Strand Specificity in the Interactions of *Escherichia coli* Primary Replicative Helicase DnaB Protein with a Replication Fork†

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ABSTRACT: The interactions of the *Escherichia coli* primary replicative helicase DnaB protein, with synthetic DNA replication fork substrates, having either a single arm or both arms, have been studied using the thermodynamically rigorous fluorescence titration techniques. This approach allows us to obtain absolute stoichiometries of the formed complexes and interaction parameters without any assumptions about the relationship between the observed signal (fluorescence) and the degree of binding. Subsequently, the formation of the complexes, with different replication fork substrates, has also been characterized using the sedimentation velocity technique. To our knowledge, this is the first quantitative characterization of interactions of a hexameric helicase with replication fork substrates. In the presence of the ATP nonhydrolyzable analog, AMP-PNP, the *E. coli* DnaB helicase preferentially binds to the 5′ arm of the single-arm fork substrate with an intrinsic affinity 6-fold higher than its affinity for the 3′ arm. ATP hydrolysis is not necessary for formation of the helicase−fork complex. The asymmetric interactions are consistent with the 5′ → 3′ directionality of the helicase activity of the DnaB protein and most probably reflects a preferential 5′ → 3′ polarity in the helicase binding to ssDNA, with respect to the ssDNA backbone. The double-stranded part of the fork contributes little to the free energy of binding. The data indicate a rather passive role of the duplex part of the fork in the binding of the helicase. This role seems to be limited to impose steric hindrance in the formation of nonproductive complexes of the enzyme with the fork. Quantitative analysis of binding of the helicase to the two-arm fork substrate shows that two DnaB hexamers can bind to the fork, with each single hexamer associated with a single arm of the fork. In this complex, the intrinsic affinity of the DnaB hexamer for the 5′ arm in a two-arm fork is not affected by the presence of the 3′ arm. Moreover, the results show that the 3′ arm is in a conformation which makes it easily available for the binding of the next DnaB hexamer. Because of the large size of the DnaB hexamer, the data indicate that the 3′ arm is separated from the 5′ arm. The separation of both arms must be to such an extent that the 3′ arm can bind an additional large DnaB hexamer. These results reveal that the 3′ arm is not engaged in thermodynamically stable interactions with the helicase hexamer, when it is bound in its stationary complex to the 5′ arm of the fork. The significance of the these results for a mechanistic model of the hexameric DnaB helicase action is discussed.

In the processes of DNA replication, recombination, and repair, the double-stranded (ds) DNA must be unwound to provide a metabolically active single-stranded (ss) intermediate. This reaction is catalyzed by a class of enzymes called helicases. These enzymes are motor proteins which use the free energy of triphosphate hydrolysis to unwind dsDNA and to translocate along the nucleic acid lattice (Matson & Kaiser-Rogers, 1990; Lohman & Bjornson, 1996; Marians, 1992; Kornberg & Baker, 1992).

DnaB is a key DNA replication protein in *Escherichia coli* and is involved in both the initiation and elongation stages of DNA replication (Lebowitz & McMacken, 1986; Matson & Kaiser-Rogers, 1990; Marians, 1992). The protein is the primary *E. coli* replicative helicase, *i.e.*, the factor responsible for unwinding the duplex DNA in front of the replication fork, with preferential unwinding of dsDNA in the 5′ → 3′ direction (Lebowitz & McMacken, 1986; Baker et al.,1987; Kornberg & Baker, 1992). The DnaB protein is the only helicase required to reconstitute DNA replication *in vitro* from the chromosomal origin of replication (oriC). The enzyme is crucial for the replication of bacterial chromosomal, phage, and plasmid DNA (Wickner et al., 1973; Kornberg & Baker, 1992; McMacken et al., 1978).

The DnaB helicase provides an outstanding model for the structure−function studies of a helicase. Native DnaB forms a hexamer composed of six identical subunits (Bujalowski et al., 1994; Reha-Krantz & Hurwitz, 1978). Analytical sedimentation studies show that the DnaB helicase exists as a stable hexamer over a large protein concentration range (Bujalowski & Klonowska, 1993; Bujalowski et al., 1994; Jezewska & Bujalowski, 1996; Jezewska et al., 1996a,b). In fact, strong stability of the DnaB hexamer distinguishes this enzyme from other well-studied hexameric helicases which exist in mixtures of various oligomeric forms (Dong et al., 1995; Egelman et al., 1995). Hydrodynamic and electron microscopy data indicate that six protomers aggregate with

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cyclic symmetry where the protomer−protomer contacts are limited to only two neighboring subunits (Bujalowski et al., 1994; San Martin et al., 1995; Yu et al., 1996).

Recently, we characterized interactions of the DnaB helicase with ssDNA using the quantitative fluorescence titration technique which eliminates any assumptions about the relationship between the observed signal change and the degree of binding (Bujalowski & Jezewska, 1995; Jezewska et al., 1996a,b; Jezewska & Bujalowski, 1996a,b). Binding of the DnaB protein to the ssDNA fluorescent derivative poly(dϵA) is accompanied by a strong increase of the nucleic acid fluorescence. We showed that in the stationary complex, in the presence of the ATP nonhydrolyzable analog AMP-PNP,[1] the DnaB hexamer binds polymer ssDNA with a site size of 20 ± 3 nucleotides per protein hexamer. Further thermodynamic and photo-cross-linking studies showed that the DnaB hexamer has a single, strong binding site for ssDNA, and that only a single subunit is primarily in contact with ssDNA (Bujalowski & Jezewska, 1995). These results preclude the possibility of extensive wrapping of the ssDNA around the hexamer and show that, in the translocation of the enzyme along the nucleic acid lattice, a limited set of subunits, most probably only one, are engaged in the interactions with ssDNA (Bujalowski & Jezewska, 1995; Jezewska et al., 1996a).

The next step of fundamental importance is the elucidation of how the determined characteristics of the DnaB helicase binding to ssDNA relates to interactions of the enzyme with a more complex substrate, a replication fork. In this communication, we report the first quantitative analysis of the DnaB helicase−replication fork complex using the rigorous fluorescence titration technique and analytical sedimentation measurements. We provide direct evidence that in the presence of the ATP nonhydrolyzable analog, AMP-PNP, the *E. coli* DnaB helicase binds preferentially to the 5′ arm of the replication fork and that ATP hydrolysis is not necessary for formation of the helicase−fork complex. Moreover, the results show that the ds part of the fork contributes little to the free energy of binding. Binding of the helicase to the two-arm fork shows that two hexamers can bind to the fork, with each hexamer associated with a single arm of the fork. Quantitative analysis of the interactions indicates that the intrinsic affinities of the hexamers bound to the 5′ and 3′ arms are not affected when both hexamers are simultaneously bound. These data provide direct evidence that the 3′ arm is not engaged in stable interactions with the helicase hexamer when it is bound in its stationary complex to the 5′ arm of the fork, thus, leaving the 3′ arm fully accessible for the binding of the second DnaB hexamer.

MATERIALS AND METHODS

Reagents and Buffers. All solutions were made with distilled and deionized >18 MΩ (Milli-Q Plus) water. All chemicals were of 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 and Nucleic Acids. The *E. coli* DnaB protein was purified, as previously described by us (Bujalowski & Klonowska, 1993, 1994). 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). All nucleic acids used in this work (Figure 1) were purchased from Midland Certified Reagents (Midland, Texas). The ss oligomers were at least >95% as judged by autoradiography on polyacrylamide gels. Concentrations of all ssDNA oligomers have been spectrophotometrically determined, using the nearest neighbor analysis (Cantor et al., 1970). The single-arm and the two-arm fork substrates were obtained by mixing the proper oligomers, warming up the mixture for 5 min at 95 °C, and slowly cooling for a period of ∼2 h.

Analytical Sedimentation Measurements. Sedimentation experiments were performed with the Optima XL-A analytical centrifuge. The analysis of the sedimentation runs was performed, as previously described by us (Bujalowski et al., 1994).

Fluorescence Measurements. All steady-state fluorescence measurements were performed using the SLM 48000S spectrofluorometer (Bujalowski & Klonowska, 1993, 1994). 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 sample.

Determination of Rigorous Thermodynamic Binding Isotherms and Absolute Stoichiometries of the DnaB Helicase Complexes with Fork Substrates. In our studies, we followed the binding of the DnaB protein to the fork substrates, by monitoring the fluorescence increase, ΔF, of their ethenoderivatives upon the complex formation. A general procedure to obtain true estimates of the average number of protein molecules bound per nucleic acid, $\sum \nu_i$, and the free protein concentration, P_F, has been previously described (Bujalowski & Klonowska, 1993, 1994). This procedure is based on the fact that the experimentally observed ΔF, in the course of titration, is related to $\sum \nu_i$ by

$$\Delta F = \sum \nu_i \Delta F_{i_{max}} \tag{1}$$

where $\Delta F_{i_{max}}$ is the maximum fluorescence increase of nucleic acid with the DnaB protein bound in complex *i*. The value of $\sum \nu_i$ and P_F is then related to the total protein concentrations, P_{T_1} and P_{T_2}, and the total nucleic acid concentrations, N_{T_1} and N_{T_2}, at which the same ΔF is obtained by

$$\sum \nu_i = (P_{T_2} - P_{T_1})/(N_{T_2} - N_{T_1}) \tag{2}$$

$$P_F = P_{T_x} - \sum \nu_i (N_{T_x}) \tag{3}$$

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

Analysis of the Binding of the DnaB Hexamer to Single-Arm Fork Substrates. As we show below, in the case of single-arm fork substrates, only one DnaB hexamer binds to the arm part of the fork. Binding of the DnaB helicase to single-arm fork substrates has been analyzed using a single-site binding isotherm as described by

$$\Delta F = \Delta F_{max} [KP_F/(1 + KP_F)] \tag{4}$$

where *K* is the binding constant and ΔF_{max} is the maximum increase of the nucleic acid fluorescence at saturation with the enzyme.

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

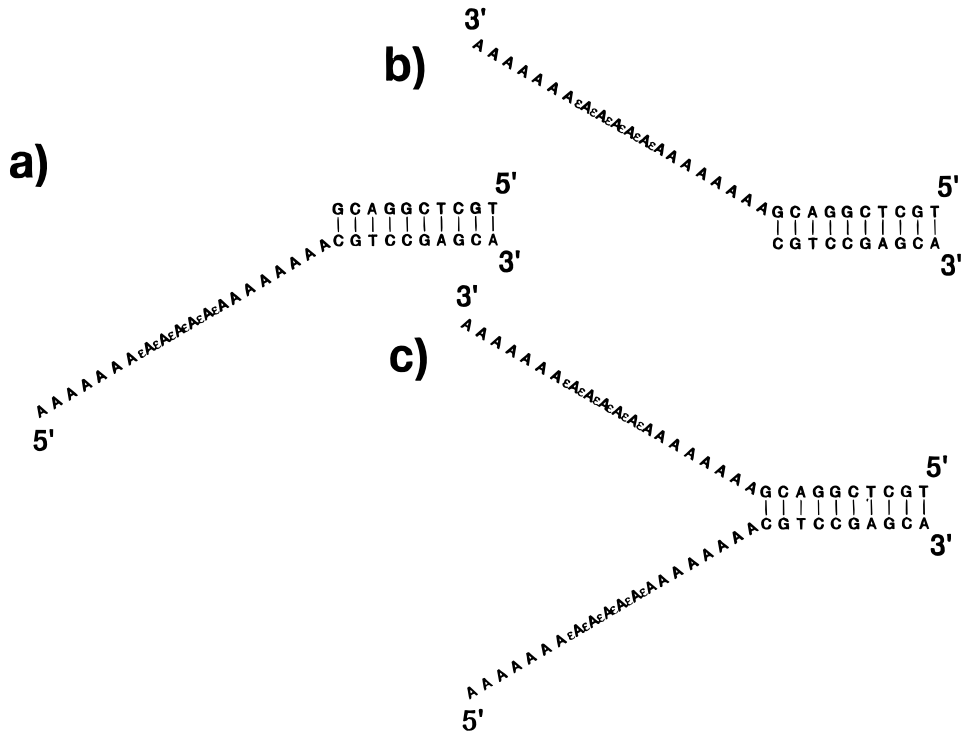

FIGURE 1: DNA substrates used in studying the interactions of the DnaB hexamer with the replication fork. (a) The 5′ single-arm fork is constructed using a 30 mer having at its 5′ end a track of 20 adenosine residues, with six middle residues replaced by fluorescent derivatives, etheno-adenosines (ϵA), providing a sequence of the arm as $dA(pA)_6(p\epsilon A)_6(pA)_7$. The remaining 10 bases of random sequence have been annealed to the complementary 10 mer, thus forming the duplex part of the fork 10 bp long. (b) The 3′ single-arm fork is constructed using an analogous 30 mer, but having at its 3′ end a track of 20 adenosine residues, with six middle residues replaced by ϵA. (c) The full two-arm fork has been constructed by annealing two corresponding 30 mers used in the construction of the single-arm forks. Analogous fork substrates were used in analytical centrifugation experiments and were constructed using 30 mers, which contain exclusively As in the part corresponding to the arm of the fork and which were modified with fluorescein at the 5′ or 3′ end at the duplex part of the fork, through the six-carbon amino linkers.

Analysis of the Binding of Two DnaB Hexamers to a Two-Arm Fork. Consider a situation in which a two-arm fork can bind two DnaB hexamers with the binding sites located on the 5′ and 3′ arms of the fork which are labeled with ϵA (Figure 1). Binding of the DnaB hexamers to the fork is characterized by intrinsic binding constants, K_1 and K_2. Because the binding sites (both arms) are very close to each other, we expect some interaction between the two large hexamers bound to the arms of the fork, even if the intrinsic binding affinities of the enzyme for each arm of the fork are not affected to any extent. These additional protein−protein interactions can be phenomenologically described by the parameter σ. The partition function Z, describing the binding of the two DnaB hexamers to the two-arm fork, is defined as

$$Z = 1 + (K_1 + K_2)P_F + K_1K_2\sigma P_F^2 \qquad (5a)$$

Let the fluorescence of the free 5′ arm and the 3′ arm be F_{F1} and F_{F2}, respectively, and the fluorescence of the 5′ arm and the 3′ arm, with the DnaB protein bound, be F_{B1} and F_{B2}. Then the fluorescence F of the two-arm fork in the presence of the helicase is

$$F = (F_{F1} + F_{F2})N_F + (F_{B1} + F_{F2})K_1N_FP_F + (F_{B2} + F_{F1})K_2N_FP_F + (F_{B1} + F_{B2})K_1K_2\sigma N_FP_F^2 \qquad (5b)$$

The total nucleic acid concentration N_T is related to the free nucleic acid concentration N_F by mass conservation equation

$$N_T = N_F(1 + K_1P_F + K_2P_F + K_1K_2\sigma P_F^2) \qquad (5c)$$

Introducing eq 5c into 5b and rearranging provides a theoretical equation relating the observed relative fluorescence increase, ΔF, to the binding of both hexamers to the two-arm fork

$$\Delta F = [(R_1\Delta F_1K_1 + R_2\Delta F_2K_2)P_F + (R_1\Delta F_1 + R_2\Delta F_2)K_1K_2\sigma P_F^2]/[Z] \qquad (6)$$

where $\Delta F = [F - (F_{F1} + F_{F2})N_T]/(F_{F1} + F_{F2})N_T)$; $R_1 = F_{F1}/(F_{F1} + F_{F2})$ and $R_2 = F_{F2}/(F_{F1} + F_{F2})$ are the contributions of each arm to the total fluorescence of the free two-arm fork; $\Delta F_1 = (F_{B1} - F_{F1})/F_{F1}$ and $\Delta F_2 = (F_{B2} - F_{F2})/F_{F2}$ are the relative increases of fluorescence of 5′ and 3′ arm, respectively, induced by the helicase binding. It should be noted that, if the binding constant K_2 is $<< K_1$ or $\sigma << 1$, there will be a characteristic intermediate plateau in the plot of the isotherm which appears at $\Delta F \approx \{R_1[K_1/(K_1 + K_2)]\}\Delta F_1 + \{R_2[K_2/(K_1 + K_2)]\}\Delta F_2$. Thus, the intermediate plateau is at a lower value than ΔF_1 and ΔF_2, due to the weighting factors $\{R_1[K_1/(K_1 + K_2)]\}$ and $\{R_2[K_2/(K_1 + K_2)]\}$. The final plateau at saturation appears at $\Delta F = R_1\Delta F_1 + R_2\Delta F_2$. The analysis described above is applicable to any cooperative or noncooperative ligand binding to discrete binding sites on a macromolecule which differ in intrinsic spectroscopic (fluorescence in our case) properties.

RESULTS

Binding of the DnaB Hexamer to Single-Arm Forks. The replication fork substrates used in these studies are depicted

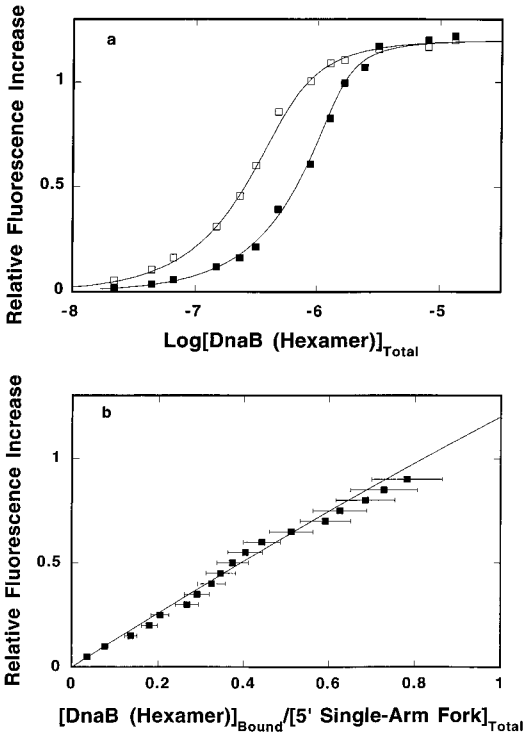

FIGURE 2: (a) Fluorescence titrations of a 5′ single-arm fork with the DnaB protein (λ_{ex} = 325 nm, λ_{em} = 410 nm) monitored by the nucleic acid fluorescence increase in buffer T2 (50 mM Tris, pH 8.1, 5 mM $MgCl_2$, 10% glycerol, 100 mM NaCl, 1 mM AMP-PNP, 10 °C) at two different nucleic acid concentrations: (□) 4.5 × 10^{-7} M; (■) 1.45 × 10^{-6} M (fork). Solid lines are computer fits of the titration curves, using a single-site binding isotherm, with intrinsic binding constant K_1 = 1.1 × 10^7 M^{-1} and relative fluorescence change $\Delta F_1 = \Delta F_{max}$ = 1.2 (eq 4). (b) Dependence of the relative fluorescence of the fork substrate, ΔF, upon the average number of bound DnaB hexamers (■). Error bars are standard deviations obtained using 4−5 independent titration curves. Solid line is the theoretical dependence of ΔF_1 upon the average number of bound DnaB hexamers, as defined by eq 4.

in Figure 1. The duplex part of each fork substrate is 10 bp long. Two of the substrates are single-arm forks, *i.e.*, they have only a 5′ or a 3′ arm. Each arm is a homooligonucleotide (dA) 20 bases long, which corresponds to the site-size of the enzyme−ssDNA complex determined in studies with polymer ssDNA (Bujalowski & Jezewska, 1995; Jezewska et al., 1996a). In the middle of each arm there is a stretch of six fluorescent derivatives of adenosine (etheno-adenosines, ϵA), which provide the fluorescence signal to monitor the binding. A full two-arm fork (Figure 1) has both arms containing the fluorescent ϵA.

Fluorescence titrations of the 5′ single-arm fork, with the DnaB helicase at two different DNA concentrations, are shown in Figure 2a. Binding of the enzyme to the fork is accompanied by a strong nucleic acid fluorescence increase (∼220%) reflecting the interactions of the helicase with ϵAs in the arm (Jezewska et al., 1996a). Application of the thermodynamically rigorous analysis (see Materials & Methods) allows us to determine the absolute stoichiometry of the complex. The dependence of the relative fluorescence increase, ΔF, as a function of the average number of the DnaB hexamers bound to the fork, is shown in Figure 2b. The absolute degree of binding of DnaB on the fork substrate could be determined up to 85% of the total binding isotherm. Short extrapolation to the maximum observed fluorescence signal shows that only a single DnaB hexamer binds to the

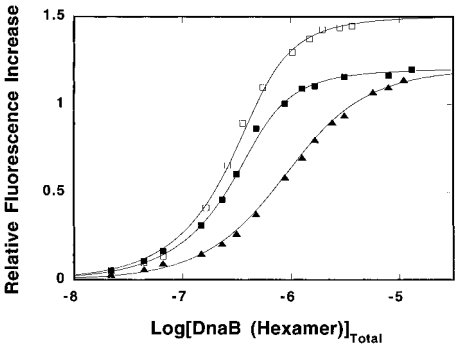

FIGURE 3: Fluorescence titrations of the 3′ single-arm fork and the 20 mer, $dA(pA)_6(p\epsilon A)_6(pA)_7$, with the DnaB helicase monitored by the nucleic acid fluorescence increase (λ_{ex} = 325 nm, λ_{em} = 410 nm) in buffer T2 (50 mM Tris, pH 8.1, 5 mM $MgCl_2$, 10% glycerol, 100 mM NaCl, 1 mM AMP-PNP, 10 °C): (▲) 4.5 × 10^{-7} M (3′ single-arm fork) and (□) 4.5 × 10^{-7} M (20 mer). For comparison, the fluorescence titration of the 5′ single-arm fork substrate, at the same nucleic acid concentration, is also included (■). Solid lines are computer fits of the titration curves, using a single-site binding isotherm (eq 4), with the intrinsic binding constant K_2 = 1.5 × 10^6 M^{-1} and $\Delta F_{max} = \Delta F_2$ = 1.2 for the 3′ single-arm fork, and K_{20} = 1.1 × 10^7 M^{-1} and $\Delta F_{max} = \Delta F_{20}$ = 1.7 for the 20 mer.

5′ arm fork at maximum saturation. The solid lines in Figure 2 are computer fits of the binding isotherms, using eq 4, which provide the binding constant $K_1 = (1.1 \pm 0.3) \times 10^7$ M^{-1}.

Fluorescence titrations of the 3′ single-arm fork and the 20 mer, $dA(pA)_6(p\epsilon A)_6(pA)_7$ oligomer, at the same nucleic acid concentrations, are shown in Figure 3. For comparison, the fluorescence titration of the 5′ single-arm fork is also included. Quantitative analysis of the binding isotherms, as described in Materials and Methods, shows that only one DnaB hexamer binds at saturation to the 20 mer and to the 3′ single-arm fork (Jezewska et al., 1996a). The solid lines in Figure 2a are computer fits of the binding isotherms, using eq 4. The binding constant of the DnaB hexamer for the 20 mer $K_{20} = (1.1 \pm 0.3) \times 10^7$ M^{-1}. Thus, it is evident that the affinities of the helicase to the 20 mer and 5′ arm fork (Figure 2a) are identical, indicating the duplex part of the fork does not contribute to the free energy of the hexamer binding in the complex with the 5′ single-arm fork. However, in the case of the 3′ single-arm fork, DnaB binds to the nucleic acid with significantly lower affinity, as compared to the 20 mer and 5′ single-arm fork, with the binding process characterized by $K_2 = (1.5 \pm 0.3) \times 10^6$ M^{-1} (Figure 3).

Nuclease protection digestion studies indicate that the active site of the helicase partially protects 3−4 base pairs of the dsDNA, when the enzyme is associated with the 5′ arm fork, but not in the complex with the 3′ arm fork (data not shown). The lower affinity for the 3′ arm fork and the lack of protection against nuclease digestion of the ds part of the fork most probably reflects binding of the helicase to the 3′ arm in a different orientation, with respect to the duplex part of the fork (see Discussion).

Binding of the DnaB Hexamer to a Two-Arm Fork. The role of both arms, in interactions with the helicase, can be determined by studying the binding of the enzyme to the full fork in which both arms are labeled with fluorescent nucleotides (Figure 1). Fluorescent titrations of the full two-arm fork, at two different concentrations of the nucleic acid, are shown in Figure 4a. A striking difference between these

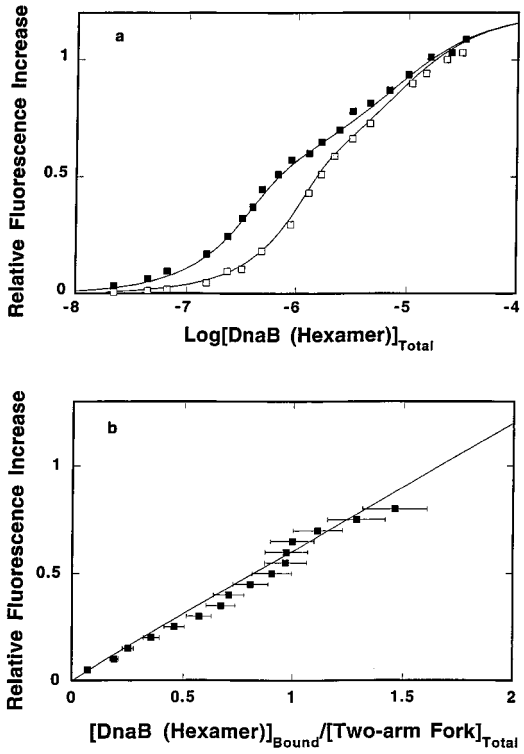

FIGURE 4: (a) Fluorescence titrations of a full two-arm fork with the DnaB protein monitored by the nucleic acid fluorescence increase ($\lambda_{ex} = 325$ nm, $\lambda_{ex} = 410$ nm) in buffer T2, at two different nucleic acid concentrations: (■) 4.5×10^{-7} M (fork); (□) 1.45×10^{-6} M (fork). Solid lines are the computer fits of two binding site isotherms (eq 6) with intrinsic binding constants $K_1 = 1 \times 10^7$ M^{-1}, $\Delta F_1 = 1.2$, $K_2 = 1.5 \times 10^6$ M^{-1}, $\Delta F_2 = 1.2$, and $\sigma = 0.08$. The fractional contribution of each arm to total intensity of the two-arm fork, $R_1 = 0.5$ and $R_2 = 0.5$, were determined from the comparison of the quantum yield of the full two-arm fork and the corresponding single-arm forks, in the absence of the helicase (see Materials and Methods). (b) Dependence of the relative fluorescence increase, ΔF, upon the average number of DnaB helicase hexamers bound per fork (■). Error bars are standard deviations obtained using four to five independent titration curves. Solid line is the theoretical dependence of ΔF upon the average number of bound DnaB hexamers per fork, at a given fork concentration, as defined by eq 6.

titrations and the binding curves obtained for the single-arm forks is a biphasic character of the isotherms. There is a strong-affinity phase, with a plateau around $\Delta F \approx 0.6$, followed by a low-affinity step with a plateau around $\Delta F \approx 1.2$. Figure 4b shows the dependence of the fluorescence increase as a function of the absolute number of the DnaB hexamers bound to the fork. The results show that at the first plateau one DnaB hexamer binds to the two-arm fork followed by the binding of the second hexamer in the second phase of the isotherm. These data show that the two-arm fork can bind two DnaB hexamers, with each single hexamer bound to a single arm of the fork. The binding of the helicase to the 5′ single arm is characterized by a higher affinity (Figure 3). Thus, the high-affinity step corresponds to the binding of the DnaB hexamer to the 5′ arm of the two-arm fork and the low-affinity step reflects the binding of the enzyme to the 3′ arm of the fork.

Binding of two DnaB hexamers to two arms of the fork indicates that the 3′ arm is available for the DnaB protein to bind, even when the first DnaB hexamer is already associated with the 5′ arm. It is important to know to what extent binding of the two DnaB hexamers reflects independent binding of a single hexamer to a single arm of the fork. The two simplest alternative models can be considered. In the first model (eq 6), the hexamers bind with the same intrinsic affinities as to the single-arm forks and with the same relative changes of the fluorescence. Due to their close proximity, when both large hexamers are simultaneously bound to the fork, any additional free energy changes in the binding process result from protein−protein interactions. In the second model, simultaneous binding of both hexamers to the two-arm fork would affect interactions of each hexamer with a corresponding arm, *i.e.*, the hexamers would not form the same complexes with the arms of the fork, as in the case of the single-arm forks. Thus, in the second model, the binding would occur with different intrinsic affinities and also with different fluorescence changes, when compared to the single-arm forks. However, comparison of the titrations of a 5′ single-arm fork (Figure 2a) and a two-arm fork (Figure 4a) shows that the binding in the high-affinity phase occurs in the same concentration range of the protein as the binding to a 5′ single-arm fork. This result shows that the affinity to the 5′ arm is not affected to any detectable extent, by the presence of the 3′ arm. Moreover, the intermediate and final plateaus occur at $\Delta F \approx 0.6$ and 1.2, respectively, as predicted, if the fluorescence changes accompanying the binding of the helicase to both arms are exactly the same as the fluorescence changes accompanying the association with the 5′ and 3′ single-arm forks. Therefore, these data indicate that intrinsic affinities, as well as the fluorescence changes accompanying the binding to each arm of the fork, are not affected by the simultaneous binding of both hexamers to a two-arm fork and strongly support the first model as defined by eq 6.

Fluorescence titrations with single-arm substrates provide the intrinsic affinities of the DnaB hexamer for the 5′ and 3′ arm of the fork, characterized by binding constants K_1 and K_2 and the corresponding relative fluorescence increases ΔF_1 and ΔF_2 (see above). The simultaneous binding of the two hexamers to the full two-arm fork is then described by the same binding constants K_1 and K_2 and the relative fluorescence increases ΔF_1 and ΔF_2 as obtained from the titrations with single-arm forks. Some additional possible interactions, resulting from the very close proximity of both large hexamers on the fork, are characterized by factor σ. Thus, from five parameters in eq 6, four parameters have been independently determined in titrations with single-arm forks.

The solid lines in Figure 4a are the computer simulations of the binding of two DnaB hexamers to the full two-arm replication fork, using K_1, K_2, ΔF_1, and ΔF_2, as obtained from the titrations with single-arm forks (eq 6, Material and Methods). The only fitted parameter which reflects additional interactions between the large hexamers brought to close proximity, when bound to the arms of the fork, is $\sigma = 0.08$. The model reproduced the biphasic experimental isotherm with remarkable precision. Thus, each arm in the two-arm fork can bind a single DnaB hexamer with the same intrinsic affinity as in the case of single-arm forks with additional protein−protein interactions resulting from the close proximity of the two hexamers in the complex.

Analytical Sedimentation Studies of the Complexes of the DnaB Hexamer with Replication Fork Substrates. We also tested the interactions of the DnaB helicase using the independent analytical centrifugation technique. For this purpose, we used replication fork substrates, analogous to

Table 1: Molecular Weights and Sedimentation Coefficients $s_{20,w}$ of the DnaB Helicase Complexes with Different Synthetic Replication Fork Substrates (Labeled with Fluorescein at the 5′ End of the Duplex Part of the Fork, Figure 1) Obtained at the Saturating Protein Concentration of 2×10^{-5} M (Hexamer)[a]

	two-arm fork−DnaB complex	5′ single-arm fork−DnaB complex	3′ single-arm fork−DnaB complex
$s_{20,w}$[b]	16.8 ± 0.3	12.5 ± 0.3	12.5 ± 0.3
molecular weight[c]	640 500 ± 30 000	325 000 ± 10 000	325 000 ± 10 000

[a] Nucleic acid concentration is 1×10^{-6} M (fork). [b] Buffer T2 (pH 8.1, 100 mM NaCl, 1 mM AMP-PNP, 20 °C), 30 000 rpm. [c] Buffer T2 (pH 8.1, 100 mM NaCl, 1 mM AMP-PNP, 20 °C), 6000 rpm. All equilibrium and sedimentation velocity scans were recorded at 495 nm. The errors are standard deviations determined using four to five independent sedimentation runs.

the ones depicted in Figure 1, labeled with fluorescein through a six-carbon amino linker at the 5′ ends of the duplex part of the forks. This approach allowed us to monitor exclusively the complex between the helicase and DNA without interference from protein and AMP-PNP absorbances. The sedimentation coefficient of the DnaB hexamer, in the presence of AMP-PNP, is $s_{20,w} = 11.7 \pm 0.3$ (Jezewska & Bujalowski, 1996a). Sedimentation experiments of the DnaB complexes with the 5′ and 3′ single-arm forks show that in both cases a single species is observed sedimenting with $s_{20,w} = 12.5 \pm 0.3$ and a molecular weight of 325 000 ± 10 000, corresponding exactly to a complex of one hexamer bound to the fork. These values of $s_{20,w}$ and molecular weights have not been affected by increased protein concentrations and provide direct evidence that the hexamer is the species which binds the replication fork.

A dramatically different behavior was observed in the case the full two-arm fork. At the protein concentrations where thermodynamically rigorous fluorescence titrations showed that only a single DnaB hexamer binds to the fork (see above), a single species with $s_{20,w} = 12.5 \pm 0.3$ and a molecular weight of 320 000 ± 10 000 was observed, which corresponds to a complex of one hexamer with the fork. However, at saturating protein concentrations, a single species, with the sedimentation coefficient of 16.8 ± 0.3 and a molecular weight of 645 000 ± 30 000, was observed, indicating the formation of the complex of two DnaB hexamers with the fork. These results are in excellent agreement with fluorescence titration studies described above. The sedimentation data are summarized in Table 1.

DISCUSSION

In vivo, the replicative helicase functions on the junction of ss and dsDNA in the replication fork (Kornberg & Baker, 1992). It is of paramount importance for our understanding of the enzyme action to elucidate the interactions of the enzyme with the replication fork substrates. The results described in this work provide the first quantitative, thermodynamic analysis of the interactions of a hexameric helicase, with the DNA substrates resembling a replication fork in a stationary complex, *i.e.*, without ATP hydrolysis. In these studies we utilized the fact that interactions of the DnaB protein with etheno-adenosine (ϵA) are accompanied by a large nucleic acid fluorescence increase (Bujalowski & Jezewska, 1995, 1996a; Jezewska et al., 1996a). Thus, the replication fork substrates contain a stretch of six ϵAs in the middle of the arms of the forks (Figure 1). The length of the arms exactly corresponds to the site-size of the DnaB hexamer−ssDNA complex, *i.e.*, each arm can accommodate only a single DnaB helicase. Thus, the binding of the helicase to the arms of the fork substrates should reflect intrinsic affinity of the enzyme for the short oligomers (including possible end effects), which constitute the arms, without major statistical factor corrections (Bujalowski & Jezewska, 1995).

The DnaB Helicase Shows Preferential Binding to the 5′ Arm of a Replication Fork. The Role of dsDNA. Quantitative analysis of DnaB interactions with single-arm fork substrates shows that, in the presence of the ATP nonhydrolyzable analog, AMP-PNP, the helicase binds with a higher affinity to the 5′ arm than to the 3′ arm of the fork. The simplest explanation of the higher affinity is that it reflects preferential polarity in the binding of the helicase, with respect to the polarity of the ssDNA phosphate-sugar backbone. Since, the 3′ arm is the same length as the 5′ arm, it can accommodate the DnaB hexamer. Thus, the preferential 5′ → 3′ polarity in the binding of the enzyme could result in an association with the 3′ arm in an opposite orientation, with respect to the duplex part of the fork, as also suggested by nuclease digestion protection studies (see above). In this complex, the active site of the helicase would face the 3′ end of the arm, in accordance with the 5′ → 3′ polarity of the helicase binding to the ssDNA. Such binding could introduce some steric hindrances, not encountered in the case of the 5′ single-arm fork substrate and the 20 mer, resulting in the lower affinity of the enzyme for the 3′ single-arm fork, as experimentally observed. On the other hand, comparison between the affinities of the enzyme for the 5′ single-arm fork and the 20 mer, corresponding to the isolated arm, indicates that the duplex part of the fork provides no contribution to the free energy of binding of the helicase. These data suggest that, in the complex with the fork, the helicase accommodates the duplex part of the fork in the active site without additional free energy changes. The binding free energy predominantly originates from interactions with the ssDNA in the fork. The effect of the dsDNA on the free energy of binding is only detectable when this accommodation is not possible, *i.e.*, when the helicase binds to the 3′ arm fork in an opposite orientation. If the duplex part of the fork was not involved in orienting the helicase in the fork, the binding of the helicase to the 3′ single-arm fork would be characterized by the same affinity as the affinity for the 5′ single-arm fork, or the isolated 20 mer, which is not experimentally observed. Thus, our data indicate that the duplex part of the fork, although not contributing to the free energy of binding of the helicase to the fork, plays a significant role in the formation of a productive complex.

The 3′ Arm of the Replication Fork Is Not Engaged in Stable Interactions with the Helicase. Structural Implications for the Hexameric Helicase−Replication Fork Complex. The high-affinity step in the binding of the DnaB hexamer to the two-arm fork occurs in exactly the same concentration range of the protein as the binding to the 5′ single-arm substrate. This result shows that the binding constant of the helicase for the 5′ arm, in the two-arm fork, is not affected by the presence of the 3′ arm. It should be noted that this conclusion is independent of any models of helicase binding to the fork. Lack of the effect of the 3′ arm on the binding of the single DnaB hexamer to the fork indicates that the 3′

arm does not form a thermodynamically stable complex with the enzyme molecule, associated with the 5′ arm of the fork, although some free energy compensation effects cannot be excluded. Moreover, quantitative analysis of the simultaneous binding of DnaB to both arms of the fork strongly suggests that the intrinsic affinity of the DnaB hexamer to the 3′ arm, in the presence of another hexamer already associated with the 5′ arm, is described by the same binding constant and the same fluorescence change as the interactions of the enzyme with the 3′ single-arm fork. These results indicate that, in spite of the binding of the first hexamer to the 5′ arm of the fork, the 3′ arm is in a conformation in which it is easily available for the binding of the next DnaB hexamer. Because of the large size of the DnaB hexamer, the data suggest that the 3′ arm is separated from the 5′ arm and that this separation could result from the binding of the first hexamer to the 5′ arm of the fork. The conformation of the 3′ arm, in the complex with the helicase, must be such that the arm can bind an additional large DnaB hexamer, with the same intrinsic affinity as in independent binding to the 3′ single-arm fork. It should be noted, the unwinding studies of dsDNA, by the DnaB helicase, indicated that the enzyme requires the presence of a 3′ arm to perform the reaction during which ATP hydrolysis takes place (LeBowitz & McMacken, 1986). Although the role of the 3′ arm in the unwinding process requires further thermodynamic and kinetic analysis, based on the data obtained in this work, we suggest that the 3′ arm is only transiently required in the unwinding reaction, and interacts with one of the conformational states of the enzyme generated during ATP hydrolysis reaction.

Current models of the hexameric helicase binding to the fork indicate that the DNA molecule goes through the cross channel of the ring-like structure of the protein, as indicated by E. M. studies of *E. coli* RuvB protein and bacteriophage T7 helicase/primase (Egelman et al., 1995; Stasiak et al., 1994). Whether or not this model applies to the DnaB helicase is still unknown. Our data show that the 3′ arm of the fork is not engaged in stable interactions with the DnaB hexamer and is separated from the 5′ arm. These data exclude any model in which both arms of the replication fork are crossing the inner channel of the hexameric ring. In such a complex, a significant separation of the arms would not exist. Moreover, it would not allow the second DnaB hexamer to associate with the 3′ arm, with unchanged intrinsic affinity, without affecting the binding of the other hexamer to the 5′ arm. Our results show that only the 5′ arm of the fork is predominately engaged in the stationary complex with the DnaB helicase. If the 5′ arm is crossing the inner channel of the hexamer, then the separation of the 3′ arm from the 5′ arm suggests that the 3′ arm is protruding in front of the enzyme. It is possible that such a helicase−fork complex is a general mode of the interactions of a hexameric helicase with a replication fork during the DNA replication process.

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