Does Single-stranded DNA Pass through the Inner Channel of the Protein Hexamer in the Complex with the *Escherichia coli* DnaB Helicase?

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Maria J. Jezewska, Surendran Rajendran, Danuta Bujalowska, and Wlodzimierz Bujalowski†

*From the Department of Human Biological Chemistry and Genetics and the Sealy Center for Structural Biology, The University of Texas Medical Branch at Galveston, Galveston, Texas 77555-1053*

The structure of the complex of the *Escherichia coli* primary replicative helicase DnaB protein with single-stranded (ss) DNA and replication fork substrates has been examined using the fluorescence energy transfer method. In these experiments, we used the DnaB protein variant, R14C, which has arginine 14 replaced by cysteine in the small 12-kDa domain of the protein using site-directed mutagenesis. The cysteine residues have been modified with a fluorescent marker which serves as a donor or an acceptor to another fluorescence label placed in different locations on the DNA substrates. Using the multiple fluorescence donor-acceptor approach, we provide evidence that, in the complex with the enzyme, ssDNA passes through the inner channel of the DnaB hexamer. This is the first evidence of the existence of such a structure of a hexameric helicase-ssDNA complex in solution. In the stationary complex with the 5’ arm of the replication fork, without ATP hydrolysis, the distance between the 5’ end of the arm and the 12-kDa domains of the hexamer (R = 47 Å) is the same as in the complex with the isolated ssDNA oligomer (R = 47 Å) having the same length as the arm of the fork. These data indicate that both ssDNA and the 5’ arm of the fork bind in the same manner to the DNA binding site. Moreover, in the complex with the helicase, the length of the ssDNA is similar to the length of the ssDNA strand in the double-stranded DNA conformation. In the stationary complex, the helicase does not invade the duplex part of the fork beyond the first 2–3 base pairs. This result corroborates the quantitative thermodynamic data which showed that the duplex part of the fork does not contribute to the free energy of binding of the enzyme to the fork. Implications of these results for the mechanism of a hexameric helicase binding to DNA are discussed.

The DnaB protein is the *Escherichia coli* primary replicative helicase, i.e. the factor responsible for unwinding the duplex DNA in front of the replication fork (1–3). The enzyme is an essential replication protein in *E. coli* (4) which is involved in both the initiation and elongation stages of DNA replication (3, 5, 6). The DnaB helicase is the only helicase required to reconstitute 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 plays an activating role for the primase in the initial stages of the priming reaction (4).

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 stabilized specifically by magnesium cations (7–9). Hydrodynamic and EM data indicate that six protomers aggregate with cyclic symmetry in which the protomer-protomer contacts are limited to only two neighboring subunits (7, 10, 11). Hydrodynamic and EM studies also provide direct evidence of the presence of long range allosteric interactions in the hexamer, encompassing all six subunits of the enzyme (7, 11, 12).

Recently, we obtained the first estimate of the stoichiometry of the DnaB helicase-ssDNA complex and the mechanism of the binding (12, 13). In the complex with ssDNA, the DnaB helicase binds the nucleic acid with a stoichiometry of 20 ± 3 nucleotides per DnaB hexamer, and this stoichiometry is independent of the type of nucleic acid base (9, 13). Our thermodynamic studies of binding of the DnaB hexamer to different ssDNA oligomers show that the enzyme has a single, strong binding site for ssDNA. Moreover, the same binding site is used in the binding to oligomers, polymer DNA, and replication fork substrates (9, 12–14). Photo-cross-linking experiments indicate that the ssDNA binding site is located predominately, if not completely, on a single subunit of the hexamer (9, 12, 13).

Our data show that, in the complex with the replication fork DNA substrates, the DnaB helicase preferentially binds to the 5’ arm of the fork (14). The 3’ arm does not form a stable complex with the DnaB hexamer associated with the 5’ arm, and the 3’ arm is in a conformation in which it is accessible for the binding of another DnaB hexamer. Moreover, the duplex part of the fork substrate does not significantly contribute to the free energy of binding which predominantly comes from interactions with the 5’ arm (14).

Formulating a physical model of a hexameric helicase mechanism requires the knowledge of the structure of the helicase-ssDNA complex. In phage T7 helicase/primase and *E. coli* RuvB protein systems, EM data indicated that, in the complex with

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1 The abbreviations used are: ss, single-stranded; ds, double-stranded; AMP-PNP, β,γ-imido-adenosine-5’-triphosphate; EM, electron microscopy; CPY, 7-diethylamino-3-(4’-maleimidophenyl)-4-methylcoumarin; FL, fluorescein; Rh, rhodamine.
the enzymes, the ssDNA passes through the inner channel of the protein hexamer (15, 16). On the other hand, an outside mode of ss nucleic acid binding has been proposed for the hexamer of the SV40 T large antigen helicase (17).

In this communication, the structure of the DnaB helicase-ssDNA complex has been studied using the fluorescence energy transfer method. We present evidence that, in the complex with the DnaB hexamer, the ssDNA oligomer, which occupies the entire total DNA binding site of the DnaB helicase, passes through the inner channel of the hexamer. The results indicate that in the stationary complex with the replication fork substrate, the helicase does not invade the duplex part of the fork beyond the first 2–3 base pairs.

**EXPERIMENTAL PROCEDURES**

**Reagents and Buffers—**All solutions were made with distilled and deionized >18 megohms (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, and 10% glycerol. The temperatures and concentrations of NaCl and AMP-PNP in the buffer are indicated in the text.

**DnaB Protein—**The E. coli DnaB protein was purified, as we described previously (18, 19). The concentration of the protein was spectrophotometrically determined using the extinction coefficient, ε₂₅₀ = 1.85 × 10⁵ M⁻¹ cm⁻¹ (hexamer) (7).

**Nucleic Acids—**All nucleic acids were purchased from Midland Certified Reagents (Midland, TX). The 20 mer dT(pT)₂₀, labeled with fluorescein at the 5’ end, or at a different location of the nucleic acid, were synthesized using fluorescein phosphoramidate (Glen Research). Labeling of 20 mers at the 3’ end with fluorescein, or labeling with rhodamine (Rh), was performed by synthesizing dT(pT)₁₈ with a nucleotide residue in a given location of the nucleic acid with the amino group on a six-carbon linker and, subsequently, modifying the amino group with fluorescein 5’-isothiocyanate or tetramethylrhodamine 6-isothiocyanate (Midland Certified Reagents). The degree of labeling was determined by absorbance at 494 nm for fluorescein (pH 9) using the extinction coefficient, ε₄₉₄ = 7.6 × 10⁴ M⁻¹ cm⁻¹, and at 555 nm for rhodamine using the extinction coefficient, ε₅₅₅ = 8.0 × 10⁴ M⁻¹ cm⁻¹ (9). Concentrations of all ssDNA oligomers have been spectrophotometrically determined, using the nearest-neighbor analysis (9, 20). The single-arm fork substrates were obtained by mixing the proper oligomers at given concentrations, warming up the mixture for 5 min at 95 °C, and slowly cooling for a period of ~2 h (14).

**Site-directed Mutagenesis of the DnaB Helicase—**Replacement of the six cysteine residues of the DnaB variant, R14C, were performed using the SLM-AMINCO 48000S and 8100 spectrofluorometers (12, 21, 22). The emission spectra have been corrected for instrument characteristics using the software provided by the manufacturer. Fluorescence anisotropy measurements were performed in the L format, using Glan-Thompson polarizers placed in the excitation and emission channels. The fluorescence anisotropy of the sample was calculated using (23)

$$ r = \frac{(I_{VH} - G I_{VP})}{(I_{VH} + 2G I_{VP})} $$

(Eq. 1)

were I is the fluorescence intensity and the first and the second subscripts refer to vertical (V) polarization of the excitation and vertical (V) or horizontal (H) polarization of the emitted light (19). The factor $G = I_{VP}/I_{VH}$ corrects for the different sensitivity of the emission monochromator for vertically and horizontally polarized light (24).

**Determination of the Average Fluorescence Energy Transfer Efficiency from Donors on the Small 12-kDa Domains of the DnaB Hexamer to an Acceptor Located on the DNA Substrates—**The efficiency of the fluorescence radiationless energy transfer, E, from donors located on the small 12-kDa domains of the DnaB protein variant R14C, to an acceptor located on a DNA substrate bound in the DNA binding site of the DnaB 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

$$ F_{DA} = (1 - \nu_D)F_D + \nu_D(1 - E_D) $$

(Eq. 2a)

where $\nu_D$ is the fraction of donors in the complex with the acceptor, and $E_D$ is the average fluorescence energy transfer from a donor to an acceptor, determined from the quenching of the donor fluorescence. Thus, the average transfer efficiency, $E_D$, obtained from the quenching of the donor fluorescence is obtained by rearranging Equation 2a

$$ E_D = \frac{1}{\nu_D} (\frac{F_D - F_{DA}}{F_D}) $$

(Eq. 2b)

The values of $\nu_D$ have been determined using the binding constants of a given DNA substrate for the DnaB helicase measured in the same solution conditions (9, 14).

In the 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 or rhodamine), excited at a wavelength where a donor predominantly absorbs, in the absence and presence of the donor. The fluorescence intensities of the acceptor in the absence, $F_A$, and presence, $F_{AD}$, of the donor are defined as

$$ F_A = I_A \varepsilon_A C_A \Phi_A $$

(Eq. 3a)

and

$$ F_{AD} = (1 - \nu_A)F_A + \nu_A \varepsilon_A C_A \Phi_A + \varepsilon_D C_D \Phi_D $$

(Eq. 3b)

where $I_A$ is the intensity of incident light, $C_A$ and $C_D$ are the total concentrations of acceptor and donor, $\nu_A$ is the fraction of acceptors in the complex with donors, $\varepsilon_A$ and $\varepsilon_D$ are the molar absorption coefficients of acceptor and donor at the excitation wavelength, respectively, $\Phi_A$ and $\Phi_D$ are the quantum yields of the free and bound acceptor, and $E_A$ is the average transfer efficiency determined by the acceptor sensitized emission. All quantities in Equations 3a and 3b can be experimentally determined. For the case considered in this work, the acceptor is practically, completely saturated with the donor, i.e., $\nu_A = 1$. Thus, for $\nu_A = 1$, dividing Equation 3b by 3a and rearranging provides the average transfer efficiency as described by

$$ E_A = \frac{1}{\nu_A} \left( \frac{\varepsilon_D C_D}{\varepsilon_A C_A} \right) \left( \frac{\Phi_A}{\Phi_D} \frac{F_{AD}}{F_A} - 1 \right) $$

(Eq. 3c)

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 which were transferred to the acceptor. The true Förster energy transfer efficiency, $E$, is a fraction of the 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 (23). The value of $E$ is related to the apparent quantities of $E_D$ and $E_A$ by

2 Jezewski, M. J., Rajendran, S., and Bujalowski, W. (1998) J. Biol. Chem. 273, 9058–9069

3 S. Rajendran, M. J. Jezewski, and W. Bujalowski, manuscript in preparation.
Thus, measurements of the transfer efficiency, using both methods, are not alternatives but parts of the entire analysis used to obtain the true efficiency of the fluorescence energy transfer. The transfer process can be described by evaluating the mutual distance of the donor and acceptor dipoles using the procedure described by Dale et al. (27). When both axial depolarization factors are positive, the mutual distance can be calculated from

\[ \kappa_{\text{max}} = \left( \frac{2}{3} \right) \left( 1 + \left[ \kappa_{\text{D}}^2 + 3\kappa_{\text{A}}^2 \right] \right) \] (Eq. 6a)

and

\[ \kappa_{\text{min}} = \left( \frac{2}{3} \right) \left( 1 - \left( \frac{1}{2} \right) \left[ \kappa_{\text{D}}^2 + \kappa_{\text{A}}^2 \right] \right) \] (Eq. 6b)

where \( \kappa_{\text{D}} \) and \( \kappa_{\text{A}} \) are the axial depolarization factors for the donor and acceptor, respectively (27). The axial depolarization factors have been calculated as square roots of the ratios of the limiting anisotropies of the donors and acceptors and their corresponding fundamental anisotropies, \( \kappa = 0.4 \) for CPM and fluorescein (19, 25, 27).

The fluorescence transfer efficiency determined for chemically identical donor-acceptor pairs, characterized by the same donor quantum yields, depends on the distance between the donor and the acceptor. The transfer process is a function of the degree of labeling and by excitation fluorescence anisotropy spectra of the fully labeled DnaB hexamer. None of these approaches showed a measurable homo-transfer, even in the case of the R14C-Fl labeled fluorescein which has a large overlap of its absorption and fluorescence spectra (36).

Fluorescence Energy Transfer from Multiple Donors to a Single Acceptor—If a set of \( m \) identical donors transfers the energy to a single acceptor, as in the cases studied in this work, the average transfer efficiency is weighted by the contributions, \( E_i \), from all donors and is defined in general as

\[ E = \left( \frac{1}{m} \right) \sum E_i \] (Eq. 7)

where \( E_i \) is the transfer efficiency from the individual donor, \( i \), to the acceptor. It should be noted that, if all individual transfer efficiencies, \( E_i \), are equal, e.g. in the case where the donors are located at the same distance from the acceptor, the experimentally determined average fluorescence transfer efficiency is then \( E = \left( \frac{1}{m} \right) \sum E_i = E \).

Quantum Yield Determinations—The quantum yields of different chromophores used in this work, \( \phi \), were determined by the comparative method (29) we previously described (19). Quinine bisulfate in 0.1 NH4SO4 and fluorescein in 0.1 NaOH were used as a standard (absolute quantum yield \( \phi = 0.7 \) and 0.92, respectively) (30, 31).

RESULTS

Distance between the 5' End of the ssDNA and the Small 12-kDa Domains of the DnaB Hexamer, Multiple Donor-Acceptor Experiments—The DnaB monomer, which has an elongated shape, is built of two structural domains (7, 10, 32). A small 12-kDa domain at the N terminus of the protein and a large 33-kDa domain at the C terminus are both connected at the hinge region. This structure of a monomer has been visualized in EM studies which also showed that, in the cyclic DnaB hexamer, all protomers are oriented with the small 12-kDa domain in the same direction (10). In R14C-CPM or R14C-Fl, each of the six small 12-kDa domains of the hexamer is labeled with a fluorescent marker, coumarin (CPM) or fluorescein at a specific site (see “Experimental Procedures”). All six fluorophores in the labeled R14C variant are at the same end of the DnaB hexamer and arranged in a ring. The schematic representation of the DnaB hexamer based on hydrodynamic and EM data is shown in Fig. 1a.

Fluorescence energy transfer from a donor to an acceptor is one of the most intensively used methods in studying macromolecular distances in solution (23). The overlap of an absorption spectrum of an acceptor with the emission spectrum of a donor is a condition for the fluorescence resonance energy transfer to occur. The fluorescence emission spectrum of R14C-CPM (\( \lambda_{ex} = 425 \text{ nm} \)) together with the absorption spectrum of 5'-Fl-dT(pT)19 and 5'-Rh-dT(pT)19, as well as the fluorescence emission spectrum of R14C-Fl (\( \lambda_{ex} = 485 \text{ nm} \)), with the absorption spectrum of 5'-Rh-dT(pT)19 in buffer T2 (pH 8.1, 20 °C), containing 100 mM NaCl and 1 mM AMP-PNP, are shown in Fig. 2, a–c. In the case of all three donor-acceptor pairs, there is a very significant spectral overlap of the donor emission with the acceptor absorption spectrum, indicating that efficient fluorescence energy transfer can occur, if the donor and acceptor are in close proximity.

Fluorescence emission spectra of R14C-CPM (\( \lambda_{ex} = 425 \text{ nm} \)), in the absence and presence of unlabeled dT(pT)19, in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP, are shown in Fig. 3. The presence of the unlabeled 20 mer has very little effect on the CPM fluorescence intensity. The situation is different in the case of the 5'-Fl-dT(pT)19 complex with R14C-CPM. The emission spectra of the labeled nuclier acid in the absence and presence of R14C-CPM, with the excitation at 485 nm, where only fluorescein absorbs, are included in Fig. 3. The presence of R14C-CPM causes an ~2 fold decrease of the fluorescence intensity of 5'-Fl-dT(pT)19, while saturation of the labeled 20 mer with the unlabeled DnaB protein causes only an ~8% decrease of the 5'-Fl-dT(pT)19 fluorescence (spectrum not shown). Thus, even in the absence of the energy transfer
process, the presence of six hydrophobic CPM residues affects the quantum yield of fluorescein located at the 5' end of the ssDNA. The ratio of quantum yields of 5'-Fl-dT(pT)19, in the complex with R14C-CPM and free in solution, $f_{BA}/f_{FA}$, has been tested over a 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. The value of $f_{BA}/f_{FA}$ is constant and equals 0.51. This result is expected because the quantum yield of fluorescein is independent of the excitation wavelength between 400 and 500 nm (23). Thus, the ratio of quantum yields, independent of excitation wavelength, reflects the change of the emission intensity of 5'-Fl-dT(pT)19 resulting from the formation of the complex with R14C-CPM, in the absence of the 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 (see Equations 3b and 3c). Analogous effect of the presence of the ring of six donor residues on the quantum yield of the acceptor at the 5' end of the 20 mer occurs in all studied complexes. The emission spectrum ($\lambda_{em} = 555$ nm) of 5' Rh-dT(pT)19, in the absence and presence of R14C-Fl, is shown in Fig. 3. The quantum yield of Rhodamine is decreased by factor 0.65 in the complex with R14C-Fl, as compared with the free oligomer, while the unlabeled DnaB protein causes only an ~9% decrease (data not shown).

The sum of the independent emission spectra ($\lambda_{exc} = 425$ nm) of R14C-CPM and 5'-Fl-dT(pT)19 in the presence of an unlabeled nucleic acid and R14C-CPM (without energy transfer), respectively, in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP, is shown in Fig. 4a. The solid line in Fig. 4a is the fluorescence emission spectrum of the complex of R14C-CPM with 5'-Fl-dT(pT)19 at the same concentrations of the protein and nucleic acid as in the case of the sum of the independent components of the complex. There is a dramatic difference between the sum of the spectra of independent components of the complex and the spectrum of the complex in Fig. 4a shows that the fluorescence intensity of the fluorescein moiety located at the 5' end of the bound 5'-Fl-dT(pT)19.

Comparison between the sum of the spectra of independent components of the complex and the spectrum of the complex in Fig. 4a shows that the fluorescence intensity of the fluorescein residue of 5'-Fl-dT(pT)19, with the peak at ~519 nm, is strongly increased in the complex with R14C-CPM. Because fluorescein does not contribute to the CPM emission band at 476 nm, we can normalize the spectrum of R14C-CPM-unla-
fluorescein on the 12-kDa domain of the DnaB protein as the donor
3
and rhodamine at the 5
3
end of the bound 20 mer is increased 8.7-fold.

Experiments using a different donor-acceptor pair, fluorescein on the 12-kDa domain of the DnaB protein as the donor and rhodamine at the 5’ end of the nucleic acid, have been obtained with labeled dT(pT)19 and the complex, as shown as a solid line. Placing the donor and acceptor in the same complex introduces a large difference in the donor and acceptor emission spectra, as compared with the sum of the independent components. In the complex with 5’-Rh-dT(pT)19, the emission intensity of R14C-Fl, with the maximum at ~519 nm, is decreased by ~23%, as compared with the protein spectrum complexed with unlabeled dT(pT)19. Also, the intensity of the sensitized emission of rhodamine is ~23-fold higher than the emission in the absence of the fluorescence energy transfer process. Both donor quenching and strong sensitized emission indicate a significant fluorescence energy transfer from fluorescein on the small 12-kDa domain of the helicase to rhodamine located at the 5’ end of the nucleic acid. The fluorescence energy transfer parameters for studied donor-acceptor pairs are included in Table I.

The large effects on the observed spectral properties of the studied complexes are reflected in the large values of the Förster transfer efficiencies, E (Table I). In the case of the R14C-Fl complex with 5’-Rh-dT(pT)19, we obtained the apparent transfer efficiencies of $E_D = 0.61 \pm 0.04$ and $E_A = 0.55 \pm 0.04$, respectively, using Equations 2b and 3c. The difference between $E_D$ and $E_A$ is within the experimental error of determination of both quantities, nevertheless, it indicates that rhodamine, at the 5’ end of the bound dT(pT)19, induces some additional nondipolar quenching of the fluorescein emission. Larger differences between $E_p$ and $E_s$ have been obtained with other studied donor-acceptor pairs with the fluorophore located at the 5’ end of the 20 mer (Table I). The true Förster fluorescence transfer efficiency from fluorescein, located on the small 12-kDa domains of the DnaB hexamer to the rhodamine resi-

beled dT(pT)19 to the spectrum of the 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 of R14C-CPM-5’-Fl-dT(pT)19 provides the sensitized emission spectrum of 5’-Fl-dT(pT)19 saturated with R14C-CPM. The emission spectrum of 5’-Fl-dT(pT)19 in the complex with R14C-CPM (without energy transfer) and the sensitized emission spectrum of 5’-Fl-dT(pT)19 in the complex with R14C-CPM, are included in Fig. 4a. In the presence of the donor, CPM, the fluorescence intensity of fluorescein at the 5’ end of the bound 20 mer is increased ~8.7-fold.

FIG. 2. Spectral overlap between donor emission and acceptor absorption spectrum in donor-acceptor pairs, studied in this work, in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl, 1 mM AMP-PNP. a, R14C-CPM emission spectrum (---), b, R14C-CPM emission spectrum (- - - -) ($\lambda_{ex} = 425$ nm), absorption spectrum of 5’-Rh-dT(pT)19 (-----), c, R14C-Fl emission spectrum (-----) ($\lambda_{ex} = 485$ nm), absorption spectrum of 5’-Rh-dT(pT)19 (-----).

FIG. 3. Fluorescence emission spectrum of the DnaB variant R14C-CPM in the absence (-----) and presence (-----) of unlabeled dT(pT)19 ($\lambda_{ex} = 425$ nm) in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP. Concentrations of R14C-CPM and the oligomer are $9.6 \times 10^{-7}$ M (hexamer) and $4.5 \times 10^{-7}$ M (oligomer), respectively. Fluorescence emission spectrum of 5’-Fl-dT(pT)19 ($\lambda_{ex} = 485$ nm) in the absence (-----) and presence (-----) of R14C-CPM (without energy transfer) in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP. Concentrations of labeled dT(pT)19 and the protein are $4.5 \times 10^{-7}$ M (oligomer) and $9.6 \times 10^{-7}$ M (hexamer), respectively. Fluorescence emission spectrum of 5’-Rh-dT(pT)19 ($\lambda_{ex} = 555$ nm) in the absence (-----) and presence (-----) of R14C-Fl (without energy transfer) in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP. Concentrations of 5’-Rh-dT(pT)19 and the protein are $4.5 \times 10^{-7}$ M (oligomer) and $9.6 \times 10^{-7}$ M (hexamer), respectively.
Structure of Hexameric Helicase-ssDNA Complex

Figure 4. a, sum of the fluorescence emission spectra (---) of DNA B R14C-CPM in the presence of unlabeled dT(pT)19 (4.5 × 10⁻⁷ M (oligomer)) and 5'-Fl-dT(pT)19 in the presence of R14C-CPM (without energy transfer) (λex = 425 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 with 5'-Fl-dT(pT)19 (λex = 425 nm) (-----) in the same buffer. Concentrations of 5'-Fl-dT(pT)19, and the protein are 4.5 × 10⁻⁷ M (oligomer) and 9.6 × 10⁻⁷ 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 (λex = 425 nm) (-----) is also included. Sensitized emission spectrum of 5'-Fl-dT(pT)19 in the presence of R14C-CPM (without energy transfer) obtained at the same excitation wavelength by multiplying the spectrum of free, labeled 20 mer by the quantum yield ratio $\phi_f/\phi_b = 0.51$, b, sum of the fluorescence emission spectra (-----) of R14C-Fl in the presence of unlabeled dT(pT)19 (4.5 × 10⁻⁷ M (oligomer)) and 5'-Rh-dT(pT)19 in the presence of R14C-Fl (without energy transfer) (λex = 425 nm) in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP and the fluorescence emission spectra of the complex of R14C-Fl with 5'-Rh-dT(pT)19 (λex = 425 nm) (-----) in the same buffer. Concentrations of 5'-Rh-dT(pT)19, and the protein are 4.5 × 10⁻⁷ M (oligomer) and 9.6 × 10⁻⁷ M (hexamer), respectively. The fluorescence emission spectrum of R14C-Fl normalized at 519 nm (peak) to the emission spectrum of the protein in the complex with 5'-Rh-dT(pT)19 (-----) is also included. Sensitized emission spectrum of 5'-Rh-dT(pT)19 in the presence of R14C-Fl (without energy transfer) (λex = 425 nm) in the complex with R14C-Fl (-----), obtained after subtraction of the normalized spectrum of R14C-Fl superimposed on the fluorescence emission spectrum of 5'-Fl-dT(pT)19 in the presence of R14C-CPM (without energy transfer) obtained at the same excitation wavelength by multiplying the spectrum of free, labeled 20 mer by the quantum yield ratio $\phi_f/\phi_b = 0.65$.

due at the 5' end of dT(pT)19, is then described by Equation 4 which provides $E = 0.59 ± 0.04$. Analogous calculations of the fluorescence energy transfer efficiency in the complex of R14C-CPM with 5'-Fl-dT(pT)19 and 5'-Rh-dT(pT)19 provide $E = 0.70 ± 0.04$ and $E = 0.55 ± 0.04$, respectively (Table I).

The large values of the Förster energy transfer efficiencies for all studied donor-acceptor pairs shows that the 5' end of the bound 20 mer, dT(pT)19, is in close proximity to the 12-kDa small domains of the DnaB hexamer. In the first approximation, assuming $\kappa^2 = 2/3$ and using the determined value of $R_{c}(2/3)$ for the studied donor-acceptor pairs (Table I), we obtained the average distances between the CPM residues on the small 12-kDa domains of the DnaB hexamer and fluorescein at the 5' end of the 20 mer $R(2/3) = 45$ Å. Analogous distances between CPM residues and 5'-Rh-dT(pT)19 or R14C-Fl and 5'-Rh-dT(pT)19 are $R(2/3) = 46$ Å and $R(2/3) = 51$ Å, respectively. The range of the possible distance for each single donor-acceptor pair, determined using the Dale et al. (27) analysis, is included in Table I. However, as we pointed out, this uncertainty in the distance estimate would apply if only a single donor-acceptor pair was used for the distance determination. In the case where different donor-acceptor pairs are used to determine the same distance, the different structures of the fluorophores introduce additional randomization of the donor-acceptor orientation not included in the Dale et al. analysis. Determination of a similar distance in the macromolecular system, using different donor-acceptor pairs, indicates the lack of a peculiar orientation of the donor and acceptor dipoles which could significantly affect the estimate of such distances through $\kappa^2$ to the extent suggested by the Dale et al. method (see “Experimental Procedures”). All studied donor-acceptor pairs in this work provide a similar distance between the donor and the acceptor, with the largest difference amounting to ~6 Å, with the average value of 47 Å (Table I). Thus, the data indicate that the obtained average distance between the 5' end of dT(pT)19, bound to the single DNA binding site on one of the DnaB helicase subunits, and the fluorophores, located at a specific site on the 12-kDa domains, is $R = 47 ± 3$ Å.

Fluorescence Energy Transfer from Donors on the 12-kDa Domains of the DnaB Hexamer and the Acceptor Placed in a Different Location along the ssDNA 20 Mer—To obtain further insight about the topology of the ssDNA-DnaB helicase complex, we performed fluorescence energy transfer measurements of the distances between the ring of donors located on the small 12-kDa domains of the DnaB hexamer and the acceptor placed in different positions along the dT(pT)19 oligomer. The ssDNA oligomers used in these studies are depicted in Fig. 1b. The fluorescence emission spectra (λex = 425 nm) of the complexes of R14C-CPM, with ssDNA oligomers, dT(pT)19, each labeled with fluorescein at different locations, at the 5' end, at the positions of 5, 10, 15, and at the 3' end, are shown in Fig. 5a. Recall, fluorescein does not contribute to CPM emission at 476 nm (Fig. 4a). To facilitate a comparison, the spectra have been normalized to the same degree of donor saturation with the protein (ip) and at 476 nm to represent a fraction of the free R14C-CPM emission at 476 nm. The corresponding sensitized emission spectra of fluorescein, located at different positions in the 20 mer, expressed as multiplicity of the corresponding spectrum of the same free, labeled oligomer in the complex with R14C-CPM (without energy transfer), are shown in Fig. 5b. The spectra in Fig. 5a show that quenching of the CPM fluorescence, as a result of the presence of the acceptor on the nucleic acid, occurs in each complex. The quenching of CPM fluorescence is particularly significant in the complexes of R14C-CPM with 5'-Fl-dT(pT)19 and dT(pT)7-Fl-(pT)10, which differ in the location of fluorescein (acceptor) by 5 nucleotide residues. Notice, the quenching of CPM in the case of 5'-Fl-dT(pT)19 and dT(pT)7-Fl-(pT)10 is almost the same, despite the fact that in dT(pT)7-Fl-(pT)10 the acceptor is located five nucleotide residues further from the 5' end of the nucleic acid. Dramatically diminished quenching is already observed in the case of dT(pT)7-Fl-(pT)10, where the acceptor is located by the distance of ten nucleotide residues further from the 5' end of...
sensitized emission shows a very similar trend, with the exception of 100 mM NaCl and 1 mM AMP-PNP. The spectra have been normalized to the same degree of donor (CPM) saturation with the acceptor (fluorescein), located at a different position along the 20 mer, dT(pT)19, in the complex with R14C-CPM (Fig. 5).

It is interesting that the energy transfer efficiency, $E$, which is only 0.11 ± 0.01 for the dT(pT)19-Fl-3' and increases to 0.70 ± 0.04 for 5'-Fl-dT(pT)19 (Table II). The corresponding average distance from the center of mass of the donors to the acceptors is 74 Å for dT(pT)19-Fl-3' and 45 Å for 5'-Fl-dT(pT)19 (see above).

The differences in spectral properties among the complexes indicate that fluorescein, located at the fifth position from the 5' end of the nucleic acid is located at the same, or even at a shorter, distance from the plane of the donor ring as when it is placed at the 5' end of the 20 mer. Recall, the donors, CPM residues located at a specific site on each of the six 12-kDa domains of R14C-CPM, form a ring whose plane is perpendicular to the longer axis of the hexamer (Fig. 1a). The same, or shorter, distance from the donors to fluorescein in dT(pT)3-Fl-(pT)16, would result if the plane of the ring of donors passes the nucleic acid axis around the third or fourth nucleotide residue from the 5' end of the 20 mer.

In such a structure, the fluorescein attached at the 5' end of the nucleic acid would be above the plane of the donor ring and separated from the plane of the ring of donors by 3–4 nucleotide residues, while fluorescein, in the fifth position from the 5' end, leading to the same or a higher energy transfer efficiency. This conclusion is further supported by the fact that the energy transfer efficiency from the donors and the next fluorescein located at position 10 from the 5' end of the 20 mer is 0.22, a dramatically lower value than 0.70 and 0.73 determined for 5'-Fl-dT(pT)19 and dT(pT)3-Fl-(pT)16, although the distance between the fifth and tenth position in the nucleic acid is very similar to the distance between the 5' end and the fifth location in the 20 mer (Fig. 1b, Table II) (see below).

Structure of the Complex of the DnaB Hexamer with ssDNA—There are two fundamentally different models which can describe the complex between the DnaB hexamer, which has a single DNA binding site on one of the protomers, and ssDNA. In the first model, the nucleic acid passes through the inner channel of the protein hexamer. In this model, every base of the bound ssDNA is, at the first approximation, at a similar distance from each protomer of the hexamer. In the second model, the nucleic acid binds to the single DNA binding site located on the outside of one of the DnaB protomers. Thus, in this complex, there are large differences among the distances between the nucleic acid and the protomers of the hexamer (see below).

EM studies show that the cyclic structure of the DnaB hexamer has a diameter of ~140 Å, with the inner channel of the hexamer having a diameter of ~40 Å (10). These dimensions indicate that the distance from the center of the hexamer to the

| Parameter | R14C-CPM | R14C-Fl | 5'-Fl-dT(pT)19 | 5'-Rh-dT(pT)19 | 5'-Rh-dT(pT)19 |
|-----------|----------|---------|----------------|---------------|---------------|
| $r_{lim}$ | 0.25 ± 0.02 | 0.12 ± 0.01 | 0.29 ± 0.02 | 0.34 ± 0.02 | 0.34 ± 0.02 |
| $\phi$    | 0.64     | 0.61    | 0.64 ± 0.04   | 0.61 ± 0.04   | 0.64 ± 0.04   |
| $J$ (m$^{-1}$ cm$^3$) | 1.96 × 10$^{-13}$ | 1.1 × 10$^{-13}$ | 2.7 × 10$^{-13}$ | 2.7 × 10$^{-13}$ | 2.7 × 10$^{-13}$ |
| $E_D$     | 0.76 ± 0.04 | 0.7 ± 0.04 | 0.57 ± 0.04   | 0.55 ± 0.04   | 0.55 ± 0.04   |
| $E_A$     | 0.57 ± 0.04 | 0.57 ± 0.04 | 0.55 ± 0.04   | 0.55 ± 0.04   | 0.55 ± 0.04   |
| $E$       | 0.7 ± 0.04 | 0.7 ± 0.04 | 0.55 ± 0.04   | 0.55 ± 0.04   | 0.55 ± 0.04   |
| $\kappa_{min}$ | 0.12     | 0.11    | 0.11 ± 0.04   | 0.22 ± 0.04   | 0.22 ± 0.04   |
| $R_c$ (2/3) (Å) | 3.1       | 3.2     | 2.4           | 2.4           | 2.4           |
| $R_c$ (2/3) (Å) | 45        | 46      | 51            | 51            | 51            |
| $\Delta R$ (Å) | 34–58     | 34–59   | 45–67         | 45–67         | 45–67         |

$^a$ Complex with R14C-CPM ($\lambda_{ex} = 425$ nm).

$^b$ Complex with R14C-CPM ($\lambda_{ex} = 425$ nm).

$^c$ Complex with R14C-Fl ($\lambda_{ex} = 485$ nm).
First, we consider the situation in which the acceptor on a nucleic acid is located in the plane of the donor ring on the hexamer. In such an arrangement, there is the shortest possible average distance between the donors and the acceptor in both considered fundamental models of the hexamer-ssDNA complex mentioned above. The simplified geometry of both models, with the acceptor located in the center of the hexamer and with the acceptor on the outside of one of the protein protomers, is depicted in Figs. 6a and 6b. The cyclic DnaB hexamer is approximated by the hexagon with the distance from the outside surface to the center of the hexamer, $R = 70 \text{ Å}$ (10). The distance between the donors and the center of the hexamer is designated as $q$. Because, in our experimental system, six donors transfer energy to a single acceptor, the average energy transfer efficiency, $E$, is described by Equation 7 (see “Experimental Procedures”). As we pointed out in the arrangement where the nucleic acid passes through the inner channel of the hexamer, the acceptor, located on the nucleic acid, is at a similar distance, $R$, from each donor in the donor ring. Therefore Equation 7 can be approximated by

$$E = \frac{R^6}{R^2 + R^6}$$  \hspace{1cm} (Eq. 8)

It should be pointed out, that with the acceptor located in the same plane as the donor ring, $R = q$.

For the model where the acceptor is located on the outside of one of the protomers, the distances between the donors and the acceptor are different, and the average energy transfer efficiency is defined by Equation 7, which for this particular case is

$$E = \frac{1}{6} \left[ \frac{R_1^6}{R_1^2 + R_2^2} + \frac{R_2^6}{R_1^2 + R_2^2} + \frac{R_3^6}{R_1^2 + R_2^2} + \frac{R_4^6}{R_1^2 + R_2^2} + \frac{R_5^6}{R_1^2 + R_2^2} + \frac{R_6^6}{R_1^2 + R_2^2} \right]$$  \hspace{1cm} (Eq. 9)

where $R_1, R_2, R_3, R_4, R_5,$ and $R_6$ are distances between a given donor and the acceptor defined as

$$R_1 = p$$  \hspace{1cm} (Eq. 10a)

$$R_2 = d$$  \hspace{1cm} (Eq. 10b)

$$R_3 = q + b$$  \hspace{1cm} (Eq. 10c)

$$R_4 = R_2$$  \hspace{1cm} (Eq. 10d)

$$R_5 = R_1$$  \hspace{1cm} (Eq. 10e)

$$R_6 = (b - q)$$  \hspace{1cm} (Eq. 10f)

where $p = (q/2)(3^{0.5}/\sin y)$, $y = 60 - \arctg((b - q)/(q + b))3^{0.5}$; $d = (q/2)(3^{0.5}/\sin \delta)$; $\delta = 30 - \arctg((b - q)/(q + b))3^{-0.5}$ (see Fig. 6c).

| Table II |
| --- |
| **Fluorescence energy transfer parameters for R14C-CPM complexes with dT(pT)19 oligomer labeled at different locations with fluorescein (Fig. 1b), in buffer T2 (pH 8.1, 10 °C) containing 100 mM NaCl and 1 mM AMP-PNP** |

| Parameter | R14C-CPM | 5'-Fl-dT(pT)19 | dT(pT)3-Fl-(dT)16 | dT(pT)8-Fl-(dT)10 | dT(pT)15-Fl-(dT)4 | dT(pT)19-Fl-3' |
| --- | --- | --- | --- | --- | --- | --- |
| $\phi$ | 0.64 | | | | | |
| $r_{\text{lim}}$ ($\text{Å}$) | 0.25 ± 0.02 | 0.29 ± 0.02 | 0.34 ± 0.02 | 0.3 ± 0.02 | 0.25 ± 0.02 | |
| $D$ ($\text{M}^{-1} \text{ cm}^2$) | 3.1 | 3.1 | 3.2 | 3.1 | 2.9 | |
| $\kappa_{\text{min}}$ | 0.12 | 0.12 | 0.12 | 0.12 | 0.14 | |
| $\kappa_{\text{max}}$ | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | |
| $E_D$ | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | |
| $E_A$ | 0.7 ± 0.04 | 0.73 ± 0.04 | 0.22 ± 0.02 | 0.14 ± 0.01 | 0.11 ± 0.01 | |
| $R_6$ ($2/3)$ (Å) | 52 | 52 | 52 | 52 | 52 | |
| $R'$ ($2/3)$ (Å) | 45 | 44 | 64 | 70 | 74 | |
| $\Delta R$ (Å) | 34–58 | 33–58 | 47–82 | 53–91 | 57–93 | |
As we pointed out, the dimensions of the DnaB hexamer and the Förster critical distance of the used donor-acceptor pairs impose the first constraints on the possible distances between the donors and acceptors and the possible values of the energy transfer efficiencies. The next constraint comes from the short length (20 nucleotides long) of the ssDNA used in our experiments. Independent measurements of the structure of the 20 mer in the complex with the DnaB helicase indicate that the length of the bound dT(pT)_{19} is ~60 Å (data not shown), thus, close to the length of one strand of dsDNA 20 base pairs long in the B form (70 Å) (33). In the case where the ss nucleic acid passes through the inner channel of the hexamer, or is bound on the outside of the protein, the nucleic acid is symbolized by a straight, thin ribbon passing through the inner channel of the hexagon.  

Notice, because the diameter of the DnaB hexamer is ~140 Å, the distance from the donors to the center of the hexamer, q, cannot be larger than ~70 Å. The theoretical dependence of the average fluorescence energy transfer efficiency, E, as a function of the distance between the donors and the center of the hexamer, q, for both considered models of the DnaB hexamer-ssDNA complex, are shown in Fig. 7, a and b. The $R_0$, selected in these simulations is 50 Å, corresponding to the $R_0$ of the studied donor-acceptor pairs (Table I). In the case where the acceptor location is not limited to the plane of the donor ring, upon the distance between the donors and the center of the hexamer (see above). It is evident that, for a cyclic hexamer, with the radius of the hexamer ~70 Å, as in case of the DnaB hexamer, and, with the critical Förster distance $R_0 = 50$ Å, the average energy transfer efficiency cannot reach the value of 0.70–0.73, which is experimentally observed for the DnaB-5'-Fl-dT(pT)_{19} and DnaB- dT(pT)_{19}-Fl-pT_{16} complexes, if the acceptor is located on the outside of one of the hexamer subunits. Only in the case where the radius of the cyclic hexagonal structure of the hexamer is reduced to the physically unrealistic value of <40 Å, corresponding to the diameter of the hexamer ~80 Å, ~two times smaller than the value determined for the DnaB helicase, and with q = 20 Å, i.e. all donors are located on the inside surface of the inner channel of the hexamer, is the energy transfer efficiency reaching the experimentally observed values of Å 0.7–0.75 (Fig. 7b).

Thus, the high values of the energy transfer efficiencies in the DnaB-5'-Fl-dT(pT)_{19} and DnaB-dT(pT)_{19}-Fl-pT_{16} complexes, and the analysis described above, provide the first strong indication that, in the complex of the DnaB hexamer with ssDNA, the nucleic acid passes through the inner channel of the protein hexamer.

We now extend our analysis to a more complex situation where a single acceptor is located in any position along a nucleic acid lattice bound to the hexamer. In other words, the acceptor location is not limited to the plane of the donor ring, but can be placed above, in, and below this plane. Such a situation corresponds to the fluorescence energy transfer process in the complexes of R14C-CPM with dT(pT)_{19} labeled with fluorescein at different locations along the nucleic acid. The approximate geometrical representations of the donor-acceptor system, according to the model in which the ssDNA passes through the inner channel of the hexamer, or is bound on the outside of the protein, are illustrated in Fig. 8, a–d. The subunits of the DnaB hexamer are represented by six spheres forming a hexagon. Six donors, located at the same specific site on each subunit, are at the same distance, q, from the center of the hexamer and arranged in a ring formed by the subunits. The nucleic acid is symbolized by a straight, thin ribbon passing through the inner channel of the hexagon or attached to the outside of one of the protomers. In both models, the 5' end of the nucleic acid is above the plane of the donor ring. The distance between the acceptor placed in an arbitrary location on the ss nucleic acid, with respect to the plane of the donor ring, is designated as x. The nucleic acid is symbolized by a straight, thin ribbon passing through the inner channel of the hexamer or attached to the outside of one of the protomers.
equals \( R \), and the average energy transfer efficiency is described by Equation 8. The theoretical dependence of the average fluorescence energy transfer efficiency, \( E \), as a function of the average distance from the acceptor to the plane of the donor ring is shown in Fig. 9a. In these calculations, we selected a distance of 10 Å (\( \sim 3-4 \) nucleotide residues) for the length of the fragment of nucleic acid protruding above the plane of the donor ring, similar to the distance indicated by our fluorescence energy transfer data (see above). The plots are partially superimposed as a result of the fact that for the same average distance of the acceptor to the donors, the transfer efficiency is the same for this geometry of the hexamer-ssDNA complex. However, for different values of \( q \), the plots span different regions of the average distance, \( R \). Thus, for \( q = 70 \) Å the maximum value of the energy transfer efficiency is only \( \sim 0.2 \), and \( R \) spans the region between 70 and 100 Å. As the donors approach the center of the hexamer, the value of \( E \) increases, reaching the value of 1 for \( q < 20 \) Å. The analogous dependence of the average energy transfer efficiency, \( E \), upon the distance of the acceptor from the plane of the donor circle, \( x \), for different values of \( q \), is shown in Fig. 9b. Recall, in this case, \( x \) spans the distance of 70 Å which is the selected length of the ss nucleic acid for all values of \( q \) in these simulations. However, the transfer efficiency is higher as the distance between the donors and the center of the hexamer decreases. The value of \( x = 0 \) corresponds to the point where the ssDNA crosses the plane of the donor ring.

The schematic arrangement of the donors and the ssDNA, when the nucleic acid binds on the outside of one of the protomers, is presented in Fig. 8, c and d. As in the previously considered model, the six donors are located at the same specific site on each subunit of the helicase at the same distance, \( q \), from the center of the hexamer. The nucleic acid, symbolized by a straight, thin ribbon, is now tangent to the outside surface of one of the helicase subunits. The geometry of this model is more complex than the one previously considered. Inspection of Fig. 8, c and d, shows that the distance between the acceptor on the nucleic acid and the donors varies significantly for each particular donor. For the model of the DnaB helicase-ssDNA complex, in which the nucleic acid binds on the outside of the hexamer, the average energy transfer efficiency is defined by Equation 7 and the average distance between the acceptor and the donors is defined as

\[
R_m = \frac{1}{6} \sum R_i \tag{Eq. 11}
\]

However, \( R_1, R_2, R_3, R_4, R_5, \) and \( R_6 \), the distances between a given donor and acceptor located on the ssDNA, are now defined as

\[
R_1 = (x^2 + p^2)^{0.5} \tag{Eq. 12a}
\]

\[
R_2 = (x^2 + b^2)^{0.5} \tag{Eq. 12b}
\]

\[
R_3 = [x^2 + (q + b)^2]^{0.5} \tag{Eq. 12c}
\]

\[
R_4 = R_2 \tag{Eq. 12d}
\]

\[
R_5 = R_1 \tag{Eq. 12e}
\]

\[
R_6 = [x^2 + (b - q)^2]^{0.5} \tag{Eq. 12f}
\]

where \( p = (q/2)(3^{0.5}/\sin \gamma) \), \( \gamma = 60 - \arctg[(b - q)/(q + b)]3^{0.5} \); \( d = (q/2)(3^{0.5}/\sin \delta) \); \( \delta = 30 - \arctg[(b - q)/(q + b)]3^{-0.5} \). The
**Structure of Hexameric Helicase-ssDNA Complex**

Fig. 9. *a*, theoretical dependence of the average fluorescence energy transfer efficiency from a ring of six donors, located at the same site on each of the protomers of a hexamer, to a single acceptor located along the ss nucleic acid bound in the inner channel of the hexamer, upon the average distance of the acceptor from the plane of the ring of donors, $R$, for a different distance of the donors from the center of the hexamer, $q$ (Fig. 8a). The maximum distance from the outside surface of the hexamer to its center, $b$, and the selected length of the nucleic acid, $x$, are 70 Å. The plane of the ring of donors passes the axis of the nucleic acid at a distance of 10 Å from its 5’ end (Fig. 8a). The Förster distance for the donor-acceptor pair is 50 Å: ( ) $q = 70$ Å; ( ) $q = 50$ Å; ( ) $q = 35$ Å; ( ) $q = 20$ Å; ( ) $q = 10$ Å. *b*, theoretical dependence of the average fluorescence energy transfer efficiency from a ring of six donors, located at the same site on each of the protomers of a hexamer, to a single acceptor, located along the ss nucleic acid lattice bound in the inner channel of the hexamer, upon the distance of the acceptor from the plane of the ring of donors, $x$, for different distances of the donors to the center of the hexamer, $q$ (Fig. 8a). All parameters and symbols are the same as in Fig. 9a above.

Fig. 10. *a*, theoretical dependence of the average fluorescence energy transfer efficiency from a ring of six donors, located at the same site on each of the protomers of a hexamer, to a single acceptor, located along the ss nucleic acid bound to the outside of one of protomers, upon the average distance of the acceptor from the plane of the ring of donors, $R$, for a different distance of the donors from the center of the hexamer, $q$ (Fig. 8c). The distance from the outside surface of the hexamer to its center and the selected length of the nucleic acid are 70 Å. The plane of the ring of donors passes the axis of the nucleic acid at a distance of 10 Å from its 5’ end (Fig. 8c). The Förster distance for the donor-acceptor pair is 50 Å: ( ) $q = 70$ Å; ( ) $q = 50$ Å; ( ) $q = 35$ Å; ( ) $q = 20$ Å; ( ) $q = 10$ Å. *b*, theoretical dependence of the average fluorescence energy transfer efficiency from a ring of six donors, located at the same site on each of the protomers of a hexamer, to a single acceptor, located along the ss nucleic acid lattice bound to the outside of one of the protomers, upon the average distance of the acceptor from the plane of the ring of donors, $x$, for different distances of the donors to the center of the hexamer, $q$ (Fig. 8c). ( ) $q = 70$ Å; ( ) $q = 50$ Å; ( ) $q = 35$ Å; ( ) $q = 20$ Å; ( ) $q = 10$ Å. All parameters are the same as in *a* above.

Theoretical dependence of the average fluorescence energy transfer efficiency, $E$, upon the average distance of the acceptor from the donors, for a different distance of the donors from the center of the hexamer, $q$, is shown in Fig. 10a, with $b = 70$ Å and $R \alpha = 50$ Å. Because of the complex relationship between the $R \alpha$ and the particular donor-acceptor distance $R$, the plots are not superimposed, contrary to the model where the ssDNA passes through the inner channel of the hexamer. In other words, the same average distance is not accompanied by the same average energy transfer efficiency. Notice, that independent of the distance between the donors and the center of the hexamer, the average energy transfer efficiency never exceeds the value of ~0.25 (Fig. 10a). This dramatically low value of $E$, as compared with the energy transfer efficiency obtained for the model in which ssDNA passes through the inner channel of the hexamer, results from the fact that the average distance between the donors and the acceptor is always significantly larger than the $R \alpha$ (50 Å) of the donor-acceptor pair for any value of $q$. The analogous dependence of the average energy transfer efficiency, $E$, upon the distance of the acceptor from the plane of the donor circle, $x$, for different values of $q$, is shown in Fig. 10b.

The computer simulations described above have been performed using dimensions of the hexamer and the nucleic acid corresponding to the dimensions of the DnaB hexamer and the ssDNA oligomer forming the complex in our fluorescence energy transfer studies. Clearly, the two fundamentally different models of the DnaB helicase-ssDNA complex differ dramatically in the value of the average fluorescence energy transfer efficiency for an arbitrary distance of the donors from the center of the DnaB hexamer, $q$. Moreover, the size of these differences well exceeds the errors due to approximations applied in the analysis (see “Discussion”).

The dependence of the experimentally determined fluores-
Structure of Hexameric Helicase-ssDNA Complex

FIG. 11. The dependence of the average fluorescence energy transfer from six CPM residues of R14C-CPM located on the small 12-kDa domains of the DnaB hexamer to the fluorescein placed at different locations of the 20 mer dt(pT)_{19} (Fig. 1b). Solid line is the computer fit of the average fluorescence energy transfer efficiency from a ring of six donors, with each donor located at the same site on a single protomer of a hexamer, to a single acceptor, located along the ssDNA 20 mer bound in the inner channel of the hexamer, upon the average distance of the acceptor from the plane of the donor ring. The distance from the outside surface of the hexamer to its center b = 70 Å, the length of the nucleic acid is 60 Å and the Förster distance for the CPM-fluorophores donor-acceptor pair is R_{0} = 52 Å. The fitted distance from the donor to the center of the hexamer is q = 44 Å. The plane of the ring of CPM residues passes the axis of the nucleic acid at a distance of 10 Å from its 5’ end, as indicated by the fluorescence energy transfer measurements of the R14C-CPM complexes with 5’-Fl-dT(pT)_{19} and dT(pT)_{3}-Fl-(pT)_{19}.

cence energy transfer efficiency upon the corresponding average distance of fluorescein, placed in different locations along the 20 mer, from the CPM residues, located on the small 12-kDa domains of the DnaB helicase, is shown in Fig. 11. The maximum of the observed E is ~0.73, thus, it is much higher than predicted by the model in which the ssDNA binds on the outside of the hexamer for any physically realistic distance of the CPM residues from the center of the DnaB hexamer. Only the model in which the nucleic acid passes through the inner channel of the hexamer can describe the experimental data. Because we know the diameter of the DnaB hexamer (140 Å) and the length of the ssDNA in the complex with the protein (~60 Å), we can approximately estimate the distance of the donors (CPMs) from the center of the DnaB hexamer. The solid line in Fig. 11 is the computer fit of the experimentally determined fluorescence energy transfer efficiency, as a function of the corresponding average distance of fluorescein from the ring of CPM residues, using b = 70 Å, x = 60 Å, and with the plane of the ring of CPMs passing the axis of the nucleic acid at 10 Å (3–4 nucleotide residues) from the 5’ end of the bound 20 mer. The model fits all experimental data points remarkably well, with the value of q = 44 Å. Thus, the data indicate that the CPM residues are at ~44 Å from the center of the inner channel. Comparison with the diameter of the inner channel, ~40 Å (10), indicates that the CPM residues are located ~24 Å from the surfaces of the DnaB protomers which form the inner channel of the hexamer.

The obtained results strongly indicate that, in the complex with the DnaB protein, the ssDNA passes through the inner channel of the protein hexamer. Our fluorescence energy transfer data indicate, for the very first time, the existence of such a hexameric helicase-ssDNA complex in solution.

Complex of the DnaB Helicase with the 5’ Arm of the Replication Fork—The DnaB helicase binds preferentially to the 5’ arm of the replication fork in a single orientation in which the small 12-kDa domains of the hexamer face the 5’ end of the arm and the large 33-kDa domains contain the entry site for the duplex part of the fork (14). The fluorescence energy transfer studies of the structure of the DnaB hexamer complex with the 5’ arm of the replication fork have been performed in an analogous way, as described above for the DnaB-labeled 20 mer complexes. In these experiments, we used R14C-CPM and the 5’ single-arm fork substrate with fluorescein at the 5’ end of the arm at the 3’ end in the duplex part of the fork, as depicted in Fig. 1c. The obtained values of the energy transfer efficiencies are included in Table III.

The Förster fluorescence transfer efficiency from CPM, located on the small 12-kDa domain to the fluorescein residue at the 5’ end of the arm of the fork substrate, is E = 0.65 ± 0.04. This value of E is very similar to the one determined for the complex with the 5’ Fl-dT(pT)_{19} ssDNA oligomer (Table II). Using R_{0} = 52 Å for this donor-acceptor pair, the obtained distance from fluorescein at the 5’ end of the arm of the fork to the CPM residues on the 12-kDa domains is 47 Å. Thus, independently of the presence of the duplex part of the fork, the helicase forms the complex with the 5’ arm in which the 5’ end of the arm is at a very similar distance from the small domains of the hexamer, as in the complex with the 20 mer. In other words, the 5’ arm occupies the same binding site and in the same way as the ssDNA 20 mer inside the inner channel of the helicase hexamer.

Quantitatively, very different behavior is observed in the case of the complex of R14C-CPM with a 5’ single-arm fork where fluorescein is located at the opposite 3’ end of the substrate on the same 30 mer (Fig. 1c; Table III). The Förster energy transfer efficiency, E = 0.04 ± 0.01, is dramatically diminished as compared with the complex where fluorescein is located at the 5’ end of the arm. Also, notice that this value of E is significantly lower than E = 0.11 ± 0.01 determined for the complex with dT(pT)_{19}-3’-Fl. The value of E = 0.04 ± 0.01 indicates that the average distance between the ring of donors on the small 12-kDa domains of the hexamer to the 3’ end of the duplex part of the replication fork substrate is ~88 Å, as compared with 74 Å determined for the 3’ end of the bound ssDNA 20 mer.

Table III

| Parameter                  | R14C-CPM | 5’ single-arm fork with fluorescein at the 5’ end of the arm | 5’ single-arm fork with fluorescein at the 3’ end of the duplex part |
|----------------------------|----------|-------------------------------------------------------------|---------------------------------------------------------------|
| r_{0} (Å)                  | 0.25 ± 0.02 | 0.29 ± 0.02                                                 | 0.21 ± 0.02                                                 |
| r (Å)                      | 0.64 ± 0.03 |                                                            |                                                             |
| J (M^{-1} cm^{2})          | 1.95 × 10^{-13} | 1.95 × 10^{-13}                                           |                                                             |
| E₀                         | 0.73 ± 0.04 | 0.4 ± 0.01                                                  | 0.4 ± 0.01                                                  |
| Eₐ                         | 0.50 ± 0.04 | 0.4 ± 0.01                                                  | 0.4 ± 0.01                                                  |
| E                          | 0.65 ± 0.04 | 0.4 ± 0.01                                                  | 0.4 ± 0.01                                                  |
| kₐ                         | 0.12 | 1.15                                                     |                                                             |
| kₐₗₘₚₗ₉             | 3.08 | 2.5                                                      |                                                             |
| R₀ (2/3) (Å)               | 52 | 52                                                       |                                                             |
| R (2/3) (Å)                | 47 | 88                                                       |                                                             |
| ΔR (Å)                     | 35–60 | 70–112                                                    |                                                             |

Notes: a λ_{ex} = 435 nm. b λ_{ex} = 485 nm.

DISCUSSION

Elucidation of fundamental aspects of the structure of a hexameric helicase complex with ssDNA in solution is of paramount importance for understanding the mechanism of the
enzyme activities. The hexamer of the E. coli primary replicative helicase DnaB protein forms a ringlike structure built of six chemically identical subunits (7). Quantitative thermodynamic measurements show that, in the complex with ssDNA, the DnaB hexamer occludes only 20 ± 3 nucleotide residues (9, 12, 22). Moreover, photo-cross-linking studies indicate that predominately, if not only, a single subunit of the hexamer is involved in interactions with the nucleic acid in a stationary complex. These studies show that the ssDNA does not wrap around the hexamer; however, they are consistent with two fundamentally different modes of DNA binding to the enzyme. In the first mode, the nucleic acid could bind on the outside of one of the hexamer protomers. Such a mode of binding has been proposed for the SV40 large tumor antigen, hexameric helicase (17). In the other mode, the DNA binds to one of the protomers while crossing the inner channel of the cyclic hexamer. This mode of binding was indicated for the phage T7 helicase/primase and E. coli RuvB proteins (15, 16). The inner channel of the DnaB protein has a diameter of ~40 Å which can accommodate a single ssDNA strand (10, 11).

A striking result of the fluorescence energy transfer measurements described in this work is the very high fluorescence energy transfer efficiency (E = 0.70–0.73) from the ring of six donors, all located on the small 12-kDa domains of the hexamer, to an acceptor placed at the 5’ end of the bound 20 mer, which encompasses the entire binding site of the enzyme. The value of E = 0.70 shows that 70% of the total absorbed energy by the donors is transferred to the acceptor. This can be possible if each donor transfers 70% of its absorbed energy to the acceptor, or if ~4.2 CPM residues are completely quenched by the acceptor, while ~1.8 CPM residues do not transfer their energy at all. The later option is physically very unlikely, in light of the fact that the ssDNA is bound to a single protomer and, moreover, each donor in the donor ring is located on an independent protomer of the large cyclic hexamer whose diameter is ~140 Å.

The shortest distance between the acceptor and donors in the donor ring would occur if the acceptor is located in the plane of the donor ring (Figs. 6 and 7). However, theoretical analysis of the energy transfer process for two possible modes of ssDNA binding to the DnaB hexamer shows that the fluorescence energy transfer efficiency from the donors to the acceptor cannot exceed the value of ~0.25 (b = 70 Å, Ro = 50 Å) for the mode in which the ssDNA is bound on the outside of one of the protomers. Thus, even in the case where the acceptor is placed in the plane of the donor ring, the value of the energy transfer efficiency is dramatically lower than the experimentally observed 0.7–0.73. Only when the acceptor is placed in the center of the hexamer is the energy transfer efficiency reaching the experimentally observed values. These results clearly show that the observed high energy transfer efficiency can occur only if the acceptor is at a similar distance from each of the donors in the donor ring, i.e. when the ssDNA is bound in the inner channel of the hexamer.

Fluorescence energy transfer experiments, with the acceptor located along the ss nucleic acid lattice, fully support the model of the hexamer-ssDNA complex in which DNA passes through the inner channel of the hexamer and provide additional information on the structure of the complex. The energy transfer efficiency, in the case of 5’-Fl-dT(pT)15 (E = 0.7), is slightly lower, as compared with dT(pT)15-Fl-(pT)15 (E = 0.73), where fluorescein is located five nucleotide residues from the 5’ end. Although this difference is still within experimental error, we have systematically obtained a slightly higher energy transfer efficiency for the complex with dT(pT)15-Fl-(pT)15, as compared with 5’-Fl-dT(pT)15. This very similar, or even higher, E value indicates that the plane of the ring of donors passes the nucleic acid axis around the 3rd–4th nucleotide from the 5’ end, resulting in a shorter distance and a higher E from the ring of donors to fluorescein in dT(pT)15-Fl-(pT)15 than to fluorescein at the 5’ end of the 20 mer. Thus, the results indicate that, in the complex, the 5’ end of the bound ssDNA oligomer protrudes approximately 3–4 nucleotide residues (~10 Å) above the plane of the donor ring. On the other hand, shifting the location of the acceptor by the same distance of the next 5 nucleotide residues from the 5’ end of the nucleic acid results in a large ~3-fold drop of the energy transfer efficiency to E = 0.22. Theoretical analysis of the energy transfer process indicates that such a large drop is predicted by the model in which ssDNA binds in the inner channel of the hexamer, but not by the outside binding mode (Figs. 9 and 10).

The experiments, using different donor-acceptor pairs, show that the distance from the acceptor located at the 5’ end of the ssDNA bound in the inner channel of the hexamer to the donor located on the 12-kDa domain of each of the DnaB protomers is 47 ± 3 Å. Because the experiments with 5’-Fl-dT(pT)19 and dT(pT)15-Fl-(pT)15 indicate that the 5’ end of the ssDNA protrudes ~10 Å above the plane of the donor ring, simple calculations (see Fig. 8a) show that each donor in the ring is at an average distance of q ~ 46 Å from the ssDNA bound in the inner channel. This result is in excellent agreement with the computer fit of the experimentally obtained dependence of the average energy transfer efficiency upon the average distance between the donor ring and the acceptor located along the nucleic acid which provides q = 44 Å, using the known diameter of the DnaB hexamer (140 Å) and the determined Förster critical distance for the CPM-fluorescein system, R0 = 52 Å (Table I). The value of q = 44–46 Å shows that the diameter of the donor ring is ~90 Å, thus, much larger than 40 Å, the diameter of the inner channel of the hexamer, indicating that the donors are located at a distance of ~25 Å from the surface of the inner channel of the hexamer.

The distance from the donor ring to the acceptor at the 3’ end of the bound 20 mer is 74 Å (Fig. 11, Table II). Notice, this distance does not include a fragment of ~10 Å of the nucleic acid and acceptor (fluorescein) at the 5’ end of the ssDNA which protrudes above the plane of the donor ring. Knowing that the ssDNA is bound in the inner channel of the hexamer, we can estimate (see Fig. 8a) the distance from the plane of the donors to the acceptor at the 3’ end of the nucleic acid, i.e. the length of the nucleic acid fragment, including the acceptor, which is 58 Å. Thus, the total length of the bound 20 mer, including the fluorescein residues at the 5’ and 3’ ends, is ~68 Å. Because the length of the fluorescein residues is comparable with the size of the nucleic base, which is approximately 3 Å, the length of the 20 mer in the complex with the DnaB helicase, without fluorescein residues, is ~62 Å. This estimate is in agreement with the independent measurement of the length of the 20 mer, using the nucleic acid labeled simultaneously with a donor and an acceptor.4 As we pointed out, the length of 62 Å is only ~11% shorter than the corresponding length of the 70 Å of one strand of dsDNA in the B form.

In our analysis of the fluorescence energy transfer data, we used the diameter of the ringlike structure of the DnaB hexamer ~140 Å which was determined in EM studies (10). It is still possible that, due to some “flattening” of the protein hexamer in EM experiments, this diameter is slightly overestimated. Thus, using our hydrodynamic data we can estimate the lower limit of the DnaB hexamer diameter as ~111 Å (7, 13).

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which would correspond to the distance of ~56 Å from the outside surface of the hexamer to its center. However, the analysis of the energy transfer process using a diameter of 56 Å for the DnaB hexamer clearly shows that the energy transfer efficiency cannot exceed the maximum value of ~0.38, for the outside binding mode of the ssDNA, even if the acceptor is assumed to be placed in the plane of the donor ring and all donors are assumed to be on the inside surface of the inner channel of the hexamer, i.e. \( q = 20 \) Å (Fig. 7b). It is evident that even in this most favorable arrangement, which gives the highest possible energy transfer efficiency for the outside binding mode, the value of \( E = 0.38 \) is much lower than the experimentally obtained 0.59 or 0.7–0.73 for the R14C-CPM complexes with the 20 mers labeled at the 5' end with different fluorescent markers (Table I).

The acceptor, located at the 5' end of the 5' arm of the replication fork substrate, is at a distance of ~47 Å from the donors located on the small 12-kDa domains of the DnaB hexamer. This distance is, within experimental accuracy, the same as the distance between the donors and the 5' end of the bound 20 mer (Tables I and III). The results indicate that both the isolated 20 mer, as well as the 5' arm of the replication fork, occupy not only the same binding site, but are also bound in the same way within the binding site which encompasses 20 ± 3 nucleotide residues (12). Thus, the 5' arm has a structure very similar in length to the single strand of a dsDNA, when associated with the helicase, and occupies most of the binding site. At this point, it should be noticed that the fluorescence energy transfer efficiency in the case of the complex of R14C-CPM with the 5' single-arm fork, where fluorescein is located at the 3' end in the duplex part of the fork, is only 0.04 ± 0.01. This value is significantly lower than 0.11 ± 0.01 obtained for the 20 mer with fluorescein located at its 3' end. Altogether, these results indicate that the enzyme, when bound to the 5' arm of the fork, does not invade the duplex part of the fork by more than 2–3 base pairs in its stationary complex, i.e. without ATP hydrolysis. This conclusion is corroborated by the fact that thermodynamic analysis of the helicase binding to different fork substrates did not show any substantial contribution of the duplex part of the fork to the free energy of binding of the helicase (14).

Based on our results, a model of the hexameric DnaB helicase, in the complex with a replication fork, is depicted in Fig. 12. The thermodynamic data show that the helicase is preferentially bound to the 5' arm of the fork, while the 3' arm is protruding in front of the enzyme (14). The DnaB is oriented toward the duplex part of the fork with the large domains of its six protomers, while the 5' arm is protruding in front of the enzyme (14). The small 12-kDa domains of the DnaB hexamer are in close proximity to the 5' end of the 5' arm, with the 5' arm of the fork passing through the inner channel of the hexamer. The binding site which encompasses 20 ± 3 nucleotide residues is shown as a groove in the protomer engaged in the interactions with DNA. For clarity, only contours of two protomers at the front of the hexamer are shown. The helicase is oriented toward the duplex part of the fork with the large domains of its six protomers. In this stationary complex, the enzyme is predominantly bound to the arm of the fork with possibly only 2–3 base pairs of the duplex part of the fork involved in the binding site. The arrow indicates the direction of translocation of the enzyme along the DNA lattice and the unwinding reaction.

The E. coli DnaB protein in solution forms a very stable hexamer (7). In fact, this property distinguishes the DnaB helicase from other well studied hexameric helicases that exist in solution as a mixture of different oligomeric forms and assemble into a hexamer when bound to DNA, or in the complex with nucleotide cofactors (7, 34). Thus, the simplest mechanism of ssDNA binding in the inner channel of a hexameric helicase, which exists in solution as a mixture of different oligomeric forms, would include assembling the enzyme into its hexameric structure around the nucleic acid. In the case of a stable hexamer, such as the DnaB protein, this simple model does not apply. Moreover, contrary to a recent suggestion (35), our data clearly show that the DnaB hexamer binds polymer, oligomer ssDNA, and DNA substrates resembling the replication fork in the presence of the ATP nonhydrolyzable analog AMP-PNP, i.e. without ATP hydrolysis which could be used “to open” the hexamer (8, 9, 12–14).

Although the exact mechanism of the DnaB helicase binding to a ssDNA is still unknown, the fact that the DNA is bound in the inner channel of the DnaB hexamer suggests that this mechanism must include a conformational change which leads to a transient opening of the hexamer, in the absence of ATP hydrolysis. On the basis of what is already known about the E. coli DnaB hexamer, the following possibilities can be pointed out. Our quantitative studies of the oligomeric structure of the DnaB protein have shown that the stability of the hexamer is controlled by specific binding of multiple magnesium cations (7). Therefore, it is possible that the transient release of Mg²⁺ from one or two protomers, at the initial stages of nucleic acid binding, induces local destabilization of one of the subunit interfaces and allows the nucleic acid to enter the inner channel. On the other hand, thermodynamic and EM studies have shown that the DnaB hexamer undergoes dramatic global conformational changes upon binding of the nucleotide cofactors (7, 11). These conformational changes, which involve large reorientations of the hexamer subunits and are controlled by nucleotide binding and release, could play a role in facilitating the entry of the ssDNA into the inner channel of the hexamer. It is also possible that coordinated binding and release of magnesium cations and nucleotide cofactors may be simultaneously involved in the “opening” of the DnaB hexamer. The mechanism of the nucleic acid binding to the DnaB heli-
case hexamer is currently being examined in our laboratory.

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