrA and Rep shared homology with RecQ helicase and fold into a globular form that is reminiscent of a crab claw (5, 8, 38). These two proteins comprise two domains. Each domain is composed of a large central parallel β-sheet flanked by α-helices. Moreover, the comparison of the secondary structure of RecQ helicase with those of PcrA and Rep determined from the crystal structures shows that most of the secondary structural elements are conserved in the RecQ, PcrA, and Rep helicases (5, 8). Thus, it is possible that RecQ helicase folds into a very similar three-dimensional structure compared with PcrA and Rep, suggesting that the globular shape may be a common feature of this protein family.

The Monomer Is the Functional Form of RecQ Helicase—To further confirm that RecQ helicase functions as a monomer in solution, we have studied both ATPase and DNA unwinding specific activities as the enzyme concentration increases. In an equilibrium condition system, increasing protein concentra-

![FIG. 7. DNA unwinding in the presence of varying concentrations of RecQ or K55A RecQ helicases. A, the rates of unwinding are plotted as a function of increasing concentrations of a mixture between wild type and mutant proteins at stoichiometric ratios. B, a plot of unwinding rate of the RecQ helicase versus the concentration of the mutant K55A RecQ. The DNA unwinding rate constants were determined from the experiments under conditions in which the concentration of wild type RecQ helicase was kept at 30 nM while the concentration of mutant K55A was increased as indicated in the figure. The line through the experimental data is the best fit to Equation 8 with n = 0.91 ± 0.1. The dashed and dotted lines represent the simulated variation of unwinding rate of RecQ helicase when the enzyme functions as a dimer (n = 2) or hexamer (n = 6), respectively. The squares show the unwinding rates determined from single-turnover kinetics.](image)
results from the fact that the wild type helicase dissociates from the DNA during the course of the unwinding reaction, and the mutant protein could bind to DNA and affect the unwinding rate. If this is true, then the specific activity of RecQ helicase should not be affected by the addition of the mutant under condition in which only single cycle unwinding is performed. As expected, a single-turnover experiment shows that the catalytic constant of wild type helicase is independent of the concentration of mutant RecQ helicase. Taken together, these functional analyses clearly indicate that no subunits interactions are involved in the unwinding processes.

Based on the observation that RecQ helicase displays positive cooperativity in ATP hydrolysis with a Hill coefficient of 3.3, Harmon and Kowalczykowski (19) proposed a multimeric structure of at least three subunits as the active state. Moreover, on the analogy of the hexameric BLM helicase structure (40), the same authors suggested an hexameric structure for RecQ helicase. Although oligomeric enzymes may show a Hill coefficient higher than 1, the reciprocal is not always true. Enzymes that display a positive cooperativity with a Hill coefficient of 1 do not always correspond to an oligomeric structure. It is well established that there is a class of monomeric enzymes that display positive cooperativity for substrate binding (41–45). This positive cooperativity has been explained by a theoretical “monomemal” model (43). This model assumes that monomeric enzyme exists mainly in one conformation state in the absence of ligands and that the binding of ligand induces a conformational transition to a new conformation state that possesses a high affinity for the substrate. Considering the fact that the conformational state of helicases is usually modulated upon binding of DNA or nucleotide cofactor (1), it is not impossible that a monomeric helicase exhibits a positive cooperativity under certain conditions. By contrast, our direct structural and functional analysis of RecQ helicase, using a wide array of biochemical and biophysical method, clearly indicated that RecQ helicase functions as a monomer.

Functional Implication of the Monomeric Nature of RecQ Helicase—Whether the active form of helicases must be oligomeric is still uncertain and under debate (1, 6). Previous biochemical studies on RecB protein, Rep, HCV RNA helicase, and PcrA helicase revealed that the enzyme exist as monomers (46–49). In addition, the available crystal structures of several helicases such as Rep, PcrA, and the hepatitis C virus RNA helicase (5, 8, 50) crystallized with DNA and/or nucleotide show that these proteins are monomeric. The work presented here further enforces the idea that helicases function as monomers (46, 49). The information available on the quaternary structure of RecQ helicase family is summarized in Table VIII. We can note that RecQ helicases adopt different quaternary structures from monomer to hexamer (40, 51, 52). This observation may suggest that (i) oligomeric structure may not be essential for the basic helicase activity or (ii) the quaternary structure of RecQ helicase family appears to be determined by the NH₂- or COOH-terminal amino acid sequences, since the central domains of the RecQ helicases display high homologies (Table VIII). The fact that helicases adopting different quaternary structures display different enzymatic properties suggests that quaternary structure plays an important role in determining the mechanism by which helicases recognize the specific DNA substrate. The quaternary structure may also influence its unwinding rate and its processivity. Thus, monomeric helicases could retain full essential activities.

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REFERENCES

1. Lohman, T. M. (1997) J. Biol. Chem. 272, 2269–2277, 1993
2. Matson, S. W., Bean, D. W., and George, J. W. (1994) Bioessays 16, 13–22
3. Hall, M. C., and Matson, S. W. (1999) Mol. Microbiol. 34, 867–877
4. Tarrant, G. T., and Geffer, M. L. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1658–1662
5. Velankar, S. S., Soultanas, P., Dillingham, M. S., Subramanya, H. S., and Wigley, D. B. (1999) Cell 97, 76–84
6. Soultanas, P., and Wigley, D. B. (2000) Curr. Opin. Struct. Biol. 10, 124–128
7. Wong, I., and Lohman, T. M. (1992) Science 256, 350–355
8. Kardel, S., Haeb, H., Gauss, G. H., Lohman, T. M., and Waksman, G. (1997) Cell 90, 635–647
9. Gangloff, S., McDonald, J. P., Bendixen, C., Arthur, L., and Rothstein, R. (1994) Mol. Cell. Biol. 14, 8391–8398
10. Stewart, E., Chapman, C. R., Al-Khoaidarly, F., Carr, A. M., and Enoch, T. (1997) EMBO J. 16, 2682–2692
11. Parham, K. L., and Blackshear, P. J. (1994) J. Biol. Chem. 269, 29838–29845
12. Ellis, N. A., Groden, J., Ye, T. Z., Straughen, J., Lennon, D. J., Ciocci, S., and Pogosian, A. Vignes for preparation of the figures. We thank Dr. B. Demeler for critical review of the manuscript and Drs. V. Croquette and D. Bensimon for comments, suggestions, and discussions.