The structure-function relationship within the DNA binding site of the Escherichia coli replicative helicase DnaB protein was studied using nuclease digestion, quantitative fluorescence titration, centrifugation, and fluorescence energy transfer techniques. Nuclease digestion of the enzyme-single-stranded DNA (ssDNA) complexes reveals large structural heterogeneity within the binding site. The total site is built of two subsites differing in structure and affinity, although both occlude ~10 nucleotides. ssDNA affinity for the strong subsite is ~3 orders of magnitude higher than that for the weak subsite.

Fluorescence energy transfer experiments provide direct proof that the DnaB hexamer binds ssDNA in a single orientation, with respect to the polarity of the sugar-phosphate backbone. This is the first evidence of directional binding to ssDNA of a hexameric helicase in solution. The strong binding subsite is close to the small 12-kDa domains of the DnaB hexamer and occludes the 5'-end of the ssDNA. The strict orientation of the helicase on ssDNA indicates that, when the enzyme approaches the replication fork, it faces double-stranded DNA with its weak subsite. The data indicate that the different binding subsites are located sequentially, with the weak binding subsite constituting the entry site for double-stranded DNA of the replication fork.

The DnaB protein is an essential replication protein in Escherichia coli (1) which is involved in both the initiation and elongation stages of DNA replication (2–4). The protein is the E. coli primary replicative helicase, i.e. the factor responsible for unwinding the duplex DNA in front of the replication fork (5, 6). The DnaB protein is the only helicase required to constitute DNA replication in vitro from the chromosomal origin of replication. In the complex with ssDNA,1 the DnaB protein forms a “mobile replication promoter.” This nucleoprotein complex is specifically recognized by the primase in the initial stages of the priming reaction (1).

In solution, the native DnaB protein exists as a stable hexamer, composed of six identical subunits (7–9). Sedimentation equilibrium, sedimentation velocity, and nucleotide cofactor binding studies show that the DnaB helicase exists as a stable hexamer in a large protein concentration range, specifically stabilized by magnesium cations (7, 8). Hydrodynamic and electron microscopy data indicate that six proteomers aggregate with cyclic symmetry in which the proteomer-proteomer contacts are limited to only two neighboring subunits (7, 10, 11). Sedimentation velocity and electron microscopy studies reveal that the DnaB hexamer undergoes dramatic conformational changes upon binding AMP-PNP and ssDNA, and provide direct evidence of the presence of long range allosteric interactions in the hexamer, encompassing all six subunits of the enzyme (8, 11).

Recently, we obtained the first estimate of the stoichiometry of the DnaB helicase-ssDNA complex and the mechanism of the binding (12–14). Using the quantitative fluorescence titration method, we determined that the DnaB helicase binds ssDNA with a stoichiometry of 20 ± 3 nucleotides/DnaB hexamer and that this stoichiometry is independent of the type of nucleic acid base (13). Our thermodynamic studies of binding of ssDNA oligomers to the DnaB hexamer show that the enzyme has a strong, single binding site for ssDNA (12). The results also show that the same binding site is used in the binding to oligomers and polymer nucleic acids (12, 13). Moreover, photo-cross-linking experiments indicate that the ssDNA binding site is located predominately, if not completely, on a single subunit of the hexamer (12, 13).

The reaction catalyzed by a helicase, the unwinding of a duplex DNA, must take place in the DNA binding site. The fact that the helicase uses the same single DNA binding site, when forming a complex with polymer ssDNAs, oligomers, and replication fork substrates, indicates a complex structure of the nucleic acid binding site that can accommodate both ssDNA and dsDNA.

In this communication, we report the analysis of interactions between the DnaB helicase and DNA within the total DNA binding site of the enzyme. We present direct evidence that the total DNA binding site of the helicase is structurally and functionally heterogeneous. The total binding site is built of two subsites, each encompassing approximately 10 nucleotide residues. We provide direct proof that the DnaB hexamer binds ssDNA in a strictly single orientation, with respect to the polarity of the sugar-phosphate backbone of the nucleic acid. The results indicate that the binding subsites are sequentially located along the nucleic acid lattice, with the weak binding subsite constituting an entry site for the duplex part of the replication fork.

MATERIALS AND METHODS

Reagents and Buffers—All solutions were made with distilled and deionized >18 megaohms (Milli-Q Plus) water. All chemicals were reagent grade. Buffer T2 is 50 mM Tris adjusted to pH 8.1 with HCl, 5 mM MgCl₂, 10% glycerol. Buffer H is 50 mM Hepes adjusted to pH 8.1 with HCl, 5 mM MgCl₂, 10% glycerol. The temperature, AMP-PNP, and
salt concentrations are indicated in the text. The fluorescent markers, CPM, and fluorescein 5'-isothiocyanate, were used in the modification, were purchased from Molecular Probes (Eugene, OR).

_DnaB Protein_—The E. coli DnaB protein was purified, as described previously by us (7, 15–17). The concentration of the protein was spectrophotometrically determined, using extinction coefficient $\varepsilon_{280} = 1.85 \times 10^4$ cm$^{-1}$ M$^{-1}$ (hexamer) (7).

_Site-directed Mutagenesis of the DnaB Helicase—_Replacement of the arginine residues at position 14 from the N terminus of the DnaB protein and obtaining the DnaB protein variant, R14C, were performed using the plasmid RLM1038, harboring the gene of the wild type DnaB helicase, generously provided by Dr. R. McMacken. The site-directed mutagenesis was accomplished in the NEIES Center facility (National Institutes of Health) directed by Dr. T. Wood.

_Labeling the DnaB R14C Variant with Fluorescent Markers—_Labeling of the 6 cysteine residues of the DnaB variant, R14C hexamer, with CPM was performed in H buffer (pH 8.1, 100 mM NaCl, 5 mM MgCl$_2$, 10% glycerol) at 4 °C. The fluorescent label was added from the stock solution to the molar ratio of the CPM/R14C—25. The mixture was incubated for 4 h, with gentle mixing. After incubation, the protein was precipitated with ammonium sulfate and dialyzed overnight against buffer T2. Any remaining free dye was removed from the modified R14C-CPM by applying the sample on a DEAE-cellulose column and eluting with buffer T2 containing 500 mM NaCl. The degree of labeling was determined by absorbance of the marker at 394 nm using the extinction coefficient of CPM, $\varepsilon_{280} = 27 \times 10^3$ cm$^{-1}$ M$^{-1}$, providing the value of 5.8 ± 0.1 of CPM per DnaB hexamer.

_Nucleic Acids—_All nucleic acids were purchased from Midland Certified Reagents (Midland, TX). The etheno-derivatives of nucleic acids were obtained by modification with chloroacetaldehyde (12, 18). Oligomer dT$_{103}$pI$_{103}$ labeled at the 5'-end with fluorescein, 5'-Fl-dT$_{103}$pI$_{103}$, was synthesized using fluorescein phosphoramidate (Glen Research). Labeling of the 3'-end was performed by synthesizing dT$_{103}$pI$_{103}$ with the last residue at the 3'-end of the oligomer having the amino group on a six-carbon linker. The amino group was subsequently modified with fluorescein 5'-isothiocyanate to obtain dT$_{103}$pI$_{103}$-Fl-3'. The degree of labeling was determined by absorbance at 494 nm (pH 9), using the extinction coefficient, $7.6 \times 10^4$ M$^{-1}$ cm$^{-1}$ (13). The same procedures were used for labeling the 5'- and 3'-ends of the d(ApA)$_n$. The concentrations of labeled oligomers were spectrophotometrically determined at 260 nm (pH 8.1), using extinction coefficients, $1.76 \times 10^4$ M$^{-1}$ cm$^{-1}$ and $11.4 \times 10^4$ M$^{-1}$ cm$^{-1}$, respectively (13). The concentrations of deApA$_n$, deApA$_n$, deApA$_n$, deApA$_n$, deApA$_n$, and deApA$_n$ were determined using extinction coefficients $37 \times 10^4$, $33.3 \times 10^4$, $29.6 \times 10^4$, $25.9 \times 10^4$, $22.2 \times 10^4$, $18.5 \times 10^4$, and $14.8 \times 10^4$ M$^{-1}$ cm$^{-1}$ at 257 nm, respectively (12, 13, 19). Labeling the 5'-ends of ssDNA oligomers with $^{32}$P was performed using the standard procedure (12).

_Sedimentation Velocity Measurements—_Analytical sedimentation experiments were performed using an Optima XL-A analytical ultracentrifuge. Analyses of the sedimentation runs were performed as we previously described (6, 9, 13). The reported values of sedimentation coefficients were corrected to standard conditions, $s_{20, w}$, for solvent density and viscosity (7).

_Fluorescence Measurements—_All steady-state fluorescence measurements were performed using the SLM-Aminco 48000S and 8100 spectrofluorometers (20). The emission spectra were corrected for a wave-length dependence of the instrument response using a software provided by the manufacturer. The binding of the DnaB protein was followed by monitoring the fluorescence of the etheno-derivatives of ssDNA oligomers ($\lambda_{ex} = 325$ nm, $\lambda_{em} = 410$ nm). All titration points were corrected for dilution and, if necessary, for inner filter effect using the formula (15),

$$F_{i+} = (F_i - B_i) e^{-V_i} \frac{V_i}{V_{total}}$$  (Eq. 1)

where $F_{i+}$ is the corrected value of the fluorescence intensity at a given point of titration i, $F_i$ is the experimentally measured fluorescence intensity, $B_i$ is the background, $V_i$ is the volume of the sample at a given titration point, $V_{total}$ is the initial volume of the sample, h is the total length of the optical path in the cuvette expressed in centimeters, and $A_{em}$ is the absorbance of the sample at the excitation wavelength. Computer fits were performed using KaleidaGraph software (Synergy Software, PA) and Mathematica (Wolfram Research, IL). The relative fluorescence increase of the nucleic acid, $\Delta F$, upon binding the DnaB protein is defined by the equation,

$$\Delta F = \frac{(F_{i+} - F_i)}{F_i}$$  (Eq. 2)

where $F_{i+}$ is defined by Equation 1, and $F_i$ is the initial value of the fluorescence of the same solution.

All steady-state fluorescence anisotropy measurements were performed in the L format, using Glan-Thompson polarizers placed in the excitation and emission channels. The fluorescence anisotropy, $r$, of the sample was calculated by the equation,

$$r = \frac{(I_{VV} - I_{HH})}{(I_{VV} + 2I_{HH})}$$  (Eq. 3)

where I is the fluorescence intensity, and the first and second subscripts represent vertical (V) polarization of the excitation and vertical (V) or horizontal (H) polarization of the emitted light (16). The factor $G = I_{V} / I_{HH}$ corrects for the different sensitivity of the emission monochromator for vertically and horizontally polarized light (21). The limiting fluorescence anisotropies of fluorophores, $r_{lim}$, were determined by measuring the anisotropy of a given sample at different solution viscosity, adjusted by sucrose or glycerol, and extrapolating to viscosity $= \infty$, using the Perrin equation (22).

_Determination of the Average Fluorescence Energy Transfer Efficiency from CPM on the Small 12-kDa Domains of the DnaB Helicase to the Fluorescin Residue Attached at the 5'- or 3'-End of the ssDNA Oligomers—_The efficiency of the fluorescence radiationless energy transfer, $E$, from CPM (donor), located on the small 12-kDa domains of the DnaB protein variant R14C, to the fluorescein (acceptor), located at the 5'- or 3'-end of dT$_{103}$pI$_{103}$, bound in the DNA binding site of the helicase, has been determined using two independent methods. The fluorescence of the donor in the presence of the acceptor, $F_{DA}$, is related to the fluorescence of the same donor, $F_D$, in the absence of the acceptor by the equation,

$$F_{DA} = (1 - r_{lim})F_D + r_{lim}(1 - E_D)$$  (Eq. 4)

where $r_{lim}$ is the fraction of donors in the complex with the acceptor, and $E_D$ is the average fluorescence energy transfer from donor to acceptor, determined from the quenching of the donor fluorescence. Thus, the average transfer efficiency, $E_D$, obtained from the quenching of the CPM fluorescence upon binding of the labeled ssDNA oligomer, is obtained by rearranging Equation 4,

$$E_D = \frac{1}{r_{lim}} \left( \frac{F_D - F_{DA}}{F_D} \right)$$  (Eq. 5)

where, in the considered case, $F_D$ and $F_{DA}$ are the values of the CPM fluorescence intensity in the absence and presence of bound 5'-Fl-dT$_{103}$pI$_{103}$ or dT$_{103}$pI$_{103}$-Fl-3'. The value of $r_{lim}$ has been determined using the binding constants of the 20- and 10-mers for the DnaB helicase measured in the same solution conditions (13).

In a second independent method, the average fluorescence transfer efficiency, $E_A$, has been determined, using a sensitized acceptor fluorescence by measuring the fluorescence intensity of the acceptor (fluorescein) excited at 435 nm, where the donor (CPM) predominantly absorbs, in the absence and presence of R14C-CPM. The fluorescence intensities of the acceptor in the absence, $F_A$, and presence, $F_{AD}$, of the donor are defined as follows,

$$F_A = I_{em} C_{AT} \phi_A$$  (Eq. 6)

and

$$F_{AD} = (1 - r_{lim})I_{em} C_{AT} \phi_A + I_{em} C_{DT} \phi_B \delta E_A$$  (Eq. 7)

where $I_{em}$ is the intensity of incident light, $C_{AT}$ and $C_{DT}$ are the total concentrations of acceptor and donor, $r_{lim}$ is the fraction of acceptors in the complex with donors, $\phi_A$ and $\phi_B$ are the molar absorption coefficients of acceptor and donor at the excitation wavelength (435 nm), respectively; $\delta F_A$ and $\phi_B$ are the quantum yields of the free and bound acceptor; and $E_A$ is the average transfer efficiency determined by acceptor-sensitized emission. All quantities in Equations 6 and 7 can be experimentally determined. For the case considered in this work, the acceptor is practically completely saturated with the donor, i.e., $r_{lim} = 1$. Thus, for $r_{lim} = 1$, dividing Equation 7 by Equation 6 and rearranging provides the average transfer efficiency as described by the following:

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Distances is then defined by using Equation 10 as follows.

$$E_D = \left( \frac{1}{n+1} \right) \left( \frac{e_C A}{x_0 C_{DA}} \right) \left( \frac{d_{\phi}}{d_{\phi_0}} \right) \left( \frac{F_{\omega}}{F_{\omega_0}} \right) - 1$$  \hspace{1cm} (Eq. 8)

It should be pointed out that the energy transfer efficiencies, $E_D$ and $E_A$, are apparent quantities. $E_D$ is a fraction of the photons absent in the donor emission as a result of the presence of an acceptor, including transfer to the acceptor and possible nondipolar quenching processes induced by the presence of the acceptor, and $E_A$ is a fraction of all photons absorbed by the donor that were transferred to the acceptor.

The true Förster energy transfer efficiency, $E$, is a fraction of photons absorbed by the donor and transferred to the acceptor in the absence of any additional nondipolar quenching resulting from the presence of the acceptor. The value of $E$ is related to the apparent quantities of $E_D$ and $E_A$ by the following (23).

$$E = \frac{E_D}{1 - E_D + E_A}$$  \hspace{1cm} (Eq. 9)

Thus, measurements of the transfer efficiency, using both methods, are not alternatives but parts of the analysis used to obtain the true efficiency of the fluorescence energy transfer process, $E$.

The fluorescence energy transfer efficiency between donor and acceptor dipoles is related to the distance, $R$, separating the dipoles by the equation,

$$E = \frac{R_0^6}{R^6 + R^6}$$  \hspace{1cm} (Eq. 10)

where $R_0 = 9790 \,(\kappa^{a} n^{-1} \phi_n J^{1/6})$ is the so called Förster critical distance (in angstroms), the distance at which the transfer efficiency is 50%; $\kappa^a$ is the orientation factor; $\phi_n$ is the donor quantum yield in the absence of the acceptor; and $n$ is the refractive index of the medium ($n = 1.4$) (22). The overlap integral, $J$, characterizes the resonance between the donor and acceptor dipoles.

The fluorescence transfer efficiency of chemically identical donor and acceptor pairs, characterized by the same quantum yields, depends on the distance between the donor and acceptor, $R$, and the factor, $\kappa^a$, describing the mutual orientation of the donor and acceptor dipoles (22). Although in the work presented in this paper we are interested in relative distances between donors and acceptors, evaluation of $\kappa^a$ allowed us to estimate the effect of the orientation factor on the differences between the studied donor-acceptor distances. The factor $\kappa^a$ cannot be experimentally determined; however, the upper ($\kappa_{\text{max}}^a$) and lower ($\kappa_{\text{min}}^a$) limits of $\kappa^a$ can be obtained from the measured limiting anisotropies of the donor and acceptor and the calculated axial depolarization factors, using the procedure described by Dale et al. (24). When both axial depolarization factors are positive, $\kappa_{\text{max}}^a$ and $\kappa_{\text{min}}^a$ can be calculated from $\kappa_{\text{max}}^a = \frac{5}{6}(1 + 3d_{\phi_{\text{up}}^d} + d_{\phi_{\text{up}}^a} + 3d_{\phi_{\text{up}}^a})$ and $\kappa_{\text{min}}^a = \frac{5}{6}(1 - \frac{1}{2}(d_{\phi_{\text{up}}^d} + d_{\phi_{\text{up}}^a} + d_{\phi_{\text{up}}^a}))$, where $d_{\phi_{\text{up}}^d}$ and $d_{\phi_{\text{up}}^a}$ are the axial depolarization factors for the donor and acceptor, respectively (24). The axial depolarization factors have been calculated as square roots of the ratios of the limiting anisotropies of the donors (CPM on the DnaB helicase) and acceptors (fluorescein at the 5′- or 3′-end of the ssDNA oligomers) and their corresponding fundamental anisotropies (17). For two chemically identical donor-acceptor pairs, characterized by the same $R_0$, the same $\kappa^a$, $\phi_n$, and $J$, the differences in the transfer efficiencies, $E_D$ and $E_A$, result exclusively from the different distances between the donor and acceptor, $R_1$ and $R_2$. The relative ratio of the two distances is then defined by using Equation 10 as follows.

$$\frac{R_1}{R_2} = \left( \frac{[1 - (1 - E_D)E_A]^{1/6}}{[1 - (1 - E_A)E_D]} \right)^{1/6}$$  \hspace{1cm} (Eq. 11)

**Determination of Rigorous Thermodynamic Binding Isotherms and Absolute Stoichiometries of the DnaB Helicase-ssDNA Complexes**—In this work, we followed the binding of the DnaB protein to the ssDNA oligomers by monitoring the fluorescence increase, $\Delta F$, of ssDNA etheno-derivatives upon the complex formation. Proteins and nucleic acids may form complexes characterized by different spectroscopic properties, particularly when multiple ligand binding processes are studied. In applying spectroscopic methods to monitor the ligand macromolecule interactions, one should not assume strict proportionality between the observed signal change and the degree of binding unless the existence of such proportionality has been shown (15). The general method to obtain thermodynamically rigorous estimates of the average degree of binding of the protein per ssDNA oligomer, $\Sigma_i$, and the free protein concentration, $P_p$, has been previously described by us (8, 15, 25).

Briefly, the experimentally observed $\Delta F$ has a contribution from each of the different possible “i” complexes of the DnaB hexamer with a nucleic acid. Thus, the observed fluorescence increase is functionally related to $\Sigma_i$ by the equation,

$$\Delta F = \Sigma_i \Delta F_{i\text{min}}$$  \hspace{1cm} (Eq. 12)

where $\Delta F_{i\text{min}}$ is the molecular parameter characterizing the maximum fluorescence increase of the nucleic acid with the DnaB protein bound in complex i. The same value of $\Delta F_{i\text{min}}$ obtained at different total nucleic acid concentrations, $N_i$ and $N_{i\text{max}}$ indicates the same physical state of the nucleic acid, i.e. the degree of binding, $\Sigma_i$, and the free DnaB protein concentration, $P_p$, must be the same. The value of $\Sigma_i$ and $P_p$ is then related to the total protein concentrations, $P_p$ and $P_p$, and the total nucleic acid concentrations, $N_i$ and $N_{i\text{max}}$ at the same value of $\Delta F$, by the following equations,

$$\Sigma_i = \frac{(P_{p_i} - P_p)}{(N_{i\text{max}} - N_i)}$$  \hspace{1cm} (Eq. 13)

$$P_p = P_{p_i} - (\Sigma_i e_i)N_i$$  \hspace{1cm} (Eq. 14)

where $x = 1$ or 2 (12, 20).

**RESULTS**

**Micrococcal Nuclease Digestion Reveals Large Structural Heterogeneity within the DNA Binding Site of the E. coli DnaB Helicase**—Quantitative fluorescence titrations and photo-cross-linking experiments, using ssDNA oligomers, showed that the DnaB hexamer has a single ssDNA binding site encompassing 20 ± 3 nucleotide residues and located predominantly on a single subunit (12–14). The first evidence of the structural heterogeneity within the DNA binding site came from nuclease digestion-protection studies of the DNA in the complex with the helicase. In the first set of experiments, the complex of the DnaB hexamer with the 20-mer d(TpT)19 labeled at its 5′-end with 32P in the presence of 1 mxi AMP-PNP was subjected to micrococcal nuclease digestion as a function of time. The protein was in molar excess over the 20-mer to ensure complete saturation of the nucleic acid. Fig. 1a shows the polyacrylamide sequencing gel of d(TpT)19 after digestion with the nuclease, at different time intervals, in the absence and presence of the helicase. In the absence of the helicase, in our solution conditions, the 20-mer was digested within 20 min. A dramatically different behavior was observed in the presence of the enzyme. The digestion process was less efficient, indicating significant protection of the nucleic acid against the nuclease by the enzyme. Moreover, at prolonged digestion times, a nucleic acid fragment of 10 or 11 nucleotide residues was strongly protected by the helicase. At the longest times, this was the major nucleic acid fragment on the gel, resistant to further nuclease action (Fig. 1a).

The size of the protected fragment was not dependent upon the length or type of base of the oligomer bound to the DnaB protein, indicating that protection against the nuclease digestion is limited to the nucleic acid bound within the single DNA binding site of the helicase. Fig. 1b shows polyacrylamide sequencing gels of d(ApA)19 after digestion with the nuclease, at different time intervals, and in the absence and presence of the helicase. As in the case of d(TpT)19, the only predominant oligomer protected by the helicase in the complex with d(ApA)19, after prolonged digestion, is a ssDNA fragment, 10 or 11 nucleotide residues long.

These data indicate that within the total DNA binding site of the DnaB helicase, approximately half of the ∼20 nucleotide residues occluded by the helicase are bound differently than the remaining half, resulting in the observed nuclease digestion pattern. Thus, these results indicate that the total DNA binding site of the DnaB helicase is built of two structurally and possibly functionally different binding subsites (see below).

**Binding of 10-mer, d(ApA)19, to the DnaB Helicase**—To de-
The concentration of the protein and the 20-mer are 1 × 10⁻⁶ M (hexamer) and 5 × 10⁻⁷ M (oligomer). Oligomers of different lengths are included in lane 1 as molecular markers. Lanes 2–6 show the different digestion times without the DnaB helicase. Lane 2, 5'-[³²P](dT)₁₀₀, 0 s; lane 3, 30 s; lane 4, 60 s; lane 5, 300 s; lane 6, 900 s; lane 7, 1800 s. Lanes 8–14 show the complex 5'-[³²P](dT)₁₀₀-DnaB helicase at different digestion times. Lane 8, 30 s; lane 9, 60 s; lane 10, 180 s; lane 11, 300 s; lane 12, 600 s; lane 13, 900 s; lane 14, 1800 s. b, autoradiogram of the 15% sequencing polyacrylamide gel electrophoresis of the 5'-[³²P](dT)₁₀₀ and DnaB protein-5'-[³²P](dT)₁₀₀ complexes, after micrococcal nuclease (MN) digestion in buffer T2 (pH 8.1, 4 °C) containing 100 mM NaCl, 1 mM CaCl₂, and 1 mM AMP-PNP. The concentration of the protein and the 20-mer are 1 × 10⁻⁶ M (hexamer) and 5 × 10⁻⁷ M (oligomer). Oligomers of different lengths are included in lane 1 as molecular markers. Lanes 2–6 show the different digestion times without the DnaB helicase. Lane 2, 5'-[³²P](dT)₁₀₀, 0 s; lane 3, 30 s; lane 4, 60 s; lane 5, 300 s; lane 6, 900 s; lane 7, 1800 s. Lanes 8–14 show the complex 5'-[³²P](dT)₁₀₀-DnaB helicase at different digestion times. Lane 8, 30 s; lane 9, 60 s; lane 10, 180 s; lane 11, 300 s; lane 12, 600 s; lane 13, 900 s; lane 14, 1800 s. b, autoradiogram of the 15% sequencing polyacrylamide gel electrophoresis of the 5'-[³²P](dT)₁₀₀ and DnaB protein-5'-[³²P](dT)₁₀₀ complexes, after micrococcal nuclease digestion in buffer T2 (pH 8.1, 4 °C) containing 100 mM NaCl, 1 mM CaCl₂, and 1 mM AMP-PNP. The concentration of the protein and the 70-mer are 1 × 10⁻⁶ M (hexamer) and 5 × 10⁻⁷ M (oligomer). Oligomers of different lengths are included in lane 1 as molecular markers. Lanes 2–6 show the different digestion times without the DnaB helicase. Lane 2, 5'-[³²P](dT)₁₀₀, 0 s; lane 3, 30 s; lane 4, 60 s; lane 5, 300 s; lane 6, 900 s; lane 7, 1800 s. Lanes 8–14 show the complex 5'-[³²P](dT)₁₀₀-DnaB helicase at different digestion times. Lane 8, 30 s; lane 9, 60 s; lane 10, 180 s; lane 11, 300 s; lane 12, 600 s; lane 13, 900 s; lane 14, 1800 s.

Fig. 1. a, autoradiogram of the 15% sequencing polyacrylamide gel electrophoresis of the 5'-[³²P](dT)₁₀₀ and DnaB protein-5'-[³²P](dT)₁₀₀ complexes, after micrococcal nuclease (MN) digestion in buffer T2 (pH 8.1, 4 °C) containing 100 mM NaCl, 1 mM CaCl₂, and 1 mM AMP-PNP. The concentration of the protein and the 20-mer are 1 × 10⁻⁶ M (hexamer) and 5 × 10⁻⁷ M (oligomer). Oligomers of different lengths are included in lane 1 as molecular markers. Lanes 2–6 show the different digestion times without the DnaB helicase. Lane 2, 5'-[³²P](dT)₁₀₀, 0 s; lane 3, 30 s; lane 4, 60 s; lane 5, 300 s; lane 6, 900 s; lane 7, 1800 s. Lanes 8–14 show the complex 5'-[³²P](dT)₁₀₀-DnaB helicase at different digestion times. Lane 8, 30 s; lane 9, 60 s; lane 10, 180 s; lane 11, 300 s; lane 12, 600 s; lane 13, 900 s; lane 14, 1800 s. b, autoradiogram of the 15% sequencing polyacrylamide gel electrophoresis of the 5'-[³²P](dT)₁₀₀ and DnaB protein-5'-[³²P](dT)₁₀₀ complexes, after micrococcal nuclease digestion in buffer T2 (pH 8.1, 4 °C) containing 100 mM NaCl, 1 mM CaCl₂, and 1 mM AMP-PNP. The concentration of the protein and the 70-mer are 1 × 10⁻⁶ M (hexamer) and 5 × 10⁻⁷ M (oligomer). Oligomers of different lengths are included in lane 1 as molecular markers. Lanes 2–6 show the different digestion times without the DnaB helicase. Lane 2, 5'-[³²P](dT)₁₀₀, 0 s; lane 3, 30 s; lane 4, 60 s; lane 5, 300 s; lane 6, 900 s; lane 7, 1800 s. Lanes 8–14 show the complex 5'-[³²P](dT)₁₀₀-DnaB helicase at different digestion times. Lane 8, 30 s; lane 9, 60 s; lane 10, 180 s; lane 11, 300 s; lane 12, 600 s; lane 13, 900 s; lane 14, 1800 s.
protein. Therefore, we used the analytical centrifugation technique to assess the affinity of DNA to the second subsite. In these experiments, we used a 10-mer, dA(pA)\textsubscript{9}, labeled at the 5' or 3' end with fluorescein (see “Materials and Methods”). This approach allowed us to monitor exclusively the nucleic acid and the protein-nucleic acid complex without the interference of the protein and AMP-PNP absorbance. The sedimentation velocity profiles (monitored at 515 nm) of the DnaB helicase-dA(pA)\textsubscript{9}-3'-Fl mixture at the nucleic acid and helicase concentrations of 9 \times 10^{-5} \text{ M} and 2 \times 10^{-5} \text{ M}, respectively, in buffer T2 (pH 8.1, 20 °C) containing 100 mM NaCl and 1 mM AMP-PNP, are shown in Fig. 3. The sedimentation run was performed at 60,000 rpm. It is clear that, initially, two independent moving boundaries exist. The slow moving boundary has a sedimentation coefficient of $s_{\text{DnaB}} = 1.4 \pm 0.2$, which is the $s_{\text{DnaB}}$ value of the free dA(pA)\textsubscript{9}-3'-Fl. The fast moving boundary contains dA(pA)\textsubscript{9}-3'-Fl in the complex with the DnaB helicase. We have previously shown that the DnaB hexamer fully preserves its hexameric structure in the complex with the ssDNA (8, 13). After the fast moving boundary reaches the cell bottom, only the slow moving boundary of the free dA(pA)\textsubscript{9}-3'-Fl still remains (dashed lines). Notice that during the sedimentation process, the boundary of the complex migrates in the field of the constant free 10-mer concentration $[T]_{\text{free}} \gg 1/K_1$, thus assuring that the enzyme always has the strong binding subsite saturated with the nucleic acid. At 515 nm, one monitors the partition function of the system, $Z$, and the degree of binding to the second subsite, $v_2$, as follows,

$$Z = K_1[T]_{\text{free}} + K_2[T]_{\text{free}}^2$$

(Eq. 15)

$$v_2 = \frac{K_2[T]_{\text{free}}^2}{Z}$$

(Eq. 16)

and $K_2$ is defined as follows.

$$K_2 = \frac{v_2}{[T]_{\text{free}}(1 - v_2)}$$

(Eq. 17)

Introducing the values of $v_2 = 0.6$ and $[T]_{\text{free}} = 5.8 \times 10^{-5}$ M, obtained from the sedimentation velocity experiments, provides the value of $K_2 = (2.6 \pm 1) \times 10^4$ M$^{-1}$. A similar value of the binding constant $K_2$ of the dA(pA)\textsubscript{9}-3'-Fl and 5'-Fl-dA(pA)\textsubscript{9}
for the weak binding subsite has been obtained using lower and higher concentrations of the nucleic acids. Thus, the data show that the affinity of ssDNA for the second subsite is ~3 orders of magnitude lower than the affinity for the strong binding subsite.

Interactions of ssDNA Oligomers Having Different Lengths with the Strong ssDNA Binding Subsite—To obtain further insight into the interactions of the DNA in the strong binding subsite, we performed quantitative fluorescence titrations of a series of ssDNA oligomers of different lengths. Fluorescence titrations of deA(pA)₅₋, deA(pA)₆₋, deA(pA)₇₋, deA(pA)₈₋, deA(pA)₉₋, and deA(pA)₁₀₋, with the DnaB helicase in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP, are shown in Fig. 4a. For comparison, the fluorescence titration of the 10-mer, deA(pA)₁₀₋, with DnaB is also included. With the decreasing number of residues, the relative maximum fluorescence changes, and the affinity decreases. In the case of 9- and 8-mers, the maximum fluorescence change, ΔFₘₐₓ, upon saturation with the helicase, is still similar to the one determined for the 10-mer. However, the affinity is lower than the affinity of the 10-mer and is characterized by binding constants Kᵢ = (1.5 ± 0.5) × 10⁷ M⁻¹ and Kᵢ = (8 ± 2) × 10⁶ M⁻¹, respectively. A dramatic drop in the affinity and maximum relative fluorescence increase is observed in the case of the 7- and 6-mer (Fig. 4a; see Table I). No detectable binding to the helicase occurs in the case of 5- and 4-mers (Fig. 4a). Titrations at very high concentrations of the 5- and 4-mer could only provide a semi-quantitative estimate of the affinities, due to the required DnaB concentration beyond the solubility of the protein; however, these experiments indicate that the binding constants for the 5- and 4-mers are not higher than 1 × 10⁷ M⁻¹ (data not shown). Fig. 4b shows the dependence of the natural logarithm of binding constants of studied oligomers to the DnaB protein as a function of the number of nucleotide residues in the ssDNA oligomer. The plot is nonlinear, a clear indication that the affinity is not a simple function of the length of the nucleic acid. The difference of the 2 residues between 10-mer and 8-mer causes only an ~0.3 kcal/mol decrease of the free energy of interactions. The difference in the 2 residues between 7-mer and 5-mer decreases the free energy of binding by at least ~3 kcal/mol, practically eliminating the binding of the 5-mer to the enzyme in studied solution conditions. These data show that, to efficiently bind to the strong DNA binding subsite, the nucleic acid must span 6 or 7 residues. Thus, the results indicate a complex structure of the ssDNA strong binding subsite where the direct contacts between the helicase and the nucleic acid, decisive in complex formation, are separated by 6 or 7 nucleotides (see “Discussion”).

Salt Effect on the Affinity of the DnaB Helicase to ssDNA Oligomers— Fluorescence titrations of deA(pA)ₙ₋ with the DnaB helicase in buffer T2 (pH 8.1, 10 °C), containing 1 mM AMP-PNP and different NaCl concentrations, are shown in Fig. 5a. As the salt concentration increases, the isotherms shift toward higher total DnaB protein concentrations, indicating a decreasing affinity of the protein-nucleic acid complex at higher salt concentrations. It should also be noted that ΔFₘₐₓ, at lower salt concentrations, decreasing from 4.3 ± 0.2 at 100 mM to 2.8 ± 0.2 at 407 mM [NaCl]. A similar decrease of the maximum fluorescence increase upon the helicase binding has been observed for all other oligomers (data not shown). The dependence of the logarithm of the intrinsic binding constants for 10-, 9-, 8-, 7-, and 6-mers upon the logarithm of [NaCl] (log-log plot) is shown in Fig. 5b. Within experimental accuracy, the plots are linear in the studied salt concentration ranges, which is different from the nonlinear behavior of the

| Stoichiometry (n) | deA(pA)₅₋ | deA(pA)₆₋ | deA(pA)₇₋ | deA(pA)₈₋ | deA(pA)₉₋ | deA(pA)₁₀₋ |
|------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Binding constant K (M⁻¹) | (1.7 ± 0.3) × 10⁷ | (1.5 ± 0.5) × 10⁷ | (8 ± 3) × 10⁶ | (3.3 ± 1) × 10⁶ | (4 ± 1) × 10⁵ | 1 |
| ΔFₘₐₓ | 4.3 ± 0.2 | 4.3 ± 0.2 | 4.2 ± 0.2 | 3.3 ± 0.2 | 2.6 ± 0.4 |
| d(log K) | −1.4 ± 0.3 | −1.5 ± 0.3 | −1.4 ± 0.3 | −1.5 ± 0.3 | −1.4 ± 0.3 |
with Respect to the Polarity of the Sugar-Phosphate Backbone of ssDNA, Using the Fluorescence Energy Transfer Method—Determination of the mutual orientations of proteins and nucleic acids in the complex should be based on a method that is sensitive to the differences in distances between different, specific regions of both macromolecules (17, 22). Fluorescence energy transfer between a donor and an acceptor, placed in specific locations on a protein and a nucleic acid, provides a very sensitive technique to assess the relative proximities between different regions of both macromolecules in the complex. The orientation of the DnaB helicase, in the complex with ssDNA, was determined by using the 20-mer, dT-PT19, labeled with fluorescein (acceptor) at its 5’- or 3’-end, respectively, and the DnaB protein variant, R14C, specifically labeled with a coumarin derivative (donor), CPM, at the small 12-kDa domain of the enzyme (see “Materials and Methods”). If the DnaB helicase binds predominantly in a single orientation, with respect to the polarity of the sugar-phosphate backbone of ssDNA, then different responses of the donor and acceptor fluorescence should be observed, depending on the different location of the acceptor on the nucleic acid.

The elongated DnaB protein monomer is built of two structural domains, small 12-kDa and large 33-kDa domains connected at the “hinge” region (28) as visualized by electron microscopy data (10, 11). In the hexamer, all protomers are oriented with their small 12-kDa domains in the same direction (10, 11). Because the protein does not have natural cysteines, we replaced arginine at position 14 from the N terminus of the protein located in the small 12-kDa domain of the enzyme with a single cysteine residue, using site-directed mutagenesis. Subsequently, this cysteine residue was specifically modified with CPM to provide R14C-CPM (see “Materials and Methods”). The selection of the modification site was directed by the fact that removal of the entire 14-amino acid fragment from the N terminus of the protein did not affect, to any extent, the biological functions of the protein (28). As a result of modification, the R14C DnaB hexamer has six CPM molecules located in the small domain of each protomer (R14C-CPM). Thus, 6 CPM residues form a ring at one end of the DnaB hexamer.

The emission spectrum of R14C-CPM strongly overlaps the absorption spectrum of the fluorescein. These spectroscopic properties of CPM make the marker an excellent fluorescence donor for fluorescein (29). The presence of unlabeled dT-PT19 causes very little change in the fluorescence emission spectra of R14C-CPM (λem = 435 nm); however, the presence of R14C-CPM causes an ~2-fold decrease of the fluorescence intensity of 5’-Fl-dT(PT)19 (λem = 485 nm), although with the excitation at 485 nm only fluorescein on the 5’-Fl-dT(PT)19 absorbs light (data not shown). Saturation of the 20-mer with the unlabeled DnaB protein causes only an ~8% decrease of the 5’-Fl-dT(PT)19 fluorescence (data not shown). It is evident that, even in the absence of the energy transfer process, the presence of 6 hydrophobic CPM residues affects the quantum yield of fluorescein at the 5’-end of the ssDNA, which already suggests close proximity between the CPMs and fluorescein. The quantum yield of fluorescein is independent of the excitation wavelength between 400 and 500 nm (22). Thus, as expected, the ratio of quantum yields of 5’-Fl-dT(PT)19 in the complex with R14C-CPM and free in solution, δδ/δo, is constant and equal to 0.51 over a tested range of excitation wavelengths between 465 and 500 nm. In this spectral range of excitation, no detectable fluorescence energy transfer from CPM residues to fluorescein occurs. Thus, this ratio of quantum yields, independent of excitation wavelength, reflects the change of the emission intensity of 5’-Fl-dT(PT)19, resulting exclusively from the formation of the complex with R14C-CPM, in the absence of the

Determination of the Orientation of the E. coli DnaB Helicase

FIG. 5. a, fluorescence titrations of deA(14w) with the DnaB protein in buffer T2 (pH 8.1, 10 °C) containing 1 mM AMP-PNP, at different NaCl concentrations as follows. □ 100 mM; ○, 194 mM; △, 304 mM; ●, 407 mM. Solid lines are computer fits using single-site binding isotherm, $\Delta F = \Delta F_{max}(K_p/P_A(1 + K_p/P_p))$, with $\Delta F_{max}$ and $K_p$ as follows. □, 4.3 and 1.5 $\times 10^7$ M$^{-1}$; ○, 3.7 and 7 $\times 10^6$ M$^{-1}$; △, 3.3 and 3.3 $\times 10^6$ M$^{-1}$; ●, 2.8 and 1.3 $\times 10^6$ M$^{-1}$. b, the dependence of the intrinsic binding constant $K_p$ for the binding of ssDNA oligomers of different lengths to the strong binding subsite of DnaB helicase upon NaCl concentrations in solution (log-log plots) in buffer T2 (pH 8.1, 10 °C) containing 1 mM AMP-PNP. □, deA(14w); ○, deA(14w)w; ●, deA(14w)w; △, deA(14w)w; ●, deA(14w)w; ○, deA(14w)w; △, deA(14w)w;

log-log plot previously determined in the case of the 20-mer, deA(14w)19 (12, 13). With increasing salt concentrations, the affinities of all oligomers decrease, indicating that the binding process is accompanied by a net release of ions with the slopes of log-log/NaCl $=-1.4 \pm 0.4$, $-1.5 \pm 0.3$, $-1.4 \pm 0.4$, $-1.5 \pm 0.4$, and $-1.4 \pm 0.4$ for 10-, 9-, 8-, 7-, and 6-mer, respectively (27) (Table I). Thus, these data indicate that a similar number of $-1.5$ ions is released upon the complex formation with each of the oligomers being long enough to provide all essential contacts with the enzyme in the binding subsite. Previously, we determined that binding of a 20-mer, deA(14w)19, which spans the entire total DNA binding site, to the DnaB helicase is accompanied by the maximum release of $-3.7$ ions (13). This number is significantly higher than the $-1.4$ obtained for the 10-mer (Table I). This comparison suggests that the interactions of ssDNA with the weak binding subsite are accompanied by a net release of $-2$ ions. Another possibility is that interactions between the strong and weak subsites, simultaneously saturated with nucleic acid in the complex with deA(14w)19, result in the net release of $-2$ additional ions. At present, we cannot exclude either of these possibilities.
energy transfer process, and can be used to obtain the spectrum of 5'-Fl-dT(pT)_{19}, in the presence of R14C-CPM, without the changes induced by the energy transfer process at any excitation wavelength (Equations 7 and 8).

The dashed line in Fig. 6a is the sum of the emission spectra of R14C-CPM (without energy transfer) \((\lambda_{ex} = 435 \text{ nm})\) in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP and the fluorescence emission spectrum of the complex of R14C-CPM and 5'-Fl-dT(pT)_{19} \((\lambda_{ex} = 435 \text{ nm})\) \((-\cdots-\cdots-)\) in the same buffer. Concentrations of 5'-Fl-dT(pT)_{19} and the protein were \(4.5 \times 10^{-7} \text{ M} \) (oligomer) and \(9.6 \times 10^{-7} \text{ M} \) (hexamer), respectively. The fluorescence emission spectrum of R14C-CPM normalized at 476 nm (peak) to the emission spectrum of the protein in the complex with 5'-Fl-dT(pT)_{19} \((-\cdots-\cdots-)\) is also included. b, sensitized emission spectrum of 5'-Fl-dT(pT)_{19} \((\lambda_{ex} = 435 \text{ nm})\) in the complex with R14C-CPM \((\cdots\cdots\cdots\cdots\cdots\cdots\cdots)\), obtained after subtraction of the normalized spectrum of R14C-CPM (see Fig. 7a) in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP superimposed on the fluorescence emission spectrum of 5'-Fl-dT(pT)_{19}, in the presence of R14C-CPM (without energy transfer) \((-\cdots-\cdots-)\) obtained at the same excitation wavelength by multiplying the spectrum of free, labeled 20-mer by the quantum yield ratio, \(\Phi_{\text{cpm}}/\Phi_{\text{flu}}\) = 0.51. Concentrations of 5'-Fl-dT(pT)_{19} and R14C-CPM are \(4.5 \times 10^{-7} \text{ M} \) (oligomer) and \(9.6 \times 10^{-7} \text{ M} \) (hexamer), respectively.

R14C-CPM at 476 nm in the complex with 5'-Fl-dT(pT)_{19} is decreased by \(\sim 35\%\), as compared with the R14C-CPM complex with unlabeled dT(pT)_{19}. The decrease of emission at 476 nm, where there is no contribution from fluorescein emission, indicates significant fluorescence energy transfer from the CPM residues located on the small 12-kDa domains of the DnaB hexamer to the fluorescein moiety placed at the 5'-end of the bound 5'-Fl-dT(pT)_{19}.

Comparison between the sum of independent components of the complex and the spectrum of the complex in Fig. 6a shows that the fluorescence intensity of the fluorescein residue of 5'-Fl-dT(pT)_{19}, with the peak at 520 nm, is strongly increased in the complex with R14C-CPM \((\lambda_{ex} = 435 \text{ nm})\). Recalling that fluorescein does not contribute to the CPM emission band at 476 nm, we can normalize the spectra of R14C-CPM-unlabeled dT(pT)_{19} and R14C-CPM-5'-Fl-dT(pT)_{19} complex at 476 nm. The difference between the normalized spectrum of R14C-CPM-unlabeled dT(pT)_{19} and the spectrum of the complex R14C-CPM-5'-Fl-dT(pT)_{19} provides the sensitized emission spectrum of the 5'-Fl-dT(pT)_{19} bound to R14C-CPM. The emission spectrum of 5'-Fl-dT(pT)_{19}, without energy transfer, with the sensitized emission spectrum of 5'-Fl-dT(pT)_{19} is shown in Fig. 6b. It is evident that in the presence of the donor, CPM, the fluorescence intensity of the fluorescein at the 5'-end of the 20-mer is increased by \(\sim 220\%\).

Analogous experiments were performed with a 20-mer, dT(pT)_{19}-Fl-3', having fluorescein located at the opposite 3'-end of the nucleic acid. Unlike the case of 5'-Fl-dT(pT)_{19}, formation of the complex with R14C-CPM causes only an \(\sim 8\%\) decrease of the fluorescence of dT(pT)_{19}-Fl-3' \((\lambda_{ex} = 485 \text{ nm})\), which is the same as observed in the presence of unlabeled protein (data not shown). This difference results from the larger distance between CPM residues on the small 12-kDa domains of the DnaB hexamer and fluorescein at the 3'-end of the 20-mer (see below). The dashed line in Fig. 7a is the sum of the fluorescence emission spectra of independent components of the complex, R14C-CPM in the presence of unlabeled dT(pT)_{19}, and the fluorescence emission spectrum of dT(pT)_{19}-Fl-3' in the presence of R14C-CPM (without energy transfer), in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP \((\lambda_{ex} = 435 \text{ nm})\). The solid line in Fig. 7a is the fluorescence emission spectrum of the R14C-CPM and dT(pT)_{19}-Fl-3' complex at the same concentrations of the protein and nucleic acid as independent components of the complex. Contrary to the situation with 5'-Fl-dT(pT)_{19}, only a small difference is observed when both the donor, CPM, on the DnaB protein, and the acceptor, fluorescein on the 3'-end of the 20-mer, are placed in the same complex as compared with the sum of the spectra of independent components of the complex. The emission intensity of R14C-CPM is only decreased by \(\sim 11\%\) as compared with \(\sim 35\%\) observed for R14C-CPM with 5'-Fl-dT(pT)_{19}, indicating a very diminished fluorescence energy transfer from CPM to the fluorescein moiety, when the acceptor is located at the 3'-end of the dT(pT)_{19}. Also, the sensitized emission of the fluorescein located at the 3'-end of the 20-mer is only increased by \(\sim 43\%\) as compared with \(\sim 220\%\) in the complex of R14C-CPM with 5'-Fl-dT(pT)_{19} (Fig. 6b).

The dramatic difference between the emission spectrum of the complex of R14C-CPM with 5'-Fl-dT(pT)_{19} and the spectrum of the complex with dT(pT)_{19}-Fl-3' clearly shows that the helicase binds ssDNA in a predominantly single orientation, with respect to the polarity of the ssDNA sugar-phosphate backbone. If the helicase could bind ssDNA in two different orientations with equal probability, then the changes in the spectra of the complexes with the 20-mer, labeled with fluores-
DNA Binding Site of a Helicase

**TABLE II**

| Property                        | ssDNA oligomer | 5'-Fl-dT(pT)19 | dT(pT)19-Fl-3’ |
|--------------------------------|----------------|----------------|---------------|
| Fluorescence anisotropy \((r_a)\)_\(^a\) | 0.28 ± 0.01    | 0.24 ± 0.01    |               |
| Limiting fluorescence           | 0.29 ± 0.01    | 0.25 ± 0.01    |               |
| Fluorescence energy transfer anisotropy \((r_e)\)_\(^b\) |               |               |               |
| Efficiency in the complex \(E_D\) | 0.77 ± 0.04    | 0.18 ± 0.02    | \(E_a\)      |
| with R14C-CPM\(^c\)             | 0.55 ± 0.04    | 0.09 ± 0.01    | \(E\)        |
| \(E_b\)                        | 0.71 ± 0.04    | 0.1 ± 0.01     | \(E_b\)      |

\(^{a}\) \(\lambda_{ex} = 485\) nm.

\(^{b}\) \(\lambda_{ex} = 485\) nm, determined using the Perrin equation (22).

\(^{c}\) \(\lambda_{ex} = 485\) nm.

transfer efficiencies shows that the 5'-end of the 20-mer, dT(pT)\(_{19}\), is in much closer proximity to the CPM residues, which are located on the small domains of the DnaB hexamer, than to the 3'-end of the nucleic acid (see “Discussion”).

The determination of exact distances between the donors (CPM) and acceptors (fluorescein) is beyond the scope of the present discussion on the mutual orientation between the DnaB helicase and the ssDNA in the complex. However, using Equation 11 we can estimate the approximate ratio of the distances between the 5'- and the 3'-end of the dT(pT)\(_{19}\) oligomer from the center of the mass of CPM donors located on the small domains of the DnaB hexamer. Introducing \(E = 0.71\) and \(E = 0.1\) into Equation 11, we obtained \(R_1/R_2 = 0.60\). Thus, the average distance of the 5'-end of the 20-mer is only 60% of the distance between the donors and the 3'-end of the nucleic acid.

Very similar behavior to the one described above has been observed when different donor-acceptor pairs have been used. These results show, for the first time, that the DnaB hexamer binds ssDNA in a single orientation, with respect to the sugar-phosphate backbone of the nucleic acid. In the complex, the small 12-kDa and the large 33-kDa domains of the enzyme face the 5'- and 3'-ends of the nucleic acid, respectively.

**DNA Mobility within the Strong and Weak DNA Binding Subsite of the DnaB Helicase—Assessment of the relative mobility of the different segments of the nucleic acid, within the DNA binding site, can be obtained by measuring the emission anisotropy of the fluorescent markers placed in different locations on the nucleic acid. To determine the relative mobility of ssDNA in two subsites of the total DNA binding site of the DnaB helicase, we determined the emission anisotropy of 5'-Fl-dT(pT)\(_{19}\) and dT(pT)\(_{19}-Fl-3’\) in the complex with the helicase. Anisotropies of both samples are constant across their emission spectra, indicating the lack of a significant local heterogeneity around the fluorescent markers (spectra not shown). However, the anisotropy of 5'-Fl-dT(pT)\(_{19}\), \(r = 0.28 ± 0.01\), is significantly higher than the anisotropy, \(r = 0.24 ± 0.01\), determined for dT(pT)\(_{19}-Fl-3’\). Because the fluorescence lifetimes of fluorescein in both complexes are very similar (4-ns, data not shown), the obtained data indicate significantly higher mobility of the nucleic acid at its 3’-end.

Analogous fluorescence energy transfer and anisotropy studies with a 10-mer, dA(pA)\(_9\), labeled with fluorescein at the 5’- or 3’-ends of the 10-mer indicate that its 5’-end is located in close proximity to the 12-kDa domain of the enzyme and has a similar strong decrease in its mobility (data not shown). As we described above, this oligomer binds exclusively to the strong subsite in the DNA binding site of the DnaB helicase. Thus,

3 Jezewska, M. J., Rajendran, S., Bujalska, and Bujalski, W. (1998) J. Biol. Chem. 273, in press.
fluorescence energy transfer and anisotropy data indicate that the nucleic acid binds with the first 10 nucleotides from its 5′-end to the strong DNA binding subsite of the total DNA binding site of the helicase.

**DISCUSSION**

The Total DNA Binding Site of a Helicase—Helicases play a key role in all aspects of DNA metabolism, and this role is related to the interactions of the enzyme with ssDNA and dsDNA controlled by binding and hydrolysis of a nucleoside triphosphate, e.g. ATP (30). Understanding the functional and structural aspects of the DNA binding site is a prerequisite for our understanding of how the enzymes perform their functions. Yet, little is known about the structure of the DNA binding site of any hexameric helicase and the functional interrelations within the binding site. In this work, we provide the first insight into the complex structure/function relationship of the DNA binding site of a hexameric replicative helicase, the E. coli DnaB protein.

Our previous studies with polymer ssDNA and ssDNA oligomers showed that in a stationary complex with the ATP-nonhydrolyzable analog, AMP-PNP, the enzyme has a single binding site located on a single subunit of the hexamer (12–14). Additionally, this single binding site is used when the enzyme binds to the DNA substrates resembling the replication fork (8, 13, 25, 26). These results indicate that the observed single binding site is, in fact, the total DNA binding site of the enzyme that, in functional complexes on the junction between ssDNA and dsDNA with the replication fork, encompasses both single- and double-stranded conformations of nucleic acid over a stretch of ~20 nucleotide residues.

The operational definition of the total binding site of the enzymes, which perform their catalysis on polymer lattices, such as helicases, should refer to the complex of the enzymes with a polymer substrate. A total binding site of an enzyme is used as a single entity that interacts with a continuous stretch of polymer substrate. This continuous fragment of the polymer substrate (DNA), within the total binding site, defines the size of the enzyme-nucleic acid complex. The total binding site can be heterogeneous, i.e. built of functionally and/or structurally different areas, subsites, specific for the catalytic functions of the enzyme. However, the location of the subsites is sequential, i.e. they are placed along the polymer substrate. The total binding site can perform the dominant catalytic process characteristic for the enzyme, e.g., unwinding of the duplex DNA. Such a binding site can be located on a single subunit of an oligomeric enzyme, such as the DnaB helicase; thus, there may be several total binding sites, but only one site (one subunit) at a time is engaged in interactions with DNA during the catalysis. A total binding site can include several subunits of an oligomeric enzyme, as in the case of DNA-dependent oligomeric polymerases.

Contrary to the total binding site of the enzyme, a subsite always interacts with a polymer DNA within the context of a total binding site. A subsite cannot be used as an independent entity in the interactions of the enzyme with polymer DNA; nor can it independently perform the catalysis.

The Total Binding Site of the DnaB Helicase Is Structurally Heterogeneous—Nuclease digestion protection studies provide a clear indication of the structural heterogeneity of the total binding site of the E. coli DnaB helicase. Only 10 or 11 nucleotide residues, within the total binding site, are strongly protected from digestion, while the remaining 9 or 10 residues are accessible to the nuclease (Fig. 1, a and b). These results indicate that the total binding site of the helicase, which occludes on ~20 nucleotide residues in the complex with polymer ssDNA, is built of two binding subsites each encompassing a similar number of ~10 nucleotides. Experiments on the binding of partial DNA ligands to the helicase showed a large difference in the affinities between the subsites and indicated that the 5′-end of the nucleic acid interacts with the strong binding subsite of the total binding site of the enzyme. The fact that the nuclease can access ~half of the total number of occluded residues within the entire binding site suggests not only a difference in the affinities between the subsites but also an open architecture of the hexamer at the subsite that encompasses the 3′-end of the nucleic acid (see below).

The Two DNA Binding Subsites of the Total Binding Site of the DnaB Helicase Have Dramatically Different Affinities for ssDNA—Direct evidence of large differences in the affinities between the DNA binding subsites of the DnaB helicase comes from the studies of the binding of a partial ligand, dA(pA)₉, to the enzyme. Using the thermodynamically rigorous method, we determined that only one 10-mer binds with significant affinity to the helicase and that the association is characterized by the binding constant $K_1 = (1.7 \pm 0.3) \times 10^7 \text{ M}^{-1}$. The affinity for the second binding subsite is characterized by $K_2 = (2.6 \pm 1) \times 10^4 \text{ M}^{-1}$; thus, it is ~3 orders of magnitude lower. It is evident that the major part of the free energy of binding of the helicase to ssDNA comes from interactions with the strong binding subsite. The very low affinity of the weak binding subsite indicates that the protein does not form efficient contacts with a single-stranded nucleic acid and suggests that this subsite of the helicase is not functionally a ssDNA binding site but rather that it fulfills a different role when the enzyme is in the complex with its physiological substrate, the replication fork (see below).

To efficiently form a complex with the strong DNA binding subsite, the nucleic acid must have a length of at least 6 or 7 nucleotide residues. No detectable affinities were observed with ssDNA oligomers shorter than 6 nucleotides in our solution conditions (Fig. 4a). It is interesting that the difference of 2 residues between 7- and 5-mer practically abolishes the affinity of the shorter oligomer for the binding site, while the same difference between the 8- and 10-mer leads to a decrease of the free energy of binding by only ~0.3 kcal/mol (Fig. 4b). A common misconception in studying protein-nucleic acid interactions is treating both a nucleic acid and a protein as interacting regular lattices. The differences between the free energy of interaction of oligomers of different lengths with the protein is then assigned to the difference in statistical effects between different oligomers, which usually has very poor quantitative justification. We point out that the nucleic acid is the only macromolecule that can be approximated by a regular lattice. The binding site on the protein can have a very complex structure, with distant regions making key contacts with the nucleic acid, hardly resemble a regular lattice. The differences between different oligomers in binding to the DnaB helicase cannot be explained by any difference in the statistical effect between the oligomers. This is particularly true for oligomers shorter than 6 or 7 residues. Rather, the results suggest that the elements of the strong binding subsite of the enzyme, which makes crucial binding contacts with the nucleic acid, are separated by a distance spanned by 6 or 7 nucleotides. The proper complex is formed only when all essential contacts are engaged in interactions with ssDNA. In this context, the similar number of ions released in the interactions of a 10-mer and a 6-mer with the helicase (~1.5) would be a result of the fact that a 6-mer can still form all essential contacts with the enzyme, although the oligomer constitutes only 60% of the length of the 10-mer (Fig. 5b).

Direct Proof That the DnaB Helicase Binds in a Single Orientation with Respect to the Sugar-Phosphate Backbone of a
ssDNA—Most of the studied helicases show preferential direction in the unwinding of dsDNA, i.e. in the 5' → 3' or the 3' → 5' direction (30). Therefore, it is often a priori assumed that the enzyme binds in strict polarity, 5' → 3' or 3' → 5', with respect to the orientation of the single-stranded nucleic acid strand. This is a natural assumption that simplifies current models, based on the still limited solution data, of how the helicase functions at the replication fork. However, one can argue that the enzyme can bind in both orientations to the nucleic acid lattice and that the proper orientation is imposed by specific interactions with dsDNA and/or multiple proteins that are building the machinery of the replication fork. In this context, it should be noted that several specific protein-protein interactions between the DnaB helicase and proteins, which are part of the primosome or replication fork complex, have been identified. Although recent electron microscopy and crystallographic data show polarity in a helicase binding to ssDNA (31, 32), the polarity in the binding of a helicase, with respect to the directionality of a ssDNA strand, has never been directly shown for any hexameric helicase in solution.

As we pointed out, the determination of the mutual orientation of the protein and nucleic acid in a complex should be based on the method that is sensitive to the differences in distances between different specific regions of both macromolecules. The fluorescence energy transfer technique is such a method. The difference in the effect of the location of the acceptor, fluorescein, at the 5'- or 3'-end of the 20-mer, dT(pT)19, on the fluorescence spectra of the complex of nucleic acid with R14CPM (excited in a predominantly donor absorption band), is dramatic (Figs. 6 and 7). These dramatic spectral differences are reflected in the large differences between the energy transfer efficiencies from CPM in the small 12-kDa domains, all located at one end of the DnaB hexamer, and the fluorescein placed at the 5'- or 3'-end of the dT(pT)19, which spans the entire DNA binding site. The efficiency, E, for the fluorescein placed at the 5'-end of the 20-mer is 0.71 ± 0.04. The efficiency of the same acceptor located at the 3'-end of the nucleic acid is only 0.10 ± 0.01. In the case of chemically identical donor-acceptor pairs, the transfer efficiency depends on two variable factors characteristic for the studied system, the distance between the donor and acceptor, R, and the orientation parameter, $\kappa^2$, which characterizes the mutual orientation of the donor absorption dipole and acceptor emission dipole (22). The value of $\kappa^2$ can theoretically assume any value between 0 and 4, but only these two extreme values would significantly affect the determined transfer efficiency. The possible range of $\kappa^2$ can be estimated by using the standard procedure (Ref. 24; Table II). The obtained ranges of $\kappa^2$ are very similar for both 5'-Fl-dT(pT)19 and dT(pT)19-Fl-3' and away from the extreme values of 0 and 4 (Table II). Another equally rigorous procedure is to perform experiments with several different donor-acceptor pairs, we obtained similar, very large differences between the fluorescence energy transfer efficiencies from the fluorophore on the 12-kDa domain of the DnaB helicase and the donor or acceptor placed at the 5'- and the 3'-end of the bound 20-mer (data not shown). The results clearly show that the large difference between the transfer efficiencies results from the large difference in the distances between the 5'-end and the 3'-end of the 20-mer and the CPM located on the small domain of the DnaB protomers.

Our data show that the DnaB helicase binds ssDNA in a predominately single orientation, with respect to the polarity of the single-stranded nucleic acid lattice. Moreover, the data show that, in the complex with ssDNA, the small domain of the protein is in close proximity to the 5'-end of the nucleic acid, while the large domain is located near the 3'-end of the bound ssDNA.

Sequential Locations of the Strong and Weak Binding Subsites of the DnaB Helicase—As determined in this work, the partial ligand, deA(pA)e, binds with overwhelming preference to the strong binding subsite of the DnaB helicase. The transfer efficiency between the 5'-end of the 10-mer, dA(pA)e, labeled with fluorescein, and CPM, located in the small domain of the DnaB protein, is very similar to the transfer efficiency between the CPM and fluorescein at the 5'-end of the 20-mer, dT(pT)19 (data not shown). These results indicate that the 5'-ends of both the 10- and 20-mers are at a similar distance from the small domain of the protein. Thus, the strong ssDNA binding subsite encompasses the bound 20-mer at its 5'-end, which is in close proximity to the small 12-kDa domain of the protein, while the weak binding subsite is located entirely on the large domain. At present, it is unknown whether or not the small domain or the hinge region of the DnaB protomer is directly involved in interactions with ssDNA. It should be noted that the isolated large 33-kDa domain of the enzyme could still bind ssDNA with some affinity, although quantitative analysis of the binding has not been performed (28). Thus, it is possible that the small domain and the hinge region constitute a part of the strong ssDNA binding subsite of the intact DnaB helicase.

The DnaB helicase binds preferentially to the 5'-arm of the replication fork (26). Because the enzyme binds in a single orientation, with respect to the polarity of the ssDNA sugar-phosphate backbone, with the small domain facing the 5'-end of the ssDNA, it is evident that in the complex with the replication fork, the helicase hexamer is oriented with the large domains of the protomers toward the duplex part of the fork,
while the 5'-end of the arm of the replication fork is located in the vicinity of the small 12-kDa domains of the protomers. Anisotropy of the probe located at the 5'-end of the 20-mer bound to the helicase is significantly higher than the anisotropy of the same fluorescein residue located at the 3'-end of the nucleic acid (Table II). A significant decrease of the anisotropy, when the probe is located at the 3'-end of the bound nucleic acid, indicates an increased mobility of the nucleic acid in the weak binding subsite and is most probably due to the lack of strong contacts between the single-stranded nucleic acid and the binding site. Recall that the micrococcal nuclease can access the part of the nucleic acid in the weak subsite of the total binding site of the DnaB helicase, suggesting a more open structure of the total binding site at the 3'-end of the bound 20-mer.

The results described in this work provide an insight into the complex structure-function relationship within the DNA binding site of a replicative hexameric helicase. A model of the single, total DNA binding site on the DnaB protomer, engaged in the complex with the replication fork and based on the data presented in this work, is schematically shown in Fig. 8. The total DNA binding site of the enzyme is built of two subsites placed sequentially along the DNA substrate in the protein-nucleic acid complex. The strong ssDNA binding subsite occludes the 5'-end of the ssDNA, is located in close proximity to the small 12-kDa domain, and is distant from the duplex part of the fork. Binding of ssDNA to this subsite leads to the significant immobilization of the nucleic acid and provides the major part of the binding free energy. The subsite, which is located at the 3'-end of the ssDNA, binds the single-stranded nucleic acid very weakly. The single orientation of the helicase in the complex with ssDNA indicates that, when the enzyme approaches the replication fork, it faces the duplex part of the fork with the weak binding subsite located entirely on the large 33-kDa domain of the protein. Thus, the weak binding subsite constitutes the entry site for the dsDNA in the fork. The more open architecture of this subsite provides a large space, which is necessary for the incoming duplex DNA.

Comparison with other hexameric helicases is difficult because, at this time, no analogous data on the structure of their nucleic acid binding sites are available. However, it is possible that similar functional and structural relationships within the DNA binding site are general for all other hexameric helicases.

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