Biochemistry

 Volume 34, Number 27 July 11, 1995

Accelerated Publications

Interactions of *Escherichia coli* Primary Replicative Helicase DnaB Protein with Single-Stranded DNA. The Nucleic Acid Does Not Wrap around the Protein Hexamer†

Wlodzimierz Bujalowski* and Maria J. Jezewska

Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch at Galveston, 301 University Boulevard, Galveston, Texas 77555-1053

Received April 28, 1995; Revised Manuscript Received May 25, 1995⊗

ABSTRACT: The interactions of the *Escherichia coli* primary replicative helicase DnaB protein with single-stranded (ss) DNA have been studied using the thermodynamically rigorous fluorescence titration technique, which allowed us to obtain absolute stoichiometries of the formed complexes and interaction parameters without any assumptions about the relationship between the observed signal change and the degree of binding. Binding of the DnaB protein to the ssDNA fluorescent derivative poly(dεA) is accompanied by a strong increase of the nucleic acid fluorescence. We show that, in the presence of the ATP nonhydrolyzable analog AMP-PNP, the DnaB helicase binds polymer ssDNA with the site-size of 20 ± 3 nucleotides per protein hexamer. This stoichiometry has been fully confirmed in the binding experiments with ssDNA oligomers of 40 and 20 residues in length. Two DnaB hexamers bind to 40-mer, and one DnaB hexamer binds to 20-mer. Thermodynamic studies of the 20-mer binding to the DnaB hexamer show that the hexamer has a single, strong binding site for ssDNA. Moreover, photo-cross-linking experiments indicate that only a single subunit is primarily in contact with ssDNA. This surprisingly very low site-size of the large hexameric helicase−ssDNA complex, the existence of only a single, strong ssDNA binding site on the hexamer, and the results of photo-cross-linking experiments preclude the possibility of extensive wrapping of the ssDNA around the hexamer and formation of the complex in which all six protomers are simultaneously bound to ss nucleic acid. Binding of the DnaB helicase to ssDNA is characterized by weak cooperativity, which indicates that the enzyme is unable to form long clusters when bound to the nucleic acid lattice. The significance of these results for a mechanistic model of the DnaB helicase is discussed.

DnaB is the key DNA replication protein in *Escherichia coli* and was originally identified on the basis of its requirement for *in vitro* φX174 phage replication (Wickner et al., 1973; McMacken et al., 1977; Ueda et al., 1978). DnaB protein is the primary *E. coli* replicative helicase, i.e., the factor responsible for unwinding the DNA duplex in front of the replication fork (LeBowitz & McMacken, 1986; Baker et al., 1987). The protein is the only helicase required to reconstitute DNA replication *in vitro* from the chromosomal origin of replication (oriC) (Kornberg & Baker, 1992). The enzyme is crucial for the replication of bacterial chromosomal, phage, and plasmid DNA and is involved in both the initiation and elongation stages of DNA replication (Matson & Kaiser-Rogers, 1990; Kornberg & Baker, 1992; Marians, 1992; Wahle et al., 1989).

† This work was supported by NIH Grant GM-46679 (to W.B.).
* Corresponding author.
⊗ Abstract published in *Advance ACS Abstracts*, July 1, 1995.

0006-2960/95/0434-8513$09.00/0

In vivo functions of the DnaB helicase are related to the ability of the protein to interact with ssDNA and dsDNA under ATP control (Arai & Kornberg, 1981b; LeBowitz & McMacken, 1986). Studies of nucleotide binding to the DnaB helicase have established that the hexamer has six nucleotide binding sites, presumably one on each protomer (Arai & Kornberg, 1981a; Bujalowski & Klonowska, 1993, 1994a,b). On the basis of thermodynamically rigorous fluorescence titrations, we have determined that the binding process is biphasic, resulting from the negative cooperative interactions limited to neighboring subunits (Bujalowski & Klonowska, 1993, 1994a).

Native DnaB forms a hexamer composed of six identical subunits (Arai & Kornberg, 1981a, Reha-Krantz & Hurwitz, 1978; Bujalowski et al., 1994). Sedimentation equilibrium, sedimentation velocity, and ligand binding studies have shown that the DnaB helicase exists as a stable hexamer over a large protein concentration range (Bujalowski et al., 1994). Magnesium ions play a crucial structural role in stabilizing the hexameric structure of the DnaB helicase. In the absence of Mg^{2+}, the DnaB protein forms a trimer which at low protein concentrations dissociates into monomers. Hydrodynamic data indicate that six protomers aggregate with cyclic symmetry in which the protomer−protomer contacts are limited to only two neighboring subunits (Bujalowski et al., 1994).

Oligomeric structure has been proposed to play an important role in the function of a helicase (Bujalowski & Klonowska, 1993; Lohman, 1993). Quantitative understanding of the interactions between the subunits of the enzyme and nucleic acids is of fundamental importance for our understanding of the mechanism of the activities of the enzyme. Knowledge of the stoichiometry and structure of the helicase−nucleic acid complex, and the mechanism of binding, is a prerequisite for formulating a model of the mechanism of enzyme translocation on the nucleic acid lattice and the catalysis of nucleic acid unwinding.

In this paper, we report the first quantitative analysis of the DnaB helicase−ssDNA complex formation using the fluorescence titration technique, which allowed us to obtain binding parameters without any assumption about the relationship between the observed signal change and the degree of binding. We present direct evidence that, in the presence of the ATP nonhydrolyzable analog AMP-PNP,[1] the DnaB helicase binds to the polymer ssDNA with the site-size of 20 ± 3 nucleotides per DnaB protein hexamer. Moreover, photo-cross-linking experiments indicate that only a single subunit of the DnaB hexamer is in contact with ssDNA in the complex with $dT(pT)_{19}$. The surprisingly very low site-size of the large hexameric helicase−ssDNA complex, the presence of only a single strong ssDNA binding site, and the results of photo-cross-linking experiments preclude any extensive wrapping of the ss nucleic acid around the hexamer and are not compatible with models of the translocation of the enzyme along the nucleic acid lattice in which all six subunits or multiple binding sites are simultaneously engaged in interactions with ssDNA.

[1] Abbreviations: AMP-PNP, β,γ-imidoadenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane.

MATERIALS AND METHODS

Reagents and Buffers. All solutions were made with distilled and deionized >18 MΩ (Milli-Q Plus) water. All chemicals were reagent grade. Buffer T2 is 50 mM Tris adjusted to pH 8.1 with HCl, 5 mM $MgCl_2$, and 10% glycerol. The temperatures and concentrations of NaCl and AMP-PNP in the buffer are indicated in the text.

DnaB Protein. The *E. coli* DnaB protein was purified as previously described by us (Bujalowski & Klonowska, 1993, 1994a). The concentration of the protein was spectrophotometrically determined using the extinction coefficient $\epsilon_{280} = 1.85 \times 10^5\ cm^{-1}\ M^{-1}$ (hexamer) (Bujalowski et al., 1994).

Nucleic Acids. Poly(dA), $dA(pA)_{39}$, and $dA(pA)_{20}$ were purchased from Midland Certified Reagents (Midland, TX). The oligomers were at least >95% pure as judged by autoradiography on polyacrylamide gels. The nucleic acids were converted into their fluorescent derivative (etheno-A) by modification with chloroacetaldehyde (Secrist et al., 1972). The concentration of the nucleic acids was determined using the extinction coefficient 3700 $M^{-1}\ cm^{-1}$ (nucleotide) at 257 nm (Ledneva et al., 1977).

Photo-Cross-Linking Experiments. The oligomer $dT(pT)_{19}$ has been labeled at the 5′ end with ^{32}P using polynucleotide kinase, according to the described protocol (Sambrook et al., 1989). The labeled ssDNA 20-mer was mixed with the DnaB helicase in buffer T2 (pH 8.1, 20 °C), containing 50 mM NaCl and 1 mM AMP-PNP (see below). After 1 h of incubation at 20 °C the samples (20 μL) were placed on parafilm and irradiated for 20 min, at a distance of 11 cm, using a mineral lamp (model UVG-11) which has a maximum output at 254 nm. The controls have been performed to determine the optimal time for efficient cross-linking, while minimizing potential degradation of the protein by prolonged UV exposure. After irradiation, the samples were loaded on an 8% SDS−polyacrylamide gel, and electrophoresis was performed at a constant voltage of 230 V. The gels were stained with Coomassie Brilliant Blue, dried, and autoradiographed.

Fluorescence Measurements. All steady-state fluorescence measurements were performed using the SLM 48000S lifetime spectrofluorometer, as previously described (Bujalowski & Klonowska, 1993, 1994a,b). The relative fluorescence increase, ΔF, is defined as $\Delta F = (F_j - F_o)/F_o$, where F_j is the fluorescence of the sample at a given titration point j and F_o is the initial value of the fluorescence of the same sample.

Determination of Rigorous Thermodynamic Binding Isotherms and Absolute Stoichiometries of the DnaB Helicase−ssDNA Complexes. In the studies described in this work, we followed the binding of the DnaB protein to the polymer and oligomer ssDNAs by monitoring the fluorescence increase, ΔF_{obsd}, of their etheno derivatives upon the complex formation. A general procedure to obtain absolute estimates of the average degree of binding of the protein per nucleotide, $\sum \nu_i$ (binding density), and the free protein concentration, P_f, has been previously described (Bujalowski & Klonowska, 1993, 1994a,b). Briefly, if there are i different complexes of DnaB hexamer with ssDNA, then the experimentally observed ΔF_{obsd} is functionally related to $\sum \nu_i$ by the equation (Bujalowski & Lohman, 1987; Bujalowski & Klonowska, 1993)

$$\Delta F_{obsd} = \sum \nu_i \Delta F_{i_{max}} \quad (1)$$

where $\Delta F_{i_{max}}$ is the maximum fluorescence increase of nucleic acid with the DnaB protein bound in complex i. Thus, ΔF_{obsd} is the degree of binding $\sum \nu_i$ weighted by the contributions to the overall fluorescence increase from the different complexes. Therefore, for the same value of ΔF_{obsd} obtained at two different total nucleic acid concentrations, M_{T1} and M_{T2}, the degree of binding $\sum \nu_i$ and the free DnaB protein concentration P_f must be the same. The value of $\sum \nu_i$ and P_f is then related to the total protein concentrations, P_{T1} and P_{T2}, and the total nucleic acid concentrations, N_{T1} and N_{T2}, at which the same ΔF_{obsd} is obtained, by the formula

$$\sum \nu_i = (P_{T2} - P_{T1})/(N_{T2} - N_{T1}) \quad (2)$$

$$P_f = P_{Tx} - \sum \nu_i (N_{Tx}) \quad (3)$$

where $x = 1$ or 2 (Bujalowski & Klonowska, 1993).

Analysis of the DnaB Hexamer−Polymer ssDNA Binding Isotherm. By detailed consideration of the appropriate conditional probabilities, McGhee and von Hippel (1974) derived two explicit equations for noncooperative and cooperative binding of a large ligand to a one-dimensional homogeneous lattice, with overlapping potential binding sites. Previously, we obtained a single generalized equation for the McGhee−von Hippel model which can be applied to both cooperative and noncooperative binding (Bujalowski et al., 1989). The Scatchard form of the generalized equation is described by the expression

$$\nu/P_f = K(1 - n\nu)\{[2\omega(1 - n\nu)]/[2\omega - 1)(1 - n\nu) + n + R]\}^{n-1}\{[1 - (n + 1)\nu + R]/2(1 - n\nu)]\}^2 \quad (4)$$

where K is the intrinsic binding constant, n is the number of nucleotides covered by the protein in the complex (site-size), ω is the parameter characterizing cooperativity, and $R = \{[1 - (n + 1)\nu]^2 + 4\omega\nu(1 - n\nu)\}^{0.5}$. Binding of the DnaB helicase to the polymer ssDNA has been analyzed using eq 4.

Statistical Thermodynamic Model of DnaB Helicase Hexamer Binding to 40-mer. Studies presented below show that, at saturation, two DnaB hexamers bind to a single molecule of dϵA(pϵA)$_{39}$. Taking into account the potential overlap of the binding sites on the short ss nucleic acid lattice, and the cooperative interactions between bound protein molecules, the partition function, Z, for the DnaB hexamer−40-mer system is given by the equation

$$Z = 1 + (N - n + 1)KP_f + \omega K^2 P_f^2 \quad (5)$$

where N is the total number of residues in the ss nucleic acid oligomer, n is the site-size of the DnaB hexamer−ssDNA complex, K is the intrinsic binding constant, and ω is the parameter characterizing the cooperative interactions between the two bound hexamers. The average number of DnaB hexamers bound per 40-mer, q, at a given free protein concentration, P_f, is then defined by the standard statistical thermodynamic expression $q = \partial \ln Z/\partial \ln P_f$ (Hill, 1985)

$$q = [(N - n + 1)KP_f + 2\omega K^2 P_f^2]/[1 + (N - n + 1)KP_f + \omega K^2 P_f^2] \quad (6)$$

In general, the relative increase of the fluorescence of the dϵA(pϵA)$_{39}$ bases engaged in the complex with the single DnaB hexamer molecule, ΔF_1, can differ from the relative fluorescence increase ($\Delta F_{max} = \Delta F_1 + \Delta F_2$) observed when two hexamers are bound per 40-mer. The observed experimentally fractional increase of the nucleic acid fluorescence, ΔF_{obsd}, is then defined in terms of intrinsic binding constant K and cooperativity parameter ω as

$$\Delta F_{obsd} = [(\Delta F_1)(N - n + 1)KP_f + (\Delta F_1 + \Delta F_2)\omega K^2 P_f^2]/[Z] \quad (7)$$

RESULTS

Absolute Stoichiometry of the DnaB Hexamer−ssDNA Complex. Our preliminary studies have shown that significant affinity of the DnaB helicase for ssDNA, necessary to perform quantitative analysis, has been observed only in the presence of ATP or ATP nonhydrolyzable analogs. Because DnaB is an efficient ATPase, even in the absence of ssDNA (Bujalowski & Klonowska, 1993), all titrations have been performed in the presence of a saturating concentration of the ATP nonhydrolyzable analog AMP-PNP. Fluorescence titrations of poly(dϵA) with the DnaB helicase at two different nucleic acid concentrations in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP are shown in Figure 1a. The selected nucleic acid concentrations differ by a factor of ~4, providing a distinct separation of the two binding isotherms, up to the fluorescence increase value of ~3. Using the fluorescence titration curves presented in Figure 1a, the absolute stoichiometry of the DnaB helicase−ssDNA complex can be determined as described in Materials and Methods. The dependence of the observed fluorescence increase of poly(dϵA) upon the average binding density $\sum \nu_i$ of the DnaB helicase, from $\sum \nu_i = 0-0.03$, is shown in Figure 1b. Within experimental accuracy, the plot is linear, indicating very similar enhancement of the nucleic acid fluorescence upon complex formation with the DnaB helicase, at the studied binding density range. Due to the entropy factor in the formation of a gap of free sites on the lattice, large enough to accommodate a protein ligand, complete saturation of the nucleic acid lattice would require extremely high concentrations of the protein (McGhee & von Hippel, 1974). The estimation of the maximum fluorescence increase upon DnaB binding has been achieved, using a series of dϵA(pϵA)$_{N-1}$ oligomers, with N ranging from 60 to 20 nucleotides, where the gap problem has been minimized or eliminated and full saturation can be reached (see below). The obtained estimation of the maximum fluorescence increase is 3.6 ± 0.3. Thus, the extrapolation of the maximum fluorescence increase for the poly(dϵA)−DnaB system, within this range of the maximum fluorescence increase, gives the value of $\sum \nu_i = 0.05 \pm 0.008$. Competition binding studies with unmodified ssDNA homopolymers [poly(dA), poly(dT), and poly(dC)] provided the same value of $\sum \nu_i = 0.05 \pm 0.01$, at saturation (Jezewska et al., manuscript in preparation). Thus, at saturation, the DnaB helicase binds to a single-stranded polymer DNA with the stoichiometry of 20 ± 3 nucleotides per hexamer. To further examine the stoichiometry of the DnaB helicase−ssDNA complex, we performed binding studies with a ssDNA oligomer, dϵA(pϵA)$_{39}$. The 40-mer should accommodate only two DnaB hexamers, and the complexity

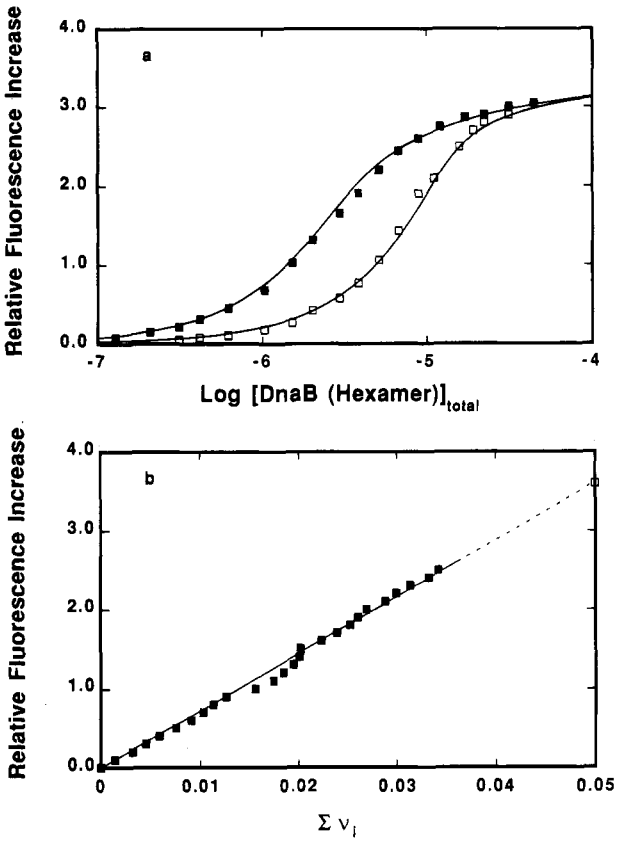

FIGURE 1: (a) Fluorescence titrations of poly(dεA) with the DnaB protein monitored by the increase of the nucleic acid fluorescence in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP at two different nucleic acid concentrations: (□) 8.4×10^{-5} M; (■) 3.5×10^{-4} M (nucleotide). Solid lines are computer fits of the binding isotherms, using the generalized McGhee–von Hippel equation (see eq 4), with intrinsic binding constant $K = 1.1 \times 10^5$ M^{-1}, cooperativity parameter $\omega = 3$, and $\Delta F_{max} = 3.6$. (b) Dependence of the relative increase of the poly(dεA) fluorescence upon the average binding density $\sum \nu_i$ of the DnaB helicase hexamer. The dashed line is the extrapolation to $\Delta F_{max} = 3.6$ (□). The absolute value of $\sum \nu_i$ has been determined, using the thermodynamically rigorous approach described in Materials and Methods.

resulting from the entropy factor related to the protein accommodation on this short lattice should be greatly reduced. Fluorescence titrations of dεA(pεA)$_{39}$ with the DnaB helicase in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP, at two different ssDNA oligomer concentrations, are shown in Figure 2a. The observed maximum increase of the dεA(pεA)$_{39}$ fluorescence is 3.6 ± 0.2. The selected concentrations of the ssDNA oligomer provide distinct separation of the two isotherms, allowing us to determine the absolute stoichiometry of the dεA(pεA)$_{39}$–DnaB complex up to ~80% of the maximum signal saturation. The dependence of the relative fluorescence increase of dεA(pεA)$_{39}$ upon the average number of DnaB hexamers bound is shown in Figure 2b. Short extrapolation to the maximum value of the fluorescence increase ($\Delta F = 3.6$, open square) shows that two DnaB hexamers are bound per one molecule of 40-mer. Thus, each hexamer binds, on average, to only 20 nucleotides, which is in excellent agreement with the stoichiometry determined using the polymer ssDNA. The plot is nonlinear, and the binding of the first DnaB hexamer is accompanied by a larger fluorescence increase than the binding of the second hexamer (see Figure 2b).

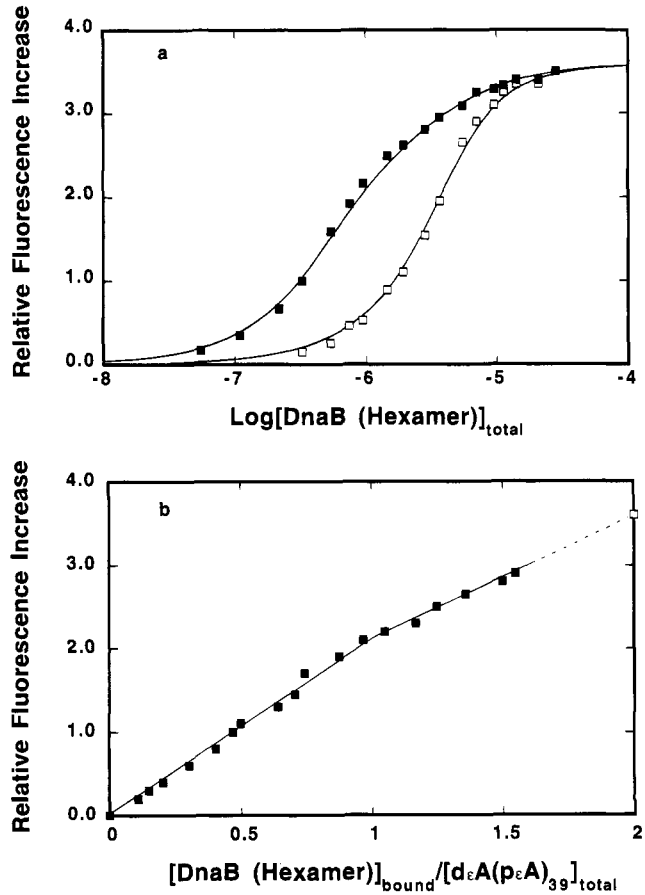

FIGURE 2: (a) Fluorescence titrations of dεA(pεA)$_{39}$ with the DnaB protein monitored by the increase of the nucleic acid fluorescence in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP at two different nucleic acid concentrations: (□) 4.9×10^{-7} M (oligomer); (■) 3.4×10^{-6} M (oligomer). Solid lines are the computer fits of the binding isotherms using the equation for the large ligand binding to the short lattice (see eqs 5–7) with intrinsic binding constant $K = 9 \times 10^6$ M^{-1}, cooperativity parameter $\omega = 0.05$, $\Delta F_1 = 2.2$, and $\Delta F_2 = 1.4$. (b) Dependence of the relative increase of the dεA(pεA)$_{39}$ fluorescence upon the average number of DnaB helicase hexamers bound per oligomer. The dashed line is the extrapolation to $\Delta F_{max} = 3.6$ (□). The absolute value of the average number of DnaB helicase hexamers bound per ssDNA oligomer has been determined using the thermodynamically rigorous approach described in Materials and Methods.

Binding of ssDNA 20-mer to the DnaB Hexamer. Figure 3a shows fluorescence titrations of dεA(pεA)$_{19}$ with the DnaB helicase in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP at three different 20-mer concentrations. The maximum fluorescence increase of this ssDNA oligomer, whose length corresponds exactly with the site-size of the DnaB–ssDNA complex, is 3.4 ± 0.2. The dependence of the observed fluorescence increase, as a function of the average number of DnaB hexamers bound per 20-mer, is linear and shows that, at saturation, one DnaB hexamer binds a single dεA(pεA)$_{19}$ molecule (Figure 3b). A 10-fold increase of the 20-mer concentration does not change the determined 1:1 stoichiometry of the complex with the DnaB helicase, indicating that the hexamer has only a single, strong ssDNA binding site (Figure 3a). If there are additional ssDNA binding sites significantly weaker than this strong site, these weaker sites must have an affinity at least 100-fold lower than the single, strong binding site (see Discussion).

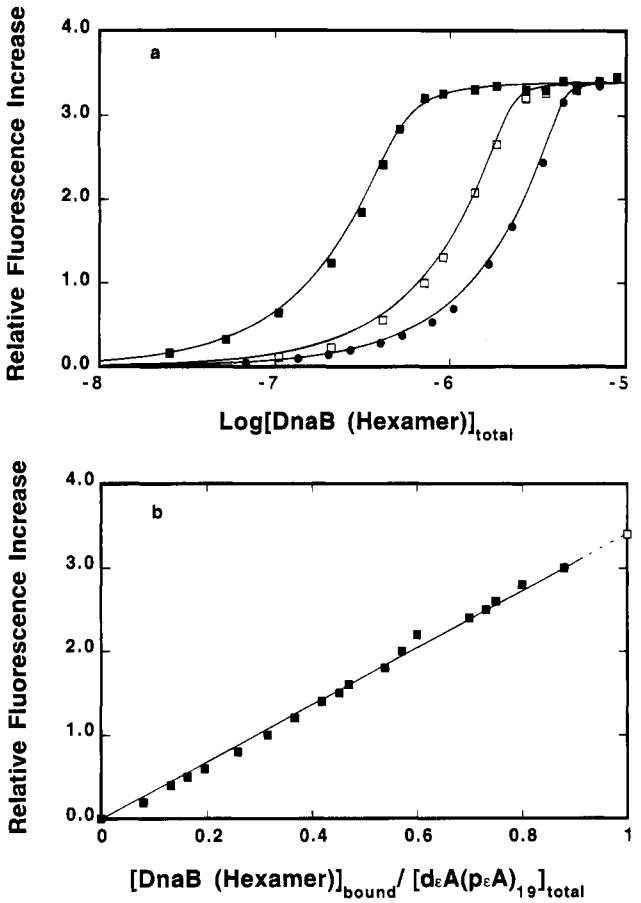

FIGURE 3: (a) Fluorescence titrations of dϵA(pϵA)$_{19}$ with the DnaB protein monitored by the increase of the nucleic acid fluorescence in buffer T2 (pH 8.1, 10 °C) containing 50 mM NaCl and 1 mM AMP-PNP at three different nucleic acid concentrations: (■) 4.9 × 10^{-7} M (oligomer); (□) 2.2 × 10^{-6} M (oligomer); (○) 4.6 × 10^{-6} (oligomer). Solid lines are the computer fits of the single binding site isotherm, $\Delta F = \Delta F_{max}[KP_f/(1 + KP_f)]$, with intrinsic binding constant $K = 4.8 \times 10^7$ M^{-1} and $\Delta F_{max} = 3.4$. (b) Dependence of the relative increase of the dϵA(pϵA)$_{19}$ fluorescence upon the average number of DnaB helicase hexamers bound per oligomer. The dashed line is the extrapolation to $\Delta F_{max} = 3.4$ (□).

Cooperativity in DnaB Helicase Binding to ssDNA. Analyses of the titration curves of poly(dϵA) with the DnaB helicase, using the site overlap model of McGhee and von Hippel, as defined by the generalized eq 5 (Bujalowski et al., 1989), provide estimations of the intrinsic free energy of binding and cooperativity. The solid lines in Figure 1a are computer best fits of experimental isotherms, using eq 4. The obtained intrinsic binding constant is $K = 1.1 \times 10^5$ M^{-1}, and cooperativity parameter $\omega = 3 \pm 0.5$ (see Figure 1a). The estimation of the cooperativity of binding can also be obtained by studying the interactions of the DnaB helicase with the ssDNA oligomer dϵA(pϵA)$_{39}$ which is capable of binding only two DnaB hexamers. The solid lines in Figure 2a are computer fits of the experimental isotherms of DnaB protein binding to the dϵA(pϵA)$_{39}$ oligomer, using eq 5. The obtained intrinsic binding constant is $K = 9 \pm 3 \times 10^6$ M^{-1}, and the cooperativity parameter is $\omega = 0.05 \pm 0.03$. The difference between the intrinsic affinity and the cooperativity in the binding to the polymer versus 40-mer may result from the possible differences in interactions of the protein with the ends of the oligomer (Bujalowski & Lohman, 1991a,b). Thus, it should be noted that the intrinsic binding constant for 20-mer [dϵA(pϵA)$_{19}$], where the end effect should be the most pronounced, is even higher ($K = 4.8 \times 10^7$ M^{-1}) than the value obtained for 40-mer. Nevertheless, both values of ω indicate that the DnaB helicase is not able to form long clusters when bound to ss nucleic acid (see Discussion).

Photo-Cross-Linking dT(pT)$_{19}$ to the DnaB Helicase Hexamer. UV irradiation has been a widely used method in studying the structure of protein−nucleic acid complexes (Williams & Konigsberg, 1991). The irradiation produces covalent linkage between nucleic acid bases and amino acid residues, with minimal perturbation in the studied complexes. The site-size of 20 ± 3 nucleotides of the DnaB helicase−ssDNA complex indicates that a limited set of hexamer subunits interacts with the ssDNA. Therefore, to obtain an estimate of how many DnaB hexamer subunits are engaged in the ssDNA binding, we have applied ultraviolet irradiation to cross-link the DnaB hexamer to dT(pT)$_{19}$, which exactly spans the size of the DnaB hexamer−ssDNA binding site. The photo-cross-linking experiments have been performed on samples containing a constant concentration of [5′-^{32}P]-(dT)$_{20}$ and varying concentrations of the DnaB helicase or on samples containing a constant DnaB concentration and varying concentrations of [5′-^{32}P](dT)$_{20}$. The autoradiogram of SDS−polyacrylamide gel (Figure 4) shows that, independently of the way of performing the experiments, only a single radioactive band, whose apparent molecular weight corresponds with the expected DnaB monomer−dT(pT)$_{19}$ complex (58 000−60 000), appears. If more subunits of the hexamer are complexed with 20-mer, then radioactive bands corresponding to the DnaB dimers, trimers, etc. should be visible; however, no such bands have been observed. Thus, these results indicate that *only one subunit* out of six subunits of the DnaB hexamer is primarily involved in ssDNA binding.

DISCUSSION

Low Site-Size of the DnaB Helicase Hexamer−ssDNA Complex. A starting point for the stoichiometry determination of the nonspecific protein−nucleic acid complex is the quantitative estimation of the site-size of the complex, using a homogeneous polymer nucleic acid (Bujalowski & Lohman, 1987, 1991a,b; McSwiggen et al., 1988). We have found that a fluorescent analog of poly(dA) [poly(dϵA)] undergoes a substantial increase [(3.6 ± 0.3)-fold] in fluorescence intensity when bound to the DnaB protein. This major change in the polynucleotide fluorescence has provided an excellent signal to monitor the interactions and to perform high-resolution measurements of the stoichiometry of the DnaB−ssDNA complex and the mechanism of binding.

Using the thermodynamically rigorous approach described in Materials and Methods, we have determined that the hexamer of the DnaB helicase binds the polymer ssDNA with the site-size of 20 ± 3 nucleotides. For comparison, the site-size of another well-studied hexameric helicase, the *E. coli* transcription termination factor Rho, has been determined to be ~80 nucleotides per hexamer, although both proteins are of similar size and similar global quaternary structure (Gogol et al., 1991; Bujalowski et al., 1994). The surprisingly low site-size of the DnaB−ssDNA complex has been confirmed in the binding experiments, with oligonucleotides 40 and 20 residues long; two DnaB hexamers bind to 40-mer at saturation, while one hexamer binds to 20-mer. It should be noted that a 10-fold increase in the concentration

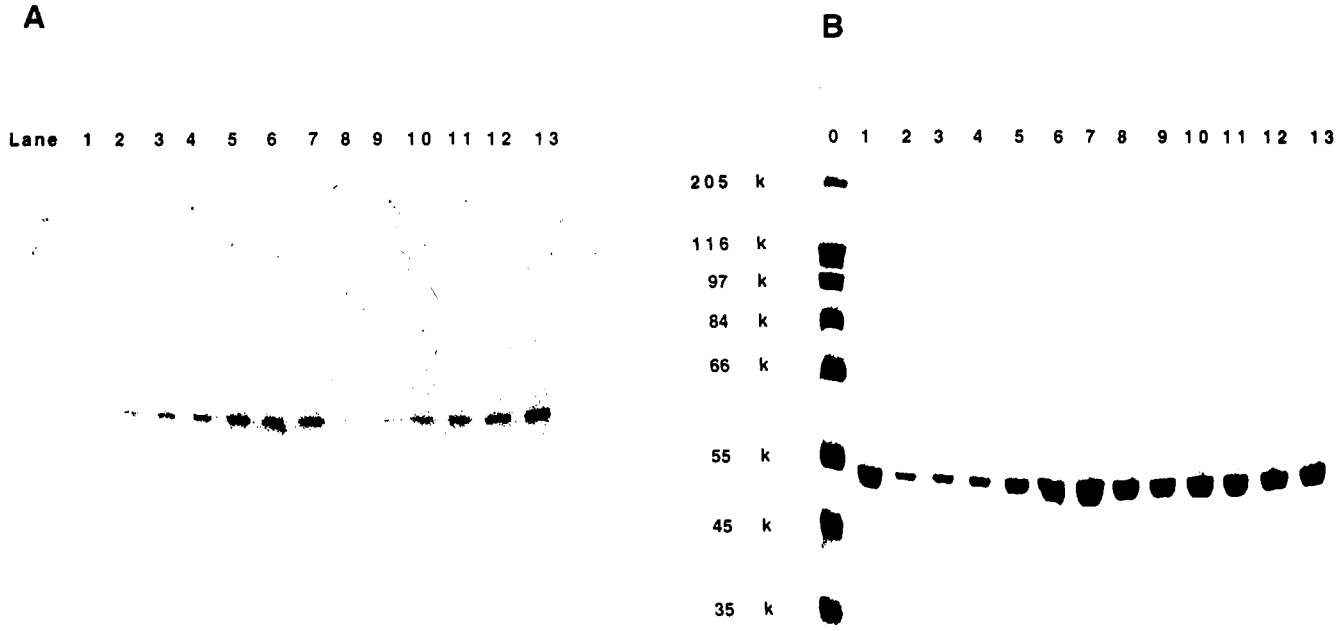

FIGURE 4: (A) Autoradiogram of the SDS−polyacrylamide gel electrophoresis of DnaB protein−[5′-^{32}P](dT)$_{20}$ complexes, after UV-mediated cross-linking of the nucleic acid to the helicase, in buffer T2 containing 50 mM NaCl and 1 mM AMP-PNP. Lane 1 shows the control sample which is an unirradiated mixture of [5′-^{32}P](dT)$_{20}$ with [DnaB] = 1×10^{-6} M (hexamer). In lanes 1−7 the concentration of the nucleic acid was kept constant ([5′-^{32}P](dT)$_{20}$ = 1×10^{-6} M), and the concentration of the helicase was varied: lane 2, 5×10^{-8} M (hexamer); lane 3, 1×10^{-7} M; lane 4, 2×10^{-7} M; lane 5, 5×10^{-7} M; lane 6, 1×10^{-6} M; lane 7, 2×10^{-6} M. In lanes 8−13, the concentration of the DnaB helicase was kept constant ([DnaB] = 1×10^{-6} M (hexamer)), and the concentration of the [5′-^{32}P](dT)$_{20}$ oligomer was varied: lane 8, 5×10^{-8} M (oligomer); lane 9, 1×10^{-7} M; lane 10, 2×10^{-7} M; lane 11, 5×10^{-7} M; lane 12, 1×10^{-6} M; lane 13, 2×10^{-6} M. (B) The same polyacrylamide gel as shown in panel A, aligned exactly with the autoradiogram, but stained with Coomassie Brilliant Blue. Lane 0 contains molecular weight markers.

of the 20-mer (up to 4.6×10^{-6} M; see Figure 3a) does not affect the 1:1 stoichiometry of the DnaB−oligomer complex, although titrations at very high 20-mer concentrations (~10^{-4} M) show the existence of additional, very weak binding sites (Bujalowski, unpublished data). It is evident that the DnaB hexamer has a *single, strong ssDNA binding site*, encompassing 20 nucleotides, and that only this site is used when the hexamer binds polymer ssDNA.

The oligonucleotide of 20 residues can span a distance of ~70 Å (Saenger, 1988). Our hydrodynamic data indicate that, in the DnaB hexamer, the protomers form a cyclic structure with each subunit in contact with only two of its neighbors (Bujalowski et al., 1994). The protomers, 52 265 Da each, have an elongated structure and, when modeled as a prolate ellipsoid, have an axial ratio of $a/b = 5.2 \pm 0.8$, which provides an estimation of the average distance between the centers of mass of adjacent DnaB protomers, within the hexamer, of at least ~30−40 Å. Thus, a single-stranded nucleic acid of 20 residues in length, bound in the single strong binding site, is only long enough to make contact with one or two subunits in the hexamer. Photo-cross-linking results obtained in this work show that indeed only one subunit of the hexamer efficiently cross-links with dT(pT)$_{19}$. However, it is still possible that the ssDNA binding site, located predominantly on one subunit, partly extends to the second subunit, but the cross-linking of dT(pT)$_{19}$ is not efficient at this part of the binding site. These results strongly indicate that only one or possibly two subunits of the hexamer are engaged in the complex with ssDNA.

As we mentioned above, the other well-studied hexameric helicase, whose site-size has been determined in quantitative equilibrium studies with ss nucleic acid, is the *E. coli* transcription termination factor Rho (McSwiggen et al., 1988). The 80 nucleotide long site-size of the Rho−ssRNA complex can span ~250 Å. The average distance between the centers of mass of adjacent Rho monomers within the hexamer is ~40 Å (Gogol et al., 1991). There are six ssRNA binding sites on Rho; thus, it appears that each subunit of the hexamer is engaged in interactions with nucleic acid, and the nucleic acid wraps around the Rho hexamer (Wang & von Hippel, 1993). The striking difference between the DnaB helicase and the Rho factor is that, in the case of the DnaB hexamer, there is a single strong ssDNA binding site, and only a limited set of subunits is engaged in the interactions with ssDNA; the site-size of ~20 ± 3 nucleotides of the DnaB−ssDNA complex is ~4 times shorter than the corresponding site-size of the Rho−ssRNA complex. Specific wrapping of the ss nucleic acid around all six subunits of the Rho hexamer has been postulated to play a key role in the functioning of Rho, particularly in the translocation of the enzyme on the ss nucleic acid (Geiselmann et al., 1993). In the case of the dimer *E. coli* helicase, Rep protein, both subunits are engaged in DNA binding (Wong & Lohman, 1993). Moreover, the involvement of DNA binding sites on both subunits, with their affinities controlled by ATP/ADP switch, was postulated to be crucial for the helicase translocation on nucleic acid and unwinding (Wong & Lohman, 1993). The results obtained in this work preclude any extensive wrapping of ssDNA around the DnaB helicase hexamer and are not compatible with models of hexameric helicase translocation along the nucleic acid lattice in which all six protomers and/or multiple binding sites are involved in ssDNA binding. Although the mechanism of translocation of the DnaB helicase on the DNA lattice is still unknown, the presence of a single, strong ssDNA binding site suggests that such a mechanism could rely on some global conformational changes in the hexamer, induced by the ATP/ADP switch and/or nucleic acid binding, rather than only on

affinity cycles of multiple DNA binding sites induced by ATP binding and hydrolysis (Wong & Lohman, 1993). The profound difference between the RNA−DNA helicase, Rho factor, and the replicative helicase, DnaB protein, in the very structure of their complexes with ss nucleic acid strongly suggests that the helicase mechanism and the translocation mechanism of these two enzymes are different.

It is possible that the binding of the hexameric helicase to ssDNA, in which only one subunit, or a limited set of subunits, is involved, as determined for the DnaB helicase in this work, is also operational for other replicative hexameric helicases. It should be noted that recent studies have suggested the existence of a limited number of ssDNA binding sites on a mutant phage T7 primase−helicase hexamer, although the precise site-size of the complex has not been determined (Hingorani & Patel, 1993).

The DnaB Helicase Does Not Form Clusters When Bound to ssDNA. It is interesting that the binding of the DnaB helicase to ssDNA is characterized by very weak cooperativity, as determined using poly(dεA) ($\omega \sim 3$), or even negative cooperativity, using the 40-mer dεA(pεA)$_{39}$ ($\omega = 0.05$). Both values of ω indicate that the DnaB helicase *is not able to form long protein clusters* on ssDNA (McGhee & von Hippel, 1974). We suggest that this result reflects a fundamental, general aspect of the function of replicative helicases. The protein is required in the initiation of DNA replication at the specific replication initiation site, oriC. In the elongation step of DNA replication, the DnaB helicase functions processively at the junction between ssDNA and dsDNA (Marians, 1992). None of these functions requires a formation of helicase clusters and/or extensive coating of long stretches of ssDNA by the protein. Thus, very weak or even negative cooperativity in the DnaB protein binding to ssDNA, as determined in this work, may be a general adaptation of replicative helicases to perform functions which require only a single enzyme molecule.

ACKNOWLEDGMENT

We thank Drs. D. Wayne Bolen and Alex Kurosky for their careful reading and helpful comments on the manuscript and Mrs. Gloria Drennan Davis for her help in preparing the manuscript.

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BI950959C