The DNA Binding Properties of the Escherichia coli RecQ Helicase*

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The RecQ helicase family is highly conserved from bacteria to men and plays a conserved role in the preservation of genome integrity. Its deficiency in human cells leads to a marked genomic instability that is associated with premature aging and cancer. To determine the thermodynamic parameters for the interaction of Escherichia coli RecQ helicase with DNA, equilibrium binding studies have been performed using the thermodynamic rigorous fluorescence titration technique. Steady-state fluorescence anisotropy measurements of fluorescein-labeled oligonucleotides revealed that RecQ helicase bound to DNA with an apparent binding stoichiometry of 1 protein monomer/10 nucleotides. This stoichiometry was not altered in the presence of AMP-PNP (adenosine 5′-(β,γ-imido) triphosphate) or ADP. Analyses of RecQ helicase interactions with oligonucleotides of different lengths over a wide range of pH, NaCl, and nucleic acid concentrations indicate that the RecQ helicase has a single strong DNA binding site with an association constant at 25 °C of $K = 6.7 \pm 0.95 \times 10^6 \text{M}^{-1}$ and a cooperativity parameter of $\alpha = 25.5 \pm 1.2$. Both single-stranded DNA and double-stranded DNA bind competitively to the same site. The intrinsic affinities are salt-dependent, and the formation of DNA-helicase complex is accompanied by a net release of 3–4 ions. Allosteric effects of nucleotide cofactors on RecQ binding to DNA were observed only for single-stranded DNA; AMPPNP, adenosine 5′-imido) triphosphate; MES, 2-(N-morpholino)ethanesulfonic acid.

DNA helicases catalyze the unwinding of duplex DNA (dsDNA)$^1$ to provide the single-stranded DNA (ssDNA) intermediate that is required for diverse DNA metabolic processes such as DNA replication, recombination, transcription, and DNA repair (1). DNA helicases function as molecular motors and use the chemical energy derived from nucleoside 5′-triphosphate binding or hydrolysis to mechanically disrupt the hydrogen bonds between the two strands in dsDNA and to translocate along DNA for processive unwinding (2).

The RecQ helicase family, which is widespread in diverse organisms from Escherichia coli to humans, has become more interesting since the discovery that several human diseases, such as Bloom’s, Werner’s, and Rothmund-Thompson syndromes, are linked with these enzymes (3–5). Analyses of these and related diseases at the cellular and molecular levels led to the conclusion that RecQ helicase plays a conserved role in the preservation of genomic integrity (6–8). An increasing amount of data has shown that the RecQ helicase family acts in concert with other proteins to perform functions in multiple processes in vivo. A number of proteins, including topoisomerases (9, 10), primase (11), polymerases (12, 13), proliferating cell nuclear antigen (10), replication protein A (14), PS3 protein (15), and FLAG endonuclease 1 (16), have been identified to interact physically and functionally with Werner and Bloom syndrome proteins and RecQ helicases. The prototypical member of the RecQ helicase family, the E. coli RecQ protein, plays an essential role both in DNA recombination and in suppression of illegitimate recombination (17–19). E. coli RecQ helicase (610 amino acids, molecular mass, 68,920 Da) displays a 3′–5′ polarity in DNA unwinding and can unwind diverse DNA substrates including DNA with blunt ends, with 5′ or 3′ overhangs, nicked or forked DNA, and three- or four-way junctions as well as G4 DNA (a guanine-rich parallel four-stranded DNA structure) (20, 21).

Elucidation of the interactions between helicase and its DNA substrate is a very important step in understanding the mechanism by which helicases bind and unwind dsDNA. Currently little is known about the thermodynamic characteristics of the binding reaction between RecQ and DNA. Additionally, the effect of solution conditions, salt, and type of salt on the intrinsic affinities and the roles of nucleotide cofactors remain unclear. Thus, more knowledge concerning the properties and kinetic mechanism of RecQ helicase binding to DNA is needed to understand the molecular mechanism underlying helicase function. Toward this goal, we have investigated the DNA binding properties of E. coli RecQ helicase under equilibrium conditions using fluorescein-labeled DNA substrates. Free fluorescein-labeled oligonucleotide (ssDNA or dsDNA) displays low fluorescence anisotropy. Its anisotropy is enhanced upon protein binding due to the reduced rotational mobility of the formed protein-DNA complex. According to the Perrin equation (22) the anisotropy of a fluorescent complex increases with an increase of its volume. Thus, the change in anisotropy of fluorescently labeled oligonucleotides can be used to measure the extent of protein binding. This approach has been used successfully in a number of studies (23–26).

In this paper we have used a steady-state fluorescence anisotropy assay to characterize the DNA binding properties of RecQ helicase from E. coli. Our results indicate that the binding site size of 10 nucleotides per monomeric enzyme does not change as oligonucleotide length increases, implying that RecQ helicase binds to DNA as a monomer. Interestingly, RecQ helicase appears to bind to DNA with high affinity and low cooperativity. In

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‡ The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; AMP-PNP, adenosine 5′-imido) triphosphate; MES, 2-(N-morpholino)ethanesulfonic acid.
addition, only the nucleotide cofactor AMPmPNP increases the affinity of the enzyme for ssDNA in a narrow concentration range, whereas ADP has no detectable effect. The formation of a helicase-DNA complex is accompanied by net release of ions. These properties offer insight into the mechanism by which RecQ helicase exercises its functions in cells.

EXPERIMENTAL PROCEDURES

Reagents and Buffers—All buffers were made with reagent grade chemicals using distilled water purified through a Milli-Q water purification system (Millipore Corp., France). ADP, ATP, and AMPmPNP were purchased from Sigma. Standard titration buffer is 20 mM Tris-HCl, pH 7.4, at 25 °C, 20 mM NaCl, 3 mM MgCl₂, and 0.1 mM dithiothreitol.

pH Measurements—pH measurements were performed with a Knick 654 pH meter and an Ingold microelectrode. For calibration three standard buffers were used, and the precision was about ±0.01 pH unit at 25 °C. To obtain a pH range from 5 to 10, 30 mM sodium citrate, MES, and Tris acetate were employed for pH intervals 5.0–6.0, 5.5–7.2, and 6.8–10, respectively. The possible effect of pH on anisotropy of DNA substrate was taken into account.

Agarose Gel Mobility Shift Assay—RecQ helicase-DNA complexes were formed in standard titration buffer in a total volume of 35 µl at room temperature. The EcoRI-restricted 3-kilobase plasmid DNA (60 M, in nucleotides) was mixed with various concentrations of protein in the range of 0–20 µg. After incubation for 30 min, 5 µl of loading buffer (40 mM Tris acetate, pH 7.5, 50% glycerol, and 0.25% (w/v) bromphenol blue) was added to each sample. The complexes were separated by electrophoresis through a 0.8% agarose gel in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3) at 100 V for 1.5 h and were visualized by ethidium bromide staining.

RecQ Helicase—His₆-tagged E. coli RecQ helicase was expressed from pET 15b expression plasmid in E. coli strain BL21 (DE3) as described (27). Briefly, the overexpressed protein was purified under native conditions by chromatography on Ni²⁺-nitrilotriacetic acid column (Qiagen) followed by fast protein liquid chromatography size exclusion chromatography columns (Superdex 200, Amersham Biosciences) and an ion-exchange chromatography (DEAE Sephadex A-50). The His tag was cleaved using biotinylated thrombin, and removal of the biotinylated thrombin was accomplished using streptavidin-agarose magnetic beads (Novagen, Madison, WI). Based on Sypro Orange-stained SDS-PAGE and electrospray mass spectrometry analyses, the purity of the RecQ preparation was >95%.

Oligonucleotides—The PAGE-purified unlabeled and fluorescein-labeled synthetic oligonucleotides were purchased from Prologo (France). Their structures, sequences, and extinction coefficients are shown in Table I. The concentrations of oligonucleotides were determined by measuring the extinction coefficients. Double-stranded oligonucleotides were made in a 20 mM Tris-HCl buffer, pH 7.2, containing 100 mM NaCl. The mixture was heated to 85 °C for 5 min, and annealing was allowed by slow cooling to room temperature.

Anisotropy Assays—The binding assay was performed by using a Beacon 2000 polarization instrument (PanVera Corp.) (28). An appropriate quantity of fluorescein-labeled ssDNA or dsDNA was added to a standard titration buffer (150 µl total volume) in a temperature-controlled cuvette at 25 °C. The anisotropy of the fluorescein-labeled DNA was measured successively until it stabilized. Then an appropriate quantity of RecQ helicase was added. After the addition of each titrant the anisotropy was measured continuously until it reached a stable plateau. To determine the concentration of the helicase-DNA complex, fluorescence signals observed in these RecQ helicase titrations were subtracted from that observed in the absence of enzyme. The increase in sample volume during the titration was taken into account in the analysis of the data. The reported values are from the average of two to three measurements.

**Table I**

| Substrate | Length | Structure and sequence | ε₂₅₀ cm⁻¹ M⁻¹ |
|-----------|--------|------------------------|---------------|
| A         | 7      | 5’-F-TAGCAGTb          | 7.1 x 10⁴     |
| B         | 10     | 5’-F-CTTCAGCATG        | 9.28 x 10⁴    |
| C         | 13     | 5’-F-AATCTCAGCATG      | 12.77 x 10⁴   |
| D         | 18     | 5’-F-GTGTTGAAAAATCTCTAGCATG | 17.73 x 10⁴ |
| E         | 21     | 5’-F-GTGTTGAAAAATCTCTAGCATG | 20.92 x 10⁴ |
| F         | 36     | 5’-F-AGACCTTTTAGCTAGTGGAAAATCTCTAGCATG | 35.84 x 10⁴ |
| G         | 45     | 5’-F-AGACCTTTTAGCTAGTGGAAAATCTCTAGCATG; | 44.91 x 10⁴ |
| H         | 21/21  | 3’-CACACCTTTTAGAGATCGCTA; | 16.7 x 10⁴ |
| F         | 36/36  | 5’-F-AGACCTTTTAGCTAGTGGAAAATCTCTAGCATG; | 36 x 10⁴ |

a The extinction coefficients of ssDNA were determined using Prologo’s software, and those of dsDNA were determined experimentally.

b F represents the fluorescein chemical group.
DNA Binding Properties of the RecQ Helicase

mined from the fit of the binding isotherms by the least squares method. For the 13-, 21-, 36- and 46-mer oligonucleotide ssDNA, the concrete expressions of \( Q \) are as follows.

\[
Q_{(13)} = \frac{1}{1 + \eta}
\]

\[
Q_{(21)} = \frac{10}{27} \times \frac{12\eta + 2\eta^2 + 4\eta^2\omega}{1 + 12\eta + \eta^2 + 2\eta^2\omega}
\]

\[
Q_{(36)} = \frac{10}{35} \times \frac{27\eta + 272\eta^2 + 34\eta^2\omega + 105\eta^3 + 126\eta^3\omega + 21\eta^3\omega^2}{1 + 27\eta + 138\eta^2 + 17\eta^2\omega + 35\eta^3 + 42\eta^3\omega + 7\eta^3\omega^2}
\]

\[
Q_{(45)} = \frac{10}{45} \times \frac{36\eta + 650\eta^2 + 52\eta^2\omega + 1680\eta^3 + 720\eta^3\omega + 48\eta^3\omega^2}{1 + 36\eta + 326\eta^2 + 26\eta^2\omega + 560\eta^3 + 240\eta^3\omega + 16\eta^3\omega^2 + 60\eta^4 + 240\eta^4\omega + 180\eta^4\omega^2 + 24\eta^4\omega^3}{1 + 15\eta^4 + 60\eta^4\omega + 45\eta^4\omega^2 + 6\eta^4\omega^3}
\]

where \( \eta = KL_\rho \), and the value of \( n \) used is 10 for RecQ (see “Results”).

RESULTS

Validity of Fluorescence Anisotropy Measurements to Determine the Binding Isotherms—We used two separate means to show that the increase in anisotropy is directly proportional to binding. First, we directly analyzed the relationship between the fractional saturation and the fluorescence anisotropy during the titration. Fig. 1A shows that, at a given concentration of fluorescein-labeled 21-mer oligonucleotide, the observed fluorescence anisotropy increases during the protein titration. If we assume that at saturation the moles of RecQ helicase added are equal to the moles of fluorescein-labeled DNA and all fluorescein-labeled DNAs are bound without precipitation of the RecQ-DNA complexes, then the fractional saturation of RecQ helicase can be derived from the anisotropy determined at each RecQ helicase concentration during titration. If the fluorescence anisotropy signal change really reflects the degree of saturation of the ligand, a linear relationship should exist between the fluorescence anisotropy and the fractional saturation of the ligand. As expected, Fig. 1B (circle) shows that the observed anisotropy increases linearly with an increase of the fractional saturation of RecQ helicase.

Secondly, to obtain thermodynamically rigorous binding parameters independent of any assumption about the relationship between the observed signal and the degree of binding, we analyzed the measured fluorescence anisotropy titration curves using the macromolecular binding density function method of Lohman and Bujalowski (31). At any given particular anisotropy value in Fig. 1A all titrations have the same binding density \( v = L_B / M_T \), where \( L_B \) is the bound ligand concentration (moles of RecQ) and \( M_T \) the total macromolecule concentration (moles of nucleotides). The relation between the total ligand concentration and free ligand concentration (\( L_F \)) should obey the law of mass conservation,

\[
L_T = L_B + L_F = vM_T + L_F
\]

For \( m \) different anisotropy values, \( m \) sets of values of \([L_T, M_T]\) could be obtained. The values of \( v \) and \( L_F \) could then be obtained from a linear plot of \( L_T versus M_T \) (Equation 10), with a slope of \( v \) and an intercept of \( L_F \). The fractional saturation (\( \theta \)) can be determined from both binding density (\( v \)) and binding site size (\( n \)) with \( \theta = vn \). Fig. 1B (square) shows the linear dependence of the observed anisotropy on the fractional saturation of RecQ helicase. The value of \( \theta \) could be determined experimentally up to 0.85, and its maximum value was derived from the extrapolation of the anisotropy data to its maximum (when \( A_{\text{max}} = 0.23, \theta = 1 \)). Both of the above analyses clearly show that the fluorescence anisotropy assay is a reliable method for binding studies.

DNA Binding Site Size and Effect of DNA Length on Helicase Binding—An agarose gel mobility shift assay was used to determine the binding site size. Under our experimental conditions a shift of the 3-kilobase DNA was observed, and its magnitude was dependent on the RecQ helicase concentration (Fig. 2, inset). This shift was stabilized when the stoichiometric ratio between DNA and RecQ helicase corresponded to 10–12 nucleotides of DNA per RecQ helicase monomer (Fig. 2). This indicates that about 11 nucleotides were occluded by a RecQ helicase monomer.

The precise value of the binding site size is important for determining the affinity (\( K \)) and cooperativity (\( \omega \)) parameters.
for interactions between helicase and DNA. We, therefore, further determined the stoichiometry value of RecQ helicase binding to an oligonucleotide using the fluorescence anisotropy method under equilibrium binding conditions. We have previously determined the apparent dissociation constants for oligonucleotides of lengths of 13-, 21-, 36-, and 45-mer. Thus, the stoichiometry for DNA binding can be obtained under conditions where the oligonucleotide concentration is kept much higher than the dissociation constant (29, 30). Fig. 3 shows the results of fluorescence anisotropy titrations of fluorescein-labeled 21- and 45-mer oligonucleotides with increasing RecQ helicase concentrations. In both cases fluorescence anisotropy increases sharply and then saturates. Binding stoichiometry was estimated empirically from the intersection of asymptotes as shown in Figs. 3, A and B. In the case of fluorescein-labeled 21-mer DNA the anisotropy increases from 0.055 to a plateau value of 0.18, demonstrating that RecQ helicase binds to DNA with 2:1 stoichiometry (Table II) and, thus, suggesting that the binding site size of RecQ helicase is 10 nucleotides. This conclusion was further supported by results obtained from global fits of the data in Fig. 3 to Equation 1.

We next titrated fluorescein-labeled oligonucleotides of lengths of 13-, 36- and 45-mer with increasing concentrations of RecQ helicase. The stoichiometry values determined by both direct inspection and global regression fits are summarized in Table II. We noted that there is a clear linear relation between the stoichiometry and the length of ssDNA used in the determination (Fig. 3C). The slope value of 11.4 ± 1.5 nucleotides indicates that the DNA binding site size is less than 11 nucleotides per enzyme. Additionally, when fluorescein-labeled 13- and 36-mer ssDNA was titrated with RecQ helicase in the presence of 1 or 2 mM AMPPNP or ADP, the stoichiometry values are essentially the same as in the absence of these nucleotide cofactors (Table II).

To further determine the number of nucleotides directly engaged in interactions with the DNA binding site of RecQ helicase, a series of ssDNA of different lengths from 7- to 45-mer were examined. Consistent with the above observation, oligonucleotides of more than 10 nucleotides in length bind tightly to the enzyme at saturating concentrations of RecQ helicase (500 nM) (Fig. 4A). However, the binding amplitude of the 7-mer oligonucleotide is lower compared with that of the 21-mer oligonucleotide. In addition, the 7-mer oligonucleotide-helicase complex is unstable and dissociates gradually as a function of time. However, the complex can be stabilized in the presence of 1.5 mM AMPPNP (Fig. 4B), indicating a possible allosteric regulation of the enzyme by the nucleotide cofactor. Therefore, we conclude that the number of nucleotides directly engaged in interactions with RecQ helicase should be 8 ≤ n ≤ 11 bases.

**Determination of Association Constants and Cooperativity**—To determine the association constant (K) and cooperativity parameter (ω) we constructed a model-free binding isotherm of $\theta$ versus $L_F$ (Fig. 5A) from the anisotropy-based binding isotherms shown in Fig. 1A (31). The binding isotherms were analyzed by the Epstein Equation, which describes more precisely the binding properties of a large ligand to a finite lattice of DNA with an arbitrary length (33). We fit the isotherms under the conditions that $n = 10$, DNA length is 21 nucleotides, and RecQ helicase binds to DNA as a monomer, as we have shown previously (27). The best fit results give an association constant $K = 6.7 \pm 0.95 \times 10^7$ M$^{-1}$ and a cooperativity parameter $\omega = 25.5 \pm 1.2$. These parameters were used to fit the binding isotherms of Fig. 1A. Similarly, the association constants and cooperativity parameters for RecQ helicases binding to fluorescein-labeled 36- and 45-mer oligonucleotides were

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**Fig. 2. Determination of binding stoichiometry (N) by agarose gel mobility shift assay.** The protein-DNA complex migration distance was determined from the gel mobility assay (inset). Increasing concentrations of RecQ protein (in the range of 0.5–20 nM) were added to 60 μM (nucleotides) of EcoRI-cut 3-kilobase plasmid DNA and run on a 0.8% agarose gel. The complexes were visualized by ethidium bromide staining. The stoichiometry (N) was determined from the intersection of asymptotic lines drawn through the steeply ascending and plateau regions of the data derived from the inset. The relative migration distance of RecQ-DNA complex ($R_f$) is calculated as $R_f = (R_m - R_o)/(R_u - R_o)$, where $R_m$ is the migration distance of the complex in the presence of RecQ protein, $R_o$ in the absence of the protein, and $R_u$ the upper limit of the migration distance.

**Fig. 3. Determination of stoichiometry by measuring fluorescence anisotropy titration of RecQ with ssDNA.** Experimental conditions and treatments of data were described under “Experimental Procedures.” In each titration the starting concentration of ssDNA was 100 nM. A and B represent titrations of 21- and 45-mer ssDNA with RecQ helicase, respectively. The stoichiometry was determined both from the intersection of asymptotic lines drawn through the steeply ascending and plateau regions of the data and from global nonlinear regression fits (Table II) of the data. C shows the linear relationship between the stoichiometry and the oligonucleotide length.
determined in the same way. The results are summarized in Table III. These data clearly indicate that the association constant and the cooperativity of RecQ helicase vary from 6.7 ± 0.95 × 10^6 to 7.5 ± 2.1 × 10^6 M⁻¹ and from 25 to 38, respectively.

We have previously determined that the DNA binding site of RecQ helicase encompasses only 10 nucleotide residues and that a 13-mer DNA binds with high affinity to RecQ helicase. Thus, the study of RecQ helicase binding to a 13-mer oligonucleotide will provide the information about helicase binding to DNA without any interference from protein-protein interactions. The model-free binding isotherm obtained with 13-mer oligonucleotide is fit best with Epstein’s non-cooperative binding equation with an association constant \( K = 6.4 ± 1.2 × 10^6 \) M⁻¹ (Fig. 5B). This result is expected because only 1 protein monomer can bind to the 13-mer oligonucleotide, and therefore, there are no protein-protein interactions.

Effect of Salt and pH on RecQ Binding to DNA—Fig. 6A demonstrates that the formation of the helicase-DNA complex is very sensitive to the concentration of sodium chloride; its formation was inhibited by sodium chloride concentrations exceeding 80 mM. About 50% of DNA-protein complexes were formed in the presence of 65 mM NaCl. However, sodium chloride was necessary for helicase binding to DNA, since in sodium chloride-free binding buffer no RecQ-DNA complex was observed. In fact, RecQ helicase aggregates and forms a tubular structure under this condition. Because the extent of complex disruption by high concentrations of NaCl reflects the affinity of a protein for DNA, we next examined the stability of the complex at high concentrations of NaCl (Fig. 6A, inset). We found that the helicase-DNA complex could not be disrupted to the same extent by the same NaCl concentration that was needed to inhibit the binding. The midpoint of salt titration for dissociation of the RecQ helicase by NaCl (130 mM) is 2-fold higher than that for inhibition by NaCl.

We next investigated the pH dependence of RecQ binding to DNA. Fig. 6B shows that RecQ helicase displays an optimum
DNA binding activity between pH 6.2 and 8.0. Similar results were obtained with dsDNA or DNA of different lengths (data not shown).

Effect of Salt Concentration on $K$ and $\omega$—The salt dependencies of the binding parameters $K$ and $\omega$ were further determined quantitatively. Fig. 7A shows the binding isotherms obtained with the 21-mer oligonucleotide in the presence of different concentrations of NaCl. The binding parameters were obtained by theoretical fits of the binding isotherms. A log-log plot of the binding constant versus the salt concentration is

| ssDNA  | $K$     | $\omega$ | $\log K/\log[\text{NaCl}]$ |
|--------|---------|----------|-----------------------------|
| 13-mer | $6.4 \pm 1.2 \times 10^5$ | -2.8 |
| 21-mer | $6.7 \pm 0.95 \times 10^6$ | -3.7 |
| 36-mer | $7.5 \pm 2.1 \times 10^6$ | -5.0 |
| 45-mer | $6.8 \pm 1.1 \times 10^6$ | -5.8 |

Table III

Thermodynamic parameters for the binding of RecQ helicase to different ssDNA oligomers

Fig. 6. A, effect of NaCl on the interaction of RecQ helicase with ssDNA. 200 nM RecQ helicase was added into the solution containing 1 nM 36-mer 3'-fluorescein-labeled oligonucleotide at different concentrations of NaCl. The reaction was performed in standard binding buffer except for the concentration of NaCl as indicated. Inset, salt titration of preformed RecQ helicase-ssDNA complex. After the RecQ-ssDNA complex was preformed at low concentrations of NaCl (5 mM), the change in anisotropy upon the addition of the indicated amount of NaCl was monitored, and the percentage of complex was determined from the anisotropy change. B, effect of pH on the interaction of RecQ helicase with ssDNA. Relative fluorescence anisotropy enhancements of RecQ-DNA complex at different pH values were measured as described under “Experimental Procedures.” 5 nM 36-mer ssDNA and 200 nM RecQ helicase were used for each pH effect study.

DNA binding activity between pH 6.2 and 8.0. Similar results were obtained with dsDNA or DNA of different lengths (data not shown).

Fig. 7. A, fluorescence anisotropy of 3'-fluorescein-labeled oligonucleotide at different NaCl concentrations. The titration curves were performed with the RecQ protein and 5 nM 21-mer ssDNA in binding buffers with different NaCl concentrations: 45 mM (○), 65 mM (■), 80 mM (▲), 100 mM (●), 120 mM (▲). The lines are computer fits of the data with the Epstein equation with binding constants ($K$) and cooperativity parameters ($\omega$): ○, $1.87 \times 10^6$ M$^{-1}$; 15.5; ■, $1.45 \times 10^6$ M$^{-1}$, 16.3; ▲, $2.76 \times 10^7$ M$^{-1}$, 20.5; ●, $1.12 \times 10^9$ M$^{-1}$, 21.5; ■, $7.5 \times 10^4$ M$^{-3}$, 20.5. B, dependence of the intrinsic binding constant ($K$) on [NaCl]. The line is a linear least-square fit that provides a slope of $\log K/\log[\text{NaCl}] = -3.7$. C, the relationship between the number of ions released upon ssDNA binding of RecQ and the oligomer length of the DNA.
well known that Mg\(^{2+}\) is required for ATP binding and hydrolysis. However, it is not clear whether Mg\(^{2+}\) also contributes to DNA binding to RecQ helicase. It was reported that Mg\(^{2+}\) ions greatly enhanced DNA binding of the Rad 51 protein, which promotes strand exchange between circular ssDNA and linear dsDNA (35). We, therefore, examined the effect of Mg\(^{2+}\) on DNA binding of RecQ helicase. A series of titrations of 21-mer ssDNA with RecQ helicase in the absence or in the presence of Mg\(_{\text{Cl}_2}\) at different concentrations is shown in Fig. 9A, in which the solid lines are the computer fits according to the Epstein equation. Fig. 9B shows the dependence of the 21-mer DNA intrinsic binding constant on [Mg\(_{\text{Cl}_2}\]) (log-log plot). The plot is linear, and the slope is $\log K_0/\log[Mg_{\text{Cl}_2}] = -0.34$, the magnitude of which is significantly lower than 3.7 obtained in the presence of NaCl alone. Because no great difference was observed between the association constants determined in the absence and presence of Mg\(^{2+}\) ions, we conclude that Mg\(^{2+}\) has only a slight effect on DNA binding of RecQ helicase.

**Effect of the Nucleotide Cofactor on the Interactions of RecQ Helicase with DNA**—We had previously observed that the binding of the 7-mer oligonucleotide to RecQ helicase was enhanced by AMPPNP (Fig. 4B). The allosteric effects were further studied here. Fig. 10A shows the fluorescence anisotropy titrations of fluorescein-labeled 21-mer ssDNA with RecQ helicase in the presence of 1.5 mM AMPPNP, 1.5 mM ADP and in the absence of nucleotide cofactor. The solid lines are computer fits using the Epstein Equation (33). The obtained intrinsic binding constants are $11.2 \pm 0.89 \times 10^6$, $6.5 \pm 1.2 \times 10^6$, and $6.8 \pm 1.1 \times 10^6$ m\(^{-1}\) for binding in the presence of AMPPNP, ADP, and in the absence of nucleotide cofactors, respectively. However, when the same experiments were performed with fluorescein-labeled dsDNA, no significant differences were observed between the intrinsic binding constants in the presence and in the absence of nucleotide cofactors (Table IV). In addition, we systematically investigated the effect of nucleotide cofactor concentration on the intrinsic binding constant obtained with ssDNA and dsDNA. Fig. 10B shows that the allosteric effect of AMPPNP was only observed when RecQ helicase binds to ssDNA in the presence of 1.5 mM AMPPNP. No detectable allosteric effect was observed with dsDNA in the presence of low concentrations of nucleotide cofactors (below 1.5 mM). Some modest inhibition effects were observed in the presence of high concentrations of nucleotide cofactors (above 1.5 mM).

**DISCUSSION**

In this report we have characterized the DNA binding properties of *E. coli* RecQ helicase, a typical member of the RecQ helicase family, using the steady-state fluorescence anisotropy assay under equilibrium binding conditions. The DNA binding assay that we used in this study is rapid and convenient since physical separation of free and DNA-bound protein is not required. This assay is also a reliable method for surveying the binding behavior of RecQ helicase under these conditions, because both direct analysis of the relationship between the fluorescence anisotropy signal and fractional saturation and the macromolecular binding density function analysis show that the fluorescence anisotropy increases linearly with the fractional saturation. Based on this assay we have performed rigorous, quantitative studies of the interactions between RecQ helicase and oligonucleotides of different lengths. To our knowledge such a protein-DNA binding study has not been previously reported for any RecQ family helicase. The major results of our studies are as follows. 1) Under optimal conditions RecQ helicase has a binding site size of 10 nucleotides and binds DNA with an intrinsic binding constant $K = 6.7 \pm 0.95 \times 10^6$ m\(^{-1}\) and low cooperativity ($\omega = 25.5 \pm 1.2$). 2) RecQ helicase can bind to ssDNA or dsDNA at the same site.
DNA binding affinity of RecQ helicase is affected by NaCl concentration; the intrinsic binding constant decreases significantly with increasing salt concentration, log $K_{NaCl}/H_1$ = 3.7. 4) RecQ helicase binding to DNA displays an optimal pH between 6.2 and 8.0. 5) In the presence of 1.5 mM AMPPNP, the non-hydrolyzable analogue of ATP, enhances RecQ helicase binding to ssDNA. These results shed some light on the understanding of molecular function of RecQ helicase family and are discussed in more details below.

The Stoichiometry of the RecQ-Helicase Complex Determined with DNA of Different Lengths Indicates That RecQ Helicase Binds the Nucleic Acid as a Monomer—The results described in this work provide an insight into the nature of the RecQ-DNA complex within the DNA binding site of the RecQ helicase. It was suggested that an oligomer or at least a dimer is absolutely required for helicase translocation and unwinding activity (36–38). However, several lines of evidences indicate that some

### Table IV

|       | $K_{NTP}$ | $K_{NDP}$ | $K_{effector}/K$ | Reference |
|-------|-----------|-----------|------------------|-----------|
| PriA  | 3.3 x 10^4 | 8 x 10^3  | 1.8 x 10^4       | 0.24      |
| DnaB  | 6 x 10^2   | 1.3 x 10^5 | 5.9 x 10^2       | 210       |
| Rep   | 2.1 x 10^6 | 0.58 x 10^6 | 0.8 x 10^6       | 0.35      |
| RecQ  | 1 x 10^7   | 3.2 x 10^6 | 1.71 x 10^7      | 1.5       |
|       | 6.8 x 10^6 | 7.3 x 10^6 | 6.5 x 10^6       | 210       |
|       | 1.12 x 10^7| 7.8 x 10^6 | 6.9 x 10^5       | 5.9       |
|       | 8.6 x 10^6 | 3.4 x 10^6 | 2.8 x 10^6       | 210       |
|       | 9.8 x 10^6 | 2.8 x 10^6 | 1.71 x 10^5      | 1.5       |

$^a$ Determined with 21-mer ssDNA and dsDNA.

$^b$ Determined with 36-mer ssDNA and dsDNA.

**FIG. 9.** A, fluorescence titrations of the 21-mer fluorescein-labeled oligonucleotide under standard conditions and in the presence of different MgCl2 concentrations: , 0.5 mM; , 1 mM; , 2 mM; , 3 mM; , 5 mM; , 7.5 mM. The lines are computer fits of the titration curves using the Epstein Equation. B, dependence of the intrinsic binding constant, $K$, on MgCl2 concentration. The line is a linear least-square fit, which provides a slope of $\log K_{MgCl2} = -0.34 \pm 0.03$.

**FIG. 10.** RecQ helicase-DNA binding isotherms obtained in the absence and in the presence of AMPPNP and ADP. A, isotherms were determined by monitoring fluorescence anisotropy change in standard binding conditions and in the presence of 1.5 mM AMPPNP (squares), 1.5 mM ADP (circles), or in the absence of nucleotide cofactor (rhombuses). The ssDNA used in this experiment is 5 nM 21-mer 3'-fluorescein-labeled oligonucleotide. The lines are computer fits of the binding isotherms using the Epstein equation with intrinsic binding constants $K = 1.02 \times 10^5$, 6.5 $\times 10^5$, 6.8 $\times 10^6$ M$^{-1}$ for isotherms obtained in the presence of AMPPNP, ADP, and in the absence of nucleotides, respectively. B, variations of intrinsic binding constant of RecQ helicase versus nucleotide cofactor concentration. The intrinsic binding constant of RecQ helicase was determined with 36-mer ssDNA in the presence of AMPPNP (■) or ADP (□) and with 36-mer dsDNA in the presence of AMPPNP (○) or ADP (●) at concentrations as indicated. The activation fold was calculated as $K_{AXP}/K$, where $K_{AXP}$ represents the intrinsic binding constant obtained in the presence of nucleotide cofactor.
helices as a monomer (39–41). The extensive structural and kinetic analysis of PcrA helicase show that the active form of this protein is a monomer (42, 43). Using a wide array of biochemical and biophysical methods we have previously shown that RecQ helicase is a monomer in solution in the absence of DNA (27). Recently, the high resolution three-dimensional structure of the RecQ protein further confirmed its monomeric nature (44). In our previous study we did not address whether RecQ helicase could undergo dimerization or higher order oligomerization upon binding to DNA, as suggested for Rep helicases that form a homodimer upon binding to either ssDNA or dsDNA (45). Our direct stoichiometry studies shown in this report indicate that the RecQ helicase binds the nucleic acid as a monomer. This conclusion is supported by the following results. (i) The stoichiometry of 13-mer DNA–RecQ helicase complex is 1, indicating that RecQ helicase binds to DNA as a monomer not as a dimer or oligomer; (ii) the stoichiometry of 1:10 of RecQ helicase–nucleotides complex remains the same independent of the DNA length (Fig. 3C), suggesting that RecQ helicase binds to DNA as a monomer whatever the length of DNA. From these observations it is clear that RecQ helicase binds sequentially to the DNA substrate as a monomer in forming the protein–nucleic acid complex. It is worth noting that RecQ helicase remains a monomer with DNA even in the presence of nucleotide cofactors (Table II).

Low Cooperativity Displayed by RecQ Helicase Is Consistent with Its Structure and Functional Properties—The most striking result of this study is that RecQ helicase displays a low cooperativity with a $\alpha$ value of $\sim 25$. First, such a behavior is expected from its quaternary structural nature. As it was mentioned above, we have previously shown that RecQ helicase is a monomer both in the absence and in the presence of DNA. Thus, no essential protein–protein interactions were observed under different conditions. Therefore, the low cooperativity exhibited by RecQ helicase is consistent with its monomeric nature. It is interesting to note that herpes simplex virus type I-coded ssDNA-binding protein, ICP8, identified as a monomer, also displayed a low cooperativity (24). Second, from the definition of the term of cooperativity, high cooperativity means that a protein will have a high affinity for long stretches of ssDNA, and the binding of molecules adjacent to each other could be greatly enhanced. Consistent with this notion, most ssDNA-binding proteins bind DNA with a relatively high cooperativity over a wide range of $\alpha$ values from $10^2$ to $10^5$ so that the protein can efficiently cover a relatively long stretch of ssDNA at low protein concentrations (46, 47). In the case of the RecQ helicase family proteins, the proteins exercise their diverse functions through heterologous protein–protein interactions. It is well established that the RecQ family proteins interact physically and/or functionally with several proteins including topoisomerases, polymerase δ, proliferating cell nuclear antigen, replication protein A, and FLAG endonuclease 1 (see the Introduction). High cooperativity would enhance the homologous protein–protein interactions and could probably inhibit the heterologous interactions with other proteins necessary for normal RecQ helicase functions. Thus, it is conceivable that low cooperativity is necessary for RecQ helicase to perform its functions in cells.

Effect of Nucleotide Cofactors and Mechanism of the Helicase Functioning—Nucleotide cofactors such as ATP not only provide energy for unwinding activity but also function as an allosteric effector to regulate helicase affinity toward DNA substrates (1, 2). It is well documented that a common feature of the well characterized helicases such as the E. coli Rep helicase, phage T7 and T4 helicases, DnaB and PriA proteins is that their affinity for ssDNA or dsDNA is modulated either by ATP or by its hydrolysis product, ADP (48–52). The binding and release of the DNA substrate as a function of nucleotide binding and hydrolysis is a central concept for models proposed so far. For example, the rolling model was proposed based on the observation that ATP favored helicase binding to ssDNA and ATP favored simultaneous binding to ssDNA and dsDNA (23). We have compared RecQ helicase with several helicases whose affinity for ssDNA and dsDNA were modulated by ATP and ADP (Table IV). Based on our studies, we find that RecQ helicase displays a special feature; its affinity for ssDNA is enhanced only by AMPPNP in a narrow concentration range of nucleotide cofactor, and ADP has no detectable effect on its affinity for either ssDNA or dsDNA. These results imply that the mechanism of RecQ helicase modulation by nucleotide cofactors may be different from other helicases. Is there a common feature for helicase binding activity modulation by nucleotide cofactors? As can be seen from Table IV, the same nucleotide cofactor may exercise different effects on different helicases. For example, AMPPNP may greatly increase the affinity of DnaB helicase for ssDNA, whereas that of Rep helicase is decreased to 35.3% by AMPPNP. Thus, it is not conceivable to give a general feature for effects of ATP or ADP on all helicases.

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