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The *Escherichia coli* PriA Helicase–Double-Stranded DNA Complex: Location of the Strong DNA-Binding Subsite on the Helicase Domain of the Protein and the Affinity Control by the Two Nucleotide-Binding Sites of the Enzyme

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Abstract

The *Escherichia coli* PriA helicase complex with the double-stranded DNA (dsDNA), the location of the strong DNA-binding subsite, and the effect of the nucleotide cofactors, bound to the strong and weak nucleotide-binding site of the enzyme on the dsDNA affinity, have been analyzed using the fluorescence titration, analytical ultracentrifugation, and photo-cross-linking techniques. The total site size of the PriA–dsDNA complex is only 5 ± 1 bp, that is, dramatically lower than 20 ± 3 nucleotides occluded in the enzyme–single-stranded DNA (ssDNA) complex. The helicase associates with the dsDNA using its strong ssDNA-binding subsite in an orientation very different from the complex with the ssDNA. The strong DNA-binding subsite of the enzyme is located on the helicase domain of the PriA protein. The dsDNA intrinsic affinity is considerably higher than the ssDNA affinity and the binding process is accompanied by a significant positive cooperativity. Association of cofactors with strong and weak nucleotide-binding sites of the protein profoundly affects the intrinsic affinity and the cooperativity, without affecting the stoichiometry. ATP analog binding to either site diminishes the intrinsic affinity but preserves the cooperativity. ADP binding to the strong site leads to a dramatic increase of the cooperativity and only slightly affects the affinity, while saturation of both sites with ADP strongly increases the affinity and eliminates the cooperativity. Thus, the coordinated action of both nucleotide-binding sites on the PriA–dsDNA interactions depends on the structure of the phosphate group. The significance of these results for the enzyme activities in recognizing primosome assembly sites or the ssDNA gaps is discussed.

Keywords

motor proteins; helicases; PriA protein; DNA binding; allosteric interactions

Introduction

The PriA protein is a key DNA replication enzyme in the *Escherichia coli* cell that is involved in major processes of the DNA metabolism, including replication, recombination, and repair.^{1–10} Classification based on the primary structure places the PriA protein in the

SF2 superfamily of helicases.¹¹ The enzyme plays a paramount role in the ordered assembly of the primosome, a large molecular machine engaged in priming the DNA during the replication process.^{2,3,10} In this capacity, the PriA protein has also been recognized as the major factor that initiates the restarting of the stalled replication fork at the damaged DNA sites, by recognizing the DNA structure at the sites.^{3,10} Trypsin digestion studies indicated that the enzyme is built of two functional domains: the large C-terminal helicase domain, which contains putative helicase motifs, and the small N-terminal domain (Fig. 1a), which shows affinity for the nucleic acid.^{8,9}

Quantitative thermodynamic studies showed that, in the complex with the single-stranded DNA (ssDNA), the total ssDNA-binding site of the PriA protein has a site size of 20 ± 3 nucleotides; that is, the bound protein occludes ~ 20 nucleotides.^{12–14} The structure of the total DNA-binding site is heterogeneous and, within the total site, the protein possesses a strong ssDNA-binding subsite, which has a high DNA affinity and can efficiently engage in interactions only $\sim 5–7$ nucleotides. Furthermore, in the absence of nucleotide cofactors, most of the intrinsic ssDNA affinity of the enzyme is generated at the strong ssDNA-binding subsite. The thermodynamic and kinetic data also indicated that the strong DNA-binding subsite is located in the central part of the enzyme molecule, on a structurally separated subdomain, as schematically depicted in Fig. 1a.^{12–15}

The PriA protein has a significant affinity for the ssDNA in the absence of the nucleotide cofactor.^{12–15} However, unlike helicases studied so far,^{16–20} the enzyme has not one but two nucleotide-binding sites, a strong and a weak binding site, which dramatically differ in their structural and dynamic properties and in their effects on the interactions with the ssDNA.^{15,19,20} Cooperative interactions between two nucleotide-binding sites reveal a profound communication between the sites and with the DNA-binding site.^{15,19,20} When both nucleotide-binding sites are specifically saturated with ADP, additional areas of the protein, possibly the N-terminal domain, become engaged in interactions with the ssDNA. This is schematically shown in Fig. 1b.^{15,19,20}

Although PriA protein interactions with the ssDNA have been intensively studied, interactions of the enzyme with the double-stranded DNA (dsDNA) have never been quantitatively examined. In fact, little is known about the quantitative energetics and dynamics of interactions of other well-studied helicases with the dsDNA, though interactions of the enzymes with the duplex conformation of the nucleic acid have been observed. The interactions with the dsDNA are particularly important in the case of the PriA protein, where, in the processes of forming the primosome and restarting the stalled replication fork, the enzyme mostly encounters the dsDNA.^{1–3,10} Our recent studies of the PriA protein association with the DNA substrates containing the ssDNA gap strongly suggested that the stoichiometry of the enzyme–dsDNA complex is significantly lower, as compared to the site size of the complex with the ssDNA and the binding process is affected by the presence of nucleotide cofactors.²¹ Nevertheless, the complexity of the gapped DNA system precluded quantitative characterization of the enzyme–dsDNA association beyond macroscopic affinities. Thus, direct quantitative examination of the PriA–dsDNA interactions and the effect of the nucleotide cofactors are of fundamental importance for elucidation of the enzyme mechanisms in DNA metabolism.

Results

The site size of the PriA–dsDNA complex in the absence of nucleotide cofactors

Similar to the interactions of the PriA helicase with the ssDNA, association of the enzyme with the dsDNA is not accompanied by adequate changes of the protein fluorescence to examine the binding process.^{12–14} Nevertheless, in the course of our studies, we found that

association of the PriA helicase with dsDNA oligomers, labeled at the 5' end of one of the ssDNA strands with fluorescein, is accompanied by a significant increase of the nucleic acid fluorescence, allowing us to perform high-resolution analyses of the enzyme–dsDNA complex formation.^{12–14} The selected dsDNA oligomers contain a random sequence of bases and 70% content of the G–C base pairs (Materials and Methods). The length of the oligomers are 10 and 15 bp, which are similar to the site size of the strong DNA-binding subsite (~5–7 nucleotides) and the total (~20 nucleotides) DNA-binding site of the PriA helicase–ssDNA complex.^{12–14}

Fluorescence titrations of the dsDNA 10-mer (Materials and Methods) with the PriA helicase at two different nucleic acid concentrations, in buffer C (pH 7.0, 50 mM NaCl, 10 °C), are shown in Fig. 2a. The maximum observed relative fluorescence increase, $\Delta F_{\max}$, is ~0.4. The titration curves, such as in Fig. 2a, have been analyzed by using the quantitative approach outlined in Materials and Methods.^{15,21–28} The dependence of the observed relative fluorescence increase, ΔF , as a function of the total average degree of binding, $\Sigma\Theta_i$, of the PriA protein on the dsDNA 10-mer is shown in Fig. 2b. The plot is clearly nonlinear, indicating the presence of two binding phases. A modest increase of ΔF to ~0.12 accompanies the binding of the first PriA molecule to the oligomer. Subsequently, the values of ΔF significantly increase in the second binding phase. Extrapolation of the second phase to the maximum value of the fluorescence increase, $\Delta F_{\max} = 0.41 \pm 0.03$, gives the maximum value of $\Sigma\Theta_i = 2.1 \pm 0.2$. Thus, at saturation, two PriA molecules bind to the dsDNA 10-mer^{12,15,19,21–28} (see Discussion).

Fluorescence titrations of the fluorescein-labeled dsDNA 15-mer with the PriA helicase, at two different nucleic acid concentrations, are more complex and are shown in Fig. 3a. The titration curves clearly show two binding phases. However, in this case, the binding phases differ in the induced changes in the nucleic acid fluorescence. In the high-affinity phase, PriA binding induces an increase of the DNA oligomer fluorescence, while in the low-affinity phase, the fluorescence quenching process is observed. Figure 3b shows the dependence of the observed relative fluorescence increase, ΔF , as a function of $\Sigma\Theta_i$ of the PriA helicase on the 15-mer. The selected DNA concentrations provide separation of titration curves up to $\Delta F \sim 0.25$. We could determine the total average degree of binding up to $\Sigma\Theta_i \sim 1.6$, indicating that two PriA molecules bind the dsDNA 15-mer in the high-affinity phase (Fig. 3b). However, the binding process continues into the low-affinity phase (Fig. 3a), indicating that additional PriA molecule(s) associate with the nucleic acid (see below).

Analytical ultracentrifugation studies

The maximum stoichiometry of the PriA–dsDNA complex has been further addressed using the sedimentation equilibrium method. The experiments were facilitated by the fact that we can exclusively monitor the nucleic acid concentration at the absorption band of fluorescein (495 nm) in the presence of the high enzyme concentration. A concentration profile of the dsDNA 10-mer, labeled at the 5' end with fluorescein (Materials and Methods) monitored at 495 nm, in the presence of the PriA helicase, as a function of the radial distance at sedimentation equilibrium, is shown in Fig. 4. The enzyme is in a large molar excess over the nucleic acid, assuring complete saturation of the DNA across the entire analyzed sedimentation equilibrium profile. The continuous line is a nonlinear least-squares fit to a single exponential function [Eq. (22)]. Including additional exponential terms does not cause significant improvement of the statistics of the fit (data not shown). The analysis of the concentration profiles provides a molecular weight of $156,000 \pm 15,000$. Since the molecular weights of the PriA helicase and the oligomer are ~81,700 and ~6300, respectively, the obtained data show that, at saturation, the PriA protein forms a 2:1 complex with the dsDNA 10-mer. Analogous experiments with the dsDNA 15-mer provided the molecular weight of

the complex, ~250,000, indicating the presence of three enzyme molecules in the complex (data not shown).

There are two key aspects of the thermodynamic and sedimentation equilibrium data. First, at saturation, two and three PriA molecules bind to the dsDNA 10- and 15-mer. These results strongly indicate that the enzyme forms a single type of complex with the dsDNA and requires only 5 bp to engage the nucleic acid. In other words, the site size of the PriA–dsDNA complex is $n=5\pm 1$ bp. This behavior is dramatically different from PriA binding to the ssDNA where the site size of the complex is ~20 nucleotides.^{12–14} Second, the maximum total degree of binding of the PriA protein on the dsDNA 10-mer is not affected by an ~10-fold increase of the oligomer concentration (data not shown). The independence of the enzyme–dsDNA stoichiometry upon the short oligomer concentrations provides strong thermodynamic evidence that only a single DNA-binding subsite of the protein engages in interactions with the nucleic acid.^{12–14,25} The ~10-fold increase of the 10-mer concentration would detect weak interactions with another DNA-binding site, characterized by the intrinsic binding constant lower by at least ~1–2 orders of magnitude than the observed affinity. Such a behavior would be manifested by a large change in the stoichiometry of the complex. However, this is not experimentally observed. Therefore, the obtained results strongly indicate that the PriA protein binds the dsDNA by using its strong DNA-binding subsite in the formed complexes^{12–14} (see below).

Statistical thermodynamic models of the PriA helicase binding to the dsDNA oligomers

The simplest statistical thermodynamic model that describes the binding of two PriA molecules to the dsDNA 10-mer is defined by the partition function, Z_{10} , as^{12–14,25,29,30}

$$Z_{10}=1+(N-n+1)K_{10}[P_F]+\omega(K_{10}[P_F])^2 \quad (1)$$

where N is the total number of base pairs in the oligomer ($N=10$), n is the site size of the PriA–dsDNA complex ($n=5$), and ω is the parameter characterizing the cooperative interactions between the bound protein molecules.^{12–14,29,30} The total average degree of binding, $\Sigma\Theta_i$, is described by the standard statistical thermodynamic formula, $\partial\ln Z_{10}/\partial\ln[P_F]$, as

$$\Sigma\Theta_i=\frac{[(N-n+1)K_{10}P_F+2\omega(K_{10}P_F)^2]}{Z_{10}} \quad (2)$$

Therefore, the observed relative fluorescence increase, ΔF , of the dsDNA 10-mer is

$$\Delta F=\Delta F_1\left[\frac{(N-n+1)K_{10}P_F}{Z_{10}}\right]+\Delta F_{\max}\left[\frac{\omega(K_{10}P_F)^2}{Z_{10}}\right] \quad (3)$$

where ΔF_1 is the relative molar fluorescence increase accompanying the binding of the first PriA molecule to the dsDNA 10-mer and $\Delta F_{\max}$ is the relative fluorescence increase induced by the binding of two helicase molecules to the oligomer.

Because of the nonlinear dependence of the observed signal as a function of the total average degree of binding, quantitative analysis of PriA binding to the dsDNA 10-mer

requires both the fluorescence titration curve (Fig. 2a) and the dependence of the observed ΔF as a function of $\Sigma\Theta_i$ (Fig. 2b).^{12–14,23–25} The value of ΔF_1 can be determined from the initial part of the plot in Fig. 2b, as, $\Delta F_1 = \partial\Delta F/\partial\Sigma\Theta_i$ which provides $\Delta F_1 = 0.12 \pm 0.03$. The value of $\Delta F_{\max}$ can be estimated from the plateau of the titration curves at high enzyme concentrations, which provides $\Delta F_{\max} = 0.41 \pm 0.03$. Thus, there are only two remaining parameters that must be determined, K_{10} and ω . The continuous lines in Fig. 2a are the nonlinear least-squares fits of the experimental titration curves to Eqs. (1)–(3), with K_{10} and ω as the fitting parameters. The model provides an excellent description of the binding process. The values of the binding and spectroscopic parameters are included in Table 1. The intrinsic binding constant, $K_{10} \sim 1.6 \times 10^7 \text{ M}^{-1}$, is significantly larger than the analogous parameter, previously obtained for the ssDNAs in similar solution conditions.^{12–14} Surprisingly, the value of $\omega \approx 10$ indicates that, unlike the binding to the ssDNA, where $\omega \approx 1–2$, interactions of the PriA protein with the dsDNA are characterized by significant positive cooperative interactions^{12–14} (see Discussion).

Because of the three enzyme molecules associating with the nucleic acid (see above), estimating the binding parameters for the PriA protein association with the dsDNA 15-mer is more complex. The binding system can be directly treated by the exact combinatorial theory for large ligand binding to a finite linear, homogeneous lattice.^{25,30} The partition function, Z_{15} , is defined as

$$Z_{15} = \sum_{k=0}^g \sum_{j=0}^{k-1} P_N(k, j) (K_{15} P_F)^k \omega^j \quad (4)$$

where g is the maximum number of ligand molecules that may bind to the finite nucleic acid lattice (for the nucleic acid lattice N residues long, $g = N/n$), K_{15} is the intrinsic binding constant, ω is the cooperative interaction parameter, k is the number of ligand molecules bound, and j is the number of cooperative contacts between the k bound ligand molecules in a particular configuration on the lattice. The combinatorial factor $P_N(k, j)$ is the number of distinct ways that k ligands bind to a lattice with j cooperative contacts and is defined by

$$P_N(k, j) = \frac{[(N - nk + 1)!(k - 1)!]}{[(N - nk + j + 1)!(k - j)!(k - j - 1)!]} \quad (5)$$

The total average degree of binding, $\Sigma\Theta_i$, is then

$$\Sigma \Theta_i = \frac{\sum_{k=1}^g \sum_{j=0}^{k-1} k P_N(k, j) (K_{15} P_F)^k \omega^j}{\sum_{k=0}^g \sum_{j=0}^{k-1} P_N(k, j) (K_{15} P_F)^k \omega^j} \quad (6)$$

The value of the relative fluorescence increase, ΔF , at any titration point, is defined as

$$\Delta F = \Delta F_1 \left[\frac{(N - n + 1)K_{15}P_F}{Z_{15}} \right] + \Delta F_2 \left[\frac{\sum_{j=0}^{k-1} P_N(k, j)(K_{15}P_F)^k \omega^j}{Z_{15}} \right] + \Delta F_3 \left[\frac{(K_{15}P_F)^2 \omega^2}{Z_{15}} \right] \quad (7)$$

where ΔF_1 , ΔF_2 , and ΔF_3 are the relative molar fluorescence increases accompanying the binding of one, two, and three PriA molecules to the 15-mer. Notice that the value of ΔF_1 can be determined from the initial part of the plot in Fig. 4b, as $\Delta F_1 = \partial \Delta F / \partial \Sigma \Theta_i$, which provides $\Delta F_1 = 0.09 \pm 0.03$. The value of $\Delta F_3 = 0.19 \pm 0.03$ can also be estimated from the plots in Fig. 4a. Thus, there are three independent parameters (ΔF_2 , K_{15} , and ω) that must be determined. The continuous lines in Fig. 3a are nonlinear least-squares fits of the titration curves using Eqs. (4)–(7).

The value of intrinsic binding constant $K_{15} = (1 \pm 0.4) \times 10^7 \text{ M}^{-1}$ and the cooperativity factor $\omega = 8 \pm 4$ are, within experimental accuracy, the same as observed for the 10-mer.^{12–14} Such a similarity of the intrinsic affinities and cooperativities indicates that the possible end effect in studied interactions is not present in the examined PriA–dsDNA systems. Similar to the 10-mer, the largest increase of the nucleic acid fluorescence occurs for the complex with two PriA molecules, with $\Delta F_2 = 0.43 \pm 0.04$. However, the value of $\Delta F_3 \approx 0.19$, which characterizes the complex with three PriA molecules, indicates that association of the third enzyme molecule induces a quenching of the nucleic acid fluorescence (Fig. 3a) (see Discussion).

Binding of the PriA helicase to unmodified dsDNA: Lattice competition titrations

Quantitative analysis of the PriA helicase binding to an unmodified dsDNA 10-mer has been performed using the Macromolecular Competition Titration method.^{25,31} In these studies, we use, as a reference fluorescent nucleic acid, the etheno derivative of the ssDNA 18-mer, dεA(pεA)₁₇, whose interactions with the PriA helicase have been extensively studied by us.^{12–14} It binds a single PriA molecule, and the association process is accompanied by a large fluorescence increase of the oligomer. For the unmodified dsDNA 10-mer, the partition function, Z_{10} , is described by Eq. (1). The partition function for the reference ssDNA 18-mer is

$$Z_{18} = 1 + K_R P_F \quad (8)$$

where K_R is the macroscopic binding constant of the PriA helicase. The concentration of the PriA protein, bound to the reference and unmodified nucleic acid, P_b , at total concentrations M_{TR} and M_{TS} , respectively, is then

$$P_b = \left(\sum \Theta_i \right)_R M_{TR} + \left(\sum \Theta_i \right)_S M_{TS} \quad (9a)$$

and

$$P_b = \left[\frac{K_R P_F}{Z_{18}} \right] M_{TR} + \left[\frac{(N - n + 1) K_{10S} P_F + 2\omega_S (K_{10S} P_F)^2}{Z_{10}} \right] M_{TS} \quad (9b)$$

where $(\Sigma\Theta_i)_R$ and $(\Sigma\Theta_i)_S$ are the total average degree of binding of the protein on the reference 18-mer and the examined unmodified dsDNA 10-mer, respectively, and K_{10S} and ω_S are the intrinsic binding constant and the cooperativity parameter for the unmodified dsDNA 10-mer. The observed relative fluorescence increase of the reference nucleic acid, ΔF , is then

$$\Delta F = \Delta F_{\max} \left[\frac{K_R P_F}{1 + K_R P_F} \right] \quad (10)$$

where

$$P_F = P_T - P_b \quad (11)$$

and P_T is the total concentration of the PriA helicase. Both $K_R = (1.6 \pm 0.3) \times 10^6 \text{ M}^{-1}$ and $\Delta F_{\max} = 1.7 \pm 0.05$ have been determined in independent fluorescence titrations in the absence of the unmodified oligomer.^{12–14}

Fluorescence titrations of dεA(pεA)₁₇, with the PriA helicase, in the absence and presence of two different concentrations of the unmodified dsDNA 10-mer, are shown in Fig. 5. A significant shift of the binding isotherm, in the presence of the dsDNA 10-mer, indicates efficient competition with dεA (pεA)₁₇ for the helicase.^{25,31} The continuous lines in Fig. 5 are nonlinear least-squares fits of the experimental titrations with K_{10S} and ω_S as independent fitting parameters, using Eqs. (8)–(11). The obtained binding parameters $K_{10S} = (8 \pm 1.5) \times 10^6 \text{ M}^{-1}$ and the cooperativity parameter $\omega_S = 6 \pm 2$. It is evident that binding of the enzyme to unmodified dsDNAs is characterized by parameters very similar to the parameters describing the enzyme binding to the fluorescein-labeled 10-mer. In other words, the presence of the fluorescein marker does not affect the enzyme interactions with the labeled dsDNA. Notice that the strong competition of the dsDNA with the ssDNA oligomer clearly shows that both nucleic acids bind to the same DNA-binding site of the enzyme (see Discussion).

Salt effect on the intrinsic affinity and cooperativity of the PriA–dsDNA 10-mer complex formation

Fluorescence titrations of the fluorescein-labeled dsDNA 10-mer with the PriA helicase, in buffer C (pH 7.0, 10 °C), containing different NaCl concentrations, are shown in Fig. 6a. The increasing salt concentration significantly decreases the affinity of the enzyme for the DNA. The continuous lines in Fig. 6a are nonlinear least-squares fits of the experimental titration curves to Eqs. (1)–(3). The dependence of the logarithm of the intrinsic binding constant, K_{10} , and cooperativity parameter, ω , upon the logarithm of the NaCl concentration (log–log plots) is shown in Fig. 6b and c.^{32,33} The plots are, within experimental accuracy, linear and characterized by the slopes $\partial \log K_{10} / \partial \log [\text{NaCl}] = -3.1 \pm 0.5$ and $\partial \log \omega / \partial \log [\text{NaCl}] = -1 \pm 0.3$, respectively. Thus, the net release of ~3 and ~1 ions accompanies the intrinsic and cooperativity interactions, respectively. The observed salt effect on the intrinsic affinity is very similar to the analogous salt effect on the enzyme complex with the

ssDNA.^{12–14} However, unlike the complex with the ssDNA, where cooperativity increases with the increase of [NaCl], the cooperativity of PriA binding to the dsDNA decreases with the increase of [NaCl] (see Discussion).^{12–14}

Temperature effect on the intrinsic and cooperative interactions of the PriA–dsDNA 10-mer

The nature of the intrinsic and cooperative interactions in the PriA–dsDNA complex was further examined by studying the temperature effect on the enzyme binding to the dsDNA 10-mer. Fluorescence titrations of the dsDNA 10-mer with the PriA helicase, performed at different temperatures, are shown in Fig. 7a. The behavior of the system is complex. In the range from 5 to 10 °C, the values of $\Delta F_{\max}$ slightly increase with the temperature. Above ~10 °C, the titration curves are characterized by lower and lower values of the maximum relative fluorescence increase with the increased temperature. In the examined temperature range (5–20 °C), the dsDNA completely preserves its duplex structure (Materials and Methods). Thus, these data provide a strong indication that the structure of the enzyme undergoes significant changes as the temperature of the sample increases. The continuous lines in Fig. 7a are nonlinear least-squares fits of the experimental titration curves using Eqs. (1)–(3).

Figure 7b shows the dependence of the natural logarithm of the intrinsic binding constant, K_{10} , upon the reciprocal of temperature (K) (van't Hoff plot).³⁴ Although the error in determining K_{10} is substantial, the plot is clearly nonlinear. The value of K_{10} decreases and reaches a minimum around ~10 °C. Above ~10 °C, the intrinsic affinity of PriA increases. Therefore, the observed nonlinear van't Hoff plot in Fig. 7b indicates that there are two temperature-dependent binding processes in the enzyme association with dsDNA 10-mer.³⁵

The simplest thermodynamic model, which can account for the observed nonlinear behavior of the van't Hoff plot in Fig. 7b, includes a conformational transition of the PriA helicase between two conformations and the binding of the dsDNA 10-mer to each conformation with different intrinsic affinities, described as

where P_L is the conformation of the PriA in the low temperature range, P_H is the corresponding conformation of the enzyme in the high temperature range, C_{11} , C_{12} , C_{21} , and C_{22} are complexes of the helicase with the 10-mer, containing one or two enzyme molecules, with the low-temperature and high-temperature conformation of the helicase, respectively. We designate K_C as the equilibrium constant characterizing the transition, $P_L \leftrightarrow P_H$, while the binding of the enzyme in the P_L and P_H conformation is described by the intrinsic binding constants K_{10L} and K_{10H} , respectively. The overall intrinsic binding constant, K_{ov} , is then defined by³⁵

$$K_{ov} = \frac{K_{10L} + K_{10H} K_C}{1 + K_C} \quad (15)$$

The temperature dependence of the particular equilibrium constants in Eq. (15) is described by

$$K_{10L} = K_{10L}(T_R) \exp \left[-\frac{\Delta H_{10L}}{R} \left(\frac{1}{T} - \frac{1}{T_R} \right) \right] \quad (16)$$

$$K_{10H} = K_{10H}(T_R) \exp \left[-\frac{\Delta H_{10H}}{R} \left(\frac{1}{T} - \frac{1}{T_R} \right) \right] \quad (17)$$

$$K_C = K_C(T_R) \exp \left[-\frac{\Delta H_C}{R} \left(\frac{1}{T} - \frac{1}{T_R} \right) \right] \quad (18)$$

The quantities $K_{10L}(T_R)$ and $K_{10H}(T_R)$ are the intrinsic binding constants of the helicase in the P_L and P_H conformations at a reference temperature, T_R , $K_C(T_R)$ is the equilibrium constant characterizing the enzyme conformational transition at T_R , and ΔH_{10L} and ΔH_{10H} are the enthalpy changes characterizing the enzyme binding in the P_L and P_H conformation, respectively. ΔH_C is the enthalpy change characterizing the transition $P_L \leftrightarrow P_H$. The reference temperature, T_R , is taken as 10 °C for the low-temperature and 20 °C for the high-temperature conformation, respectively. The reference temperature for the $P_L \leftrightarrow P_H$ transition is taken at 10 °C.

The values of $K_{10L}(T_R)$ and $K_{10H}(T_R)$ are known. Other parameters in Eqs. (15)–(18) can be estimated from the plot in Fig. 7b. The slopes in the low- and high-temperature range provides the estimates of $\Delta H_{10L} = 14 \pm 6$ kcal/mol and $\Delta H_{10H} = -27 \pm 11$ kcal/mol, respectively. Thus, there are only two unknown parameters, $K_C(T_R)$ and ΔH_C . The continuous line in Fig. 7b is the nonlinear least-squares fit of the plot to Eqs. (15)–(18), which gives $K_C(10 \text{ °C}) = 0.1 \pm 0.05$ and $\Delta H_C = 60 \pm 30$ kcal/mol. No doubt, there is a significant error in these parameters. Nevertheless, the value of ΔH_C clearly indicates that the PriA protein undergoes a significant structural transition, characterized by a large positive enthalpy change. The value of $K_C(10 \text{ °C})$ indicates that below ~10 °C, the PriA helicase exists in the P_L state. On the other hand, introducing the value of ΔH_C into Eq. (18) gives the value of $K_C \approx 4$ already at 20 °C, indicating that the enzyme is in the P_H state in the high-temperature region (see Discussion).

The dependence of the natural logarithm of the cooperativity parameter, ω , upon the reciprocal of temperature, shown in Fig. 7c, is different from the intrinsic affinity plot (Fig. 7b). Within experimental accuracy, the plot is linear and the values of ω decrease with the temperature increase (see Discussion). The slope of the plot provides $\Delta H_\omega = -29 \pm 10$ kcal/mol. Such a behavior of the plot provides an additional indication that a process, different from the intrinsic association, is observed. The cooperative interactions strongly decrease with temperature. In fact, the value of ΔH_ω indicates that at temperature higher than 20 °C,

the enzyme binding to the dsDNA is characterized by negative cooperativity (see Discussion).

Interactions of the PriA helicase with the dsDNA in the presence of nucleotide cofactors

As mentioned above, the PriA helicase possesses two strong and weak nucleotide-binding sites, which dramatically affect the enzyme interactions with the ssDNA, particularly in the presence of ADP (Fig. 1b).^{15,19–21} In the next set of experiments, we address the effect of ADP and the ATP nonhydrolyzable analog, ATP γ S, on the enzyme association with the dsDNA conformation of the nucleic acid. Fluorescence titrations of the dsDNA 10-mer with the PriA helicase at two different nucleic acid concentrations, in buffer C (pH 7.0, 10 °C), containing 1×10^{-5} M ADP, are shown in Fig. 8a. At this ADP concentration, only the strong nucleotide-binding site is saturated with the cofactor.^{15,19–21} The observed maximum relative fluorescence increase, $\Delta F_{\max}$, is ~ 0.3 , which is lower than ~ 0.4 obtained in the absence of cofactors (Table 1). The dependence of the relative fluorescence increase, ΔF , as a function of the total average degree of binding, $\Sigma\Theta_i$, of the PriA protein on the dsDNA 10-mer is shown in Fig. 8b. Within experimental accuracy, the plot is linear, which is also different from the behavior observed in the absence of the cofactors (Fig. 2b). We could determine the value of $\Sigma\Theta_i$, up to ~ 1.2 . Extrapolation of the plot to the maximum value of the relative fluorescence increase, $\Delta F_{\max} = 0.29 \pm 0.03$, gives the maximum value of $\Sigma\Theta_i = 2.2 \pm 0.2$. Thus, at saturation, two PriA molecules, with the strong nucleotide-binding site associated with ADP, bind to the 10-mer.

The spectroscopic titration curves in Fig. 8a have been analyzed using the binding model defined by Eqs. (1)–(3). The linear dependence of ΔF as a function of $\Sigma\Theta_i$ indicates that $\Delta F_1 = \Delta F_{\max}$. The continuous lines in Fig. 8a are the nonlinear least-squares fits of the titration curves to Eqs. (1)–(3) with K and ω as fitting parameters (Table 1). Saturation of only the strong nucleotide-binding site with ADP has a very modest effect on the value of the intrinsic binding constant, K_{10} , which is diminished by a factor of ~ 2 . However, the most dramatic effect is observed in the case of the cooperativity parameter, which increases by a factor of ~ 5 to $\omega \sim 45$ (Table 1). Nevertheless, the different values of ΔF_1 and $\Delta F_{\max}$, as compared to the same parameters obtained in the absence of the nucleotide cofactors, indicate that the orientation of the enzyme on the dsDNA is affected by the bound cofactor (see Discussion).

Very different behavior is observed when both nucleotide-binding sites of the PriA helicase are saturated with ADP. Fluorescence titrations of the dsDNA 10-mer with the PriA helicase at two different nucleic acid concentrations, in buffer C (pH 7.0, 10 °C), containing 3×10^{-3} M ADP, are shown in Fig. 9a. At this ADP concentration, both the strong and the weak nucleotide-binding sites are saturated with the nucleotide.^{15,19–21} The observed maximum relative fluorescence increase, $\Delta F_{\max}$, is ~ 0.9 , which is profoundly higher than ~ 0.4 and ~ 0.3 obtained in the absence of cofactors, or with only the strong nucleotide-binding site saturated with ADP (Table 1). The dependence of the relative fluorescence increase, ΔF , as a function of the total average degree of binding, $\Sigma\Theta_i$, of the PriA protein on the dsDNA 10-mer is shown in Fig. 9b. The plot is nonlinear, as observed in the absence of the cofactors, indicating the presence of two binding phases (Fig. 2b). We could determine the value of $\Sigma\Theta_i$, up to ~ 1.6 . Binding of the first PriA molecule to the dsDNA 10-mer is accompanied by ΔF_1 to 0.21 ± 0.03 . Subsequently, ΔF strongly increases in the second binding phase and extrapolation of the second phase of the plot to $\Delta F_{\max} = 0.93 \pm 0.03$ gives the maximum value of $\Sigma\Theta_i = 2.0 \pm 0.2$. Thus, saturation of both nucleotide-binding sites with ADP does not affect the stoichiometry of the PriA–dsDNA complex^{15,19–21} (see Discussion).

Analysis of the titration curves in Fig. 9a has been performed as described above for the analogous studies in the absence of the cofactors. With ΔF_1 and $\Delta F_{\max}$ known (see above),

the continuous lines in Fig. 9a are the nonlinear least-squares fits of the experimental titration curves to Eqs. (1)–(3), with K_{10} and ω as the fitting parameters (Table 1). Saturation of both the strong and the weak nucleotide-binding site with ADP dramatically increases the intrinsic binding constant, K_{10} to $\sim 1.3 \times 10^8 \text{ M}^{-1}$. When corrected for the difference in salt concentrations, this affinity is ~ 2 and ~ 1 orders higher than the intrinsic affinities obtained for homo-purine and homo-pyrimidine ssDNAs, respectively.^{12,13,15} It is also by more than an order of magnitude higher than the dsDNA intrinsic affinity of the enzyme with ADP bound to only the strong nucleotide-binding site (Table 1). On the other hand, $\omega \sim 2$ is strongly diminished by a factor of ~ 23 as compared to $\omega \sim 45$, when only the strong nucleotide-binding site is engaged in interactions with ADP (see Discussion). Moreover, the values of the spectroscopic parameters ΔF_1 and ΔF_{max} are significantly higher than their values obtained in the absence of the nucleotide cofactors, or low [ADP], an indication that the orientation of the enzyme on the dsDNA is affected when both nucleotide sites are saturated with ADP (see Discussion).

Still, different behavior is observed when the nucleotide-binding sites of the PriA protein are engaged in interactions with ATP nonhydrolyzable analog, ATP γ S. The PriA helicase does not hydrolyze ATP γ S on the time scale of the reported binding experiments, both in the absence and in the presence of the dsDNA^{15,19–21}. Fluorescence titrations of the dsDNA 10-mer with the PriA helicase in buffer C (pH 7.0, 10 °C), containing $1 \times 10^{-4} \text{ M}$ or $3 \times 10^{-3} \text{ M}$ ATP γ S, are shown in Fig. 10. For comparison, titrations of the 10-mer, at the same nucleic acid concentration, in the presence of low and high concentrations of ADP are also included in Fig. 10. Analysis of the titration curves in the presence of ATP γ S has been performed as described above, using Eqs. (1)–(3) (Table 1). The difference between the effects of the saturation of the two nucleotide-binding sites with the nucleoside diphosphate or nucleoside triphosphate is staggering. Both the low- and high-ATP γ S concentrations induce, within experimental accuracy, the same maximum relative increases of the nucleic acid fluorescence. Also, ΔF_1 is virtually the same and similar to the emission changes accompanying the enzyme binding in the absence of any cofactor or in the presence of a low-ADP concentration (Table 1). Saturation of only the strong nucleotide-binding site with ATP γ S lowers the intrinsic binding constant, K_{10} , by a factor of ~ 2.5 , while saturation of both nucleotide-binding site lowers the intrinsic affinity by a factor ~ 5 . Thus, in the presence ATP γ S, the enzyme has the lowest intrinsic affinity of all examined enzyme states with or without the cofactors. However, ATP γ S does not affect the cooperativity of PriA binding, which remains the same or slightly higher than in the absence of nucleotide cofactors (Table 1).

Photo-cross-linking of the PriA helicase to the ssDNA

The involvement of the domains of the PriA protein in interactions with the ssDNA and the dsDNA has been addressed using the UV irradiation method.^{36–39} UV irradiation produces covalent linkage between nucleic acid bases and amino acid residues, resulting in a “zero-length” cross-linking with minimal perturbation to the protein–nucleic acid complex.³⁶ Among the nucleic acid bases, thymine is by far the most reactive in the photo-cross-linking reactions. Therefore, parallel with the thermodynamic studies, we performed photo-cross-linking experiments of the PriA protein complex with the radioactive [³²P]-dT₂₀ (Materials and Methods). Recall that, in the absence of cofactors, only a single PriA protein molecule associates with the ssDNA 20-mer, using its strong DNA-binding subsite.^{12–14} Both the ssDNA and the dsDNA compete for the same binding site; that is, the dsDNA also binds to the strong DNA-binding subsite (see above). However, the location of the strong DNA-binding subsite within the domain structure of the enzyme is unknown. The reason for the use of the ssDNA, [³²P]-dT₂₀, is dictated by the fact that it photo-cross-links with the PriA protein more efficiently than the dsDNA.

We applied the following strategy to address the problem of the domain location of the strong DNA-binding subsite of the enzyme. Trypsin digestion of the PriA protein results in formation of the dominant fragment of the protein (~500 amino acids), containing the large helicase domain and two smaller fragments containing the small N-terminal domain, which are well separated on the polyacrylamide gel.^{8,9} The digestion experiments have been performed using the UV photo-cross-linked PriA-[³²P]-dT₂₀ complex, at the protein concentration, which completely saturates the nucleic acid.¹²⁻¹⁴ Figure 11a shows the polyacrylamide gel, stained with Coomassie Brilliant Blue, of the appearance of the trypsin digestion products of the UV-irradiated PriA protein-[³²P]-dT₂₀ sample, in the presence of trypsin, as a function of time. The PriA band at ~82,000 diminishes, while the dominant band, corresponding to the helicase domain (~53,000) and two fragments containing the N-terminal domain (~27,000 and ~35,000), appear on the gel. Figure 11b shows the autoradiogram of the same gel of the PriA-[³²P]-dT₂₀ complex, subjected to trypsin digestion after irradiation, as a function of time. Two dominant radioactive bands appear on the gel, at molecular weights of ~85,000, and ~55,000, corresponding to the PriA protein and the helicase domain complexes with [³²P]-dT₂₀. The small nucleic acid oligomer only slightly affects the SDS gel mobility of the cross-linked PriA protein—and helicase domain—DNA complexes. Thus, it is evident that the strong DNA-binding subsite, which exclusively engages both the ssDNA and the dsDNA in the absence of high [ADP] (see above), is located on the large helicase domain of the PriA protein. This is in excellent agreement with the proposed location of the strong DNA-binding subsite, based on quantitative thermodynamic and kinetic studies¹²⁻¹⁴ (see Discussion).

Discussion

The site size of the PriA-dsDNA complex and the competition studies with the ssDNA indicate that the helicase engages only the strong DNA-binding subsite in interactions with the dsDNA

The strong DNA-binding subsite of the PriA occludes only 5–7 nucleotides (Fig. 1a).¹²⁻¹⁴ Nevertheless, the orientation of the bound PriA molecule on the ssDNA is such that the enzyme always occludes ~20 nucleotides. On the other hand, the total site size of the PriA-dsDNA complex, $n = 5 \pm 1$ bp, determined in this work, is dramatically lower than the total site size of the PriA-ssDNA complex.¹²⁻¹⁴ There are two important aspects of this result. First, the site size of the complex is, within experimental accuracy, identical with the site size of the strong DNA-binding subsite, which indicates that the helicase exclusively engages the strong DNA-binding subsite in interactions with the dsDNA. This is also shown by the strong competition of the dsDNA and the ssDNA for the enzyme in absence of high [ADP], where the enzyme associates with the ssDNA only using its strong DNA-binding subsite.¹²⁻¹⁵ Second, to achieve such a small total site size, the helicase must assume a very different orientation in the complex, as compared to the complex with the ssDNA (see below). The total site size of the PriA-dsDNA complex may seem different from the observed binding of the single enzyme molecule to 10 bp extensions of DNA substrates, containing the ssDNA gap.²¹ However, the difference simply results from the fact that the PriA molecule bound to the ssDNA gap and forming the gap complex engages parts of the dsDNAs adjacent to both sides of the gap, enabling association of only a single PriA molecule with each of the duplex DNA parts.²¹

Notice that the nonlinear dependence of ΔF as a function of $\Sigma \Theta_i$ can easily be understood in the context of the considered statistical thermodynamic model, which provides excellent description of the binding process (Fig. 2b). The first bound enzyme molecule experiences multiple potential binding sites and, on average, is at some distance from the fluorescence marker located at the 5' end of the DNA. The second PriA molecule binds in a closer distance from the marker, inducing a further change of its emission intensity.

Photo-cross-linking data show that the strong DNA-binding subsite is located on the large helicase domain of the PriA protein

Previous thermodynamic analysis indicated that the strong DNA-binding subsite is centrally located within the PriA molecule on a protruding domain and, in the absence of the high-ADP concentration, exclusively engages the ssDNA (Fig. 1a).^{12–14} These studies were performed before the domain structure of the enzyme was known. Photo-cross-linking data describe here unambiguously show that the strong DNA-binding subsite is located on the large helicase domain of the PriA protein. Moreover, the lack of any radioactive band corresponding to the location of the small N-terminal domain (Fig. 11b) indicates that, in the absence of the high [ADP], the N-terminal domain does not engage the nucleic acid. This is in excellent agreement with thermodynamic analysis, which indicated that the DNA-binding subsite on the N-terminal domain efficiently engages the DNA only when the weak nucleotide-binding site of the helicase is associated with ADP.¹⁵

The PriA helicase preferentially binds to the double-stranded conformation over the single-stranded conformation of the DNA

Another surprising result is that the PriA helicase has a significantly higher intrinsic affinity for the dsDNA, as compared to its intrinsic affinity for the ssDNA.^{12–14} The PriA protein shows a strong preference for the homo-pyrimidine ssDNAs.¹³ However, even in this case, the highest intrinsic binding constant for the homo-thymine nucleic acid is $\sim 1.5 \times 10^6 \text{ M}^{-1}$, as compared to $\sim 0.8\text{--}1.6 \times 10^7 \text{ M}^{-1}$ for the dsDNA in similar solution conditions.^{12–14} This difference is diminished only in the presence of NTP analogs.¹⁵ In other words, the strong DNA-binding subsite of the PriA protein seems to be better geared to accept the dsDNA than the ssDNA (see below). When corrected for the difference in salt concentrations in solution and the statistical effect, the dsDNA affinity of the enzyme ($\sim 2 \times 10^7 \text{ M}^{-1}$), for the duplex extensions of previously examined gapped DNAs, is similar to the values reported here, in the absence of the nucleotide cofactors.²¹ Moreover, the enzyme affinity for the dsDNA is additionally amplified by significant positive cooperative interactions. Such a high dsDNA affinity is unexpected if one only considers the helicase activity of the protein, where usually a significantly higher intrinsic affinity for the ssDNA than for the dsDNA, particularly in the presence of NTPs, is postulated.^{16–18} Rather, the high dsDNA affinity of the PriA protein reflects the complex role of the protein in the recognition of the damaged DNA and the primosome assembly sites (PASs) (see below).

Strong positive cooperative interactions provide additional indication of specific orientation of the PriA helicase in the complex with the dsDNA

A peculiar aspect of the PriA binding to the dsDNA is the presence of significant, positive cooperative interactions (Table 1). Interactions with the ssDNA, in the same solution conditions, are characterized by rather weak cooperative interactions.^{12–14} The dramatic difference in the nature of enzyme interactions with two different conformations of the DNA indicates the presence of very different orientations of the bound helicase in each of these complexes (see above). Similar to the intrinsic interactions, the cooperative interactions in the PriA–dsDNA complex are weakened by the increase of the salt concentration in solution (Fig. 6c). The negative slope of the log–log plot, $\partial \log \omega / \partial \log [\text{NaCl}] = -1 \pm 0.3$, shows that a net release of a single ion accompanies the engagement of the interacting areas of the bound protein molecules. Once again, this is very different behavior from the salt effect on the cooperative interactions with the ssDNA, where the increase of the values of ω is observed at higher salt concentrations, indicating a net uptake of ~ 2.5 ions.^{32,33} This different thermodynamic response of cooperativity parameters reinforces the notion that interactions of a very different nature, that is, engagement of different areas of the protein, resulting from different orientations, are observed in the complex with the dsDNA *versus* the ssDNA.

Temperature effect on the PriA–dsDNA interactions indicates a significant conformational flexibility of the enzyme

The simplest thermodynamic model that can account for the observed nonlinear character of the van't Hoff plot (Fig. 7b) includes two conformations of the protein, P_L and P_H , with different temperature dependences of the intrinsic affinities for the dsDNA. Above 15 °C, the enzyme exists predominantly in the P_H conformation, whose apparent enthalpy of intrinsic interactions with the nucleic acid is strongly negative with the $\Delta H_{10H} \approx -27$ kcal/mol, while below 10 °C, the helicase assumes the P_L conformation and the intrinsic interactions are characterized by a positive $\Delta H_{10L} \approx 14$ kcal/mol. Only a large conformational transition of the entire protein could induce such drastically different changes of the apparent enthalpy. This conclusion is supported by the large and positive enthalpy of the $P_L \leftrightarrow P_H$ transition, $\Delta H_C \sim 60$ kcal/mol. The strong temperature dependence of the dsDNA affinities of two enzyme conformations indicates that the intrinsic PriA–dsDNA interaction is effectively enthalpy-driven process at temperatures above ~20 °C and entropy-driven process below ~10 °C. This remarkable behavior indicates that the enzyme can exist in different conformations, which very differently interact with the dsDNA, although they possess identical intrinsic affinities.

Binding of ADP exclusively to the strong nucleotide-binding site increases the positive cooperativity of the PriA interactions with the dsDNA

Saturation of the strong nucleotide-binding site with ADP induces only a very modest change of the intrinsic affinity of the PriA helicase for the dsDNA (Table 1). However, the presence of ADP in the strong nucleotide-binding site has a dramatic effect on the cooperativity parameter, ω , which increases from the value of ~10 to ~45. First, such an increase of the cooperativity compensates for the slightly diminished intrinsic affinity and the enzyme preserves the overall, high affinity for the dsDNA. Second, this high overall affinity is now achieved not through protein–nucleic acid but through protein–protein interactions, and the enzyme is able to form long clusters on the nucleic acid lattice. The value of ΔF_1 remains similar to the value determined in the absence of ADP, indicating similar intrinsic interactions and similar orientation of a single enzyme molecule on the DNA (Table 1). However, $\Delta F_{\max}$ is significantly lower than observed in the absence of ADP, indicating a changed orientation of the enzyme now engaged in strong cooperative interactions.

Saturation of the weak nucleotide-binding site with ADP increases the intrinsic affinity and dramatically decreases the cooperativity of the PriA protein binding to the dsDNA

Diametrically different behavior, as compared to the enzyme associated with ADP in the strong nucleotide-binding site, is observed when both the strong and the weak site are saturated with ADP (Table 1; Figs. 8 and 9). The enzyme has a very strong preference for the dsDNA over the ssDNA. However, this is achieved not through protein–protein but through the intrinsic protein–nucleic acid interactions. The very high intrinsic affinity is reflected in the values of ΔF_1 and $\Delta F_{\max}$, which are significantly higher than determined in the free helicase, or for the enzyme with ADP in the strong nucleotide-binding site (Table 1). On the other hand, the dependence of ΔF upon the total average degree of binding value is biphasic, as observed in the absence of ADP (Figs. 2b and 9b), indicating similar orientations of the bound enzyme molecules in the states characterized by weak cooperative interactions.

Recall that when both nucleotide-binding sites are saturated with ADP, the PriA helicase binds an additional fragment of the ssDNA, presumably through the engagement of the N-terminal domain in interactions with the nucleic acid (Fig. 1b).^{15,19,20} This is not the case here. The site size of the PriA–dsDNA complex is not affected by the presence of the bound

ADP to both sites. These data strongly suggest that the different orientation of the enzyme on the dsDNA and the stiffness of the dsDNA prevent the domain from entering into interactions with the DNA.^{15,19,20}

Binding of ATP γ S to the strong and weak nucleotide-binding sites controls only the intrinsic affinity of the PriA interactions with the dsDNA

Unlike the effect of ADP binding to both nucleotide-binding sites of the PriA helicase, analogous association of the triphosphate analog, ATP γ S, affects only the intrinsic affinity of the enzyme. This is particularly seen for the complex with the weak nucleotide-binding site associated with the cofactor, where the intrinsic affinity is ~ 2 and ~ 1 order of magnitude lower than that observed for the analogous state in the presence of ADP or in the absence of any cofactor, respectively (Table 1).

Functional implications

The functional role of the discovered low site size, strong affinity and cooperativity in the PriA protein binding to the dsDNA can be understood in the context of the enzyme activities in the *E. coli* chromosomal DNA and plasmid replication.^{1–3} The PriA helicase recognizes the ssDNA gap in the process of restarting DNA replication at the damaged DNA sites.^{1,3,10} Moreover, the enzyme specifically recognizes the structure of the PASs in the formation of the primosome, originally described for phage Φ 174.^{2,5} In these activities, the PriA protein must specifically recognize the ssDNA gap, as well as the PAS-like structures, where short stretches of the dsDNA constitute specific parts of the DNA structure. Notice that these are recognition processes, which should be geared for high affinity and efficiency, without involving mechanical translocation of the enzyme. This can be achieved through the exclusive engagement of the strong DNA-binding subsite with the site size of 5 ± 1 bp. Moreover, the two nucleotide-binding sites of the protein control the intrinsic affinity and cooperativity of the binding process, without affecting the site size of the complex and without involving free-energy transduction.

This is schematically depicted in Fig. 12a and b. In the case of the PAS-like DNA structures (Fig. 12a), short stretches of the dsDNA (~ 3 – 10 bp) are proposed to be embedded among small ssDNA loops and bulges. Both the dsDNA and the dsDNA–ssDNA junctions can constitute PriA binding sites or be parts of the binding sites.²¹ In the recognition process, binding of ADP to the strong nucleotide-binding site, or hydrolysis of the bound ATP and release of ADP from the weak nucleotide-binding site, allows the bound protein to engage in a significant positive cooperativity, which could reinforce the overall affinity. The size of the PAS-like DNA fragments and our data strongly suggest that more than one PriA molecule may participate in the initial recognition of the PAS-like structures. With both nucleotide-binding sites saturated with ADP, the cooperativity is strongly diminished, but the intrinsic affinity for the dsDNA is strongly increased, preserving the specificity of the recognition of the PAS structures.

In the case of the ssDNA gap at the stalled replication fork (Fig. 12b), the gap is embedded between two dsDNA parts. The recognition site includes both the dsDNA and the dsDNA–ssDNA junctions, but not the ssDNA of the gap.²¹ Mostly, the PriA protein with both nucleotide-binding sites saturated with ADP seems to be involved, although the enzyme molecule without nucleotide cofactors may also participate in the recognition process.²¹ Low cooperativity of the binding process, with two nucleotide-binding sites of the protein saturated with ADP, indicates that a single PriA molecule participates in the ssDNA gap recognition.²¹

Notice that the discussed recognition processes would not involve the PriA protein associated with ATP, as this cofactor only slightly affects the interactions with the dsDNA. In fact, early biochemical studies indicated that the initial steps of the primosome assembly do not require ATP or ATP hydrolysis.^{40,41} Our data indicate that, in the presence of ATP, the ATP hydrolysis is only necessary to provide ADP, which controls the intrinsic affinity and the cooperativity. Nevertheless, once the ssDNA gap in a damaged DNA is recognized, the protein must change its mode of interactions and engage the single-stranded conformation of the nucleic acid.²¹ This transition is a subject of current studies in our laboratory.

The presence of two nucleotide-binding sites on the PriA protein dramatically changes current thinking regarding the nature and the mechanism of interplay between the nucleotide cofactors and the conformation of the nucleic acid bound to the helicase. The data indicate different roles of both nucleotide-binding sites in the PriA helicase binding to the dsDNA.^{15,19,20} Moreover, this role is also different from the nucleotide effect on the enzyme–ssDNA interactions.^{12–14} This difference must reflect separation between recognition processes, predominantly occurring in complexes with the dsDNA and/or the dsDNA–ssDNA junctions, and mechanical translocation occurring in complexes with the ssDNA.

Materials and Methods

Reagents and buffers

All solutions were made with distilled and deionized >18 M Ω (Milli-Q Plus) water. All chemicals were reagent grade. Buffer C is 10 mM sodium cacodylate adjusted to pH 7.0 with HCl, 1 mM DTT, 5 mM MgCl₂, and 25% glycerol (w/v).^{12–14} Temperatures and concentrations of salts in the buffer are indicated in the text.

PriA protein

The *E. coli* PriA protein has been purified as we described before.^{12–15,19–21} The enzyme was >98% pure as judged by polyacrylamide electrophoresis with Coomassie Brilliant Blue staining. The concentration of the protein was spectrophotometrically determined, with the extinction coefficient $\epsilon_{280} = 1.06 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$, obtained using an approach based on Edelhoch's method.^{12–14,42,43}

Nucleotide and nucleic acids

ATP γ S, ADP, trypsin, and trypsin inhibitor were from Sigma (St. Louis, MO). Modified and unmodified ssDNA oligomers were purchased from Midland Certified Reagents (Midland, TX). Nucleic acids were at least >95% pure as judged by electrophoresis on polyacrylamide gels. The modified ssDNA oligomers contain a fluorescent label, fluorescein (Fl), attached to the 5' end through phosphoramidate chemistry. The sequences of the labeled oligomers are: Fl-CTGACGTGCG and Fl-CTGACGTGCGGATGC, respectively. Concentrations of all ssDNA oligomers have been spectrophotometrically determined as previously described by us.^{44–50} The dsDNA substrates were obtained by mixing the labeled ssDNA oligomers with complementary unmodified oligomers at given concentrations, warming the mixture for 5 min at 95 °C, and slowly cooling for a period of ~3–4 h.^{44–50} The integrity of the dsDNA oligomers has been checked by UV melting and analytical ultracentrifugation techniques.^{44–51} The melting temperature of the examined dsDNA oligomers is ~54 °C or higher in the studied solution conditions. The etheno derivative, of the ssDNA 18-mer, d ϵ A(p ϵ A)₁₇, was obtained by modification with chloroacetaldehyde.^{12–14,52–54} The concentration of d ϵ A (p ϵ A)₁₇ was determined using the extinction coefficient $\epsilon_{257} = 3700 \text{ M}^{-1} \text{ cm}^{-1}$ (nucleotide).^{12–14,52–54}

Fluorescence measurements

All steady-state fluorescence titrations were performed using the Fluorolog F-11 spectrofluorometer (Jobin Yvon).^{12–14,44–50} The PriA protein binding was followed by monitoring the emission of the fluorescein-labeled nucleic acid ($\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$) or the emission of dεA(pεA)₁₇ ($\lambda_{\text{ex}} = 325 \text{ nm}$, $\lambda_{\text{em}} = 410 \text{ nm}$). Computer fits were performed using Mathematica (Wolfram, IL) and KaleidaGraph (Synergy Software, PA).

Determination of quantitative binding isotherms of the PriA–dsDNA complexes

In this work, we followed the binding of the PriA helicase to the DNA oligomers by monitoring the fluorescence increase, ΔF , of the FI-labeled DNA or etheno derivative of the nucleic acid. To obtain quantitative estimates of the total average degree of binding, $\Sigma\Theta_i$ (average number of bound PriA molecules per DNA oligomer), and the free protein concentration, P_F , independent of any assumption about the relationship between the observed spectroscopic signal and $\Sigma\Theta_i$, we applied an approach previously described by us.^{12–14,24–28} Briefly, each different possible “*i*” complex of the PriA protein with the dsDNA contributes to the experimentally observed fluorescence increase, ΔF . Thus, ΔF is functionally related to $\Sigma\Theta_i$ by

$$\Delta F = \sum \Theta_i \Delta F_i \quad (19)$$

where ΔF_i is the maximum fluorescence increase of the nucleic acid with the PriA bound in complex “*i*”. The same value of ΔF , obtained at two different total nucleic acid concentrations, M_{T1} and M_{T2} , indicates the same physical state of the nucleic acid; that is, the total average degree of binding, $\Sigma\Theta_i$, and the free PriA concentration, P_F , must be the same. The value of $\Sigma\Theta_i$ and P_F is then related to the total protein concentrations, P_{T1} and P_{T2} , and the corresponding total nucleic acid concentrations, M_{T1} and M_{T2} , at the same value of ΔF_{obs} , by

$$\sum \Theta_i = \frac{(P_{T2} - P_{T1})}{(M_{T2} - M_{T1})} \quad (20)$$

$$P_F = P_{Tx} - \left(\sum \Theta_i \right) M_{Tx} \quad (21)$$

where $x = 1$ or 2 .^{12–14,24–28}

Analytical ultracentrifugation measurements

Analytical ultracentrifugation experiments were performed with an Optima XL-A analytical ultracentrifuge (Beckman Inc., Palo Alto, CA), as we previously described.^{55–59}

Sedimentation equilibrium scans were collected at the absorption band of the fluorescein of the labeled dsDNAs (495 nm) at a large excess of the protein to assure the complete saturation of the DNA. The sedimentation was considered to be at equilibrium when consecutive scans, separated by time intervals of 8 h, did not indicate any changes. For the *n*-component system, the total concentration at radial position *r*, c_r , is defined by⁶⁰

$$c_r = \sum_{i=1}^n c_{bi} \exp \left[\frac{(1 - \bar{v}_i \rho) \omega^2 M_i (r^2 - r_b^2)}{2RT} \right] + b \quad (22)$$

where c_{bi} , $\bar{v}_i$, and M_i are the concentration at the bottom of the cell, partial specific volume, and molecular weight of the “i” component, respectively, ρ is the density of the solution, ω is the angular velocity, and b is the baseline error term. Equilibrium sedimentation profiles were fitted to Eq. (22) with M_i and b as fitting parameters.^{55,56}

Photo-cross-linking and trypsin digestion experiments

Time-dependent digestion of the PriA helicase ssDNA complex with trypsin has been performed in the same buffer as the binding experiments. The ssDNA oligomer, dT(pT)₁₉ has been labeled at the 5' end with [³²P], using polynucleotide kinase.^{37,38} The complex was mixed with the protease at the 37:1 PriA–trypsin molar ratio and the reaction was stopped by adding trypsin inhibitor, subsequently adding SDS to 10% concentration, and immersing the sample in ~100 °C water bath. Several different PriA protein–trypsin molar ratios were examined to obtain the optimal time dependence of the digestion reaction. The samples (total volume, ~40 µl) were placed on Parafilm, immersed in 10 °C, and irradiated for 20 min, at a distance of 11 cm, using a mineral lamp (model UVG-11) with a maximum output of 254 nm. The controls were performed to determine the optimal time for cross-linking and to avoid possible degradation of the protein by prolonged exposure to UV light. The samples collected at different time intervals of the protease reaction were loaded on 10% SDS polyacrylamide gel and electrophoresis was performed at a constant voltage. The gels were stained with Coomassie Brilliant Blue and scanned, using the phosphorimager SI (Molecular Dynamics).

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Abbreviations used

dsDNA	double-stranded DNA
ssDNA	single-stranded DNA
PAS	primosome assembly site

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Fig. 1.

(a) Schematic model of the PriA helicase-ssDNA complex, in the absence of nucleotide cofactors, in the presence of ATP analogs, or at low ADP concentration.^{12–15} The strong DNA-binding subsite of the enzyme, which encompasses only 5–7 nucleotides, is located in the center of the enzyme molecule on a protruding domain. Data obtained in this work show that the strong DNA-binding site is located on the helicase domain of the enzyme (see above). The enzyme occludes the total site size of ~20 nucleotides. Yellow ovals symbolize the strong and weak nucleotide-binding sites of the helicase. (b) Schematic model of the PriA helicase complex with the ssDNA in the presence of high concentrations of ADP, which saturates both nucleotide-binding sites of the enzyme. The helicase, bound to the ssDNA through its strong DNA-binding subsite, engages an additional fragment of the nucleic acid, presumably through the binding subsite located on the N-terminal domain of the enzyme.^{12–15}

Fig. 2.

(a) Fluorescence titrations of the dsDNA 10-mer, labeled at the 5' end of one of the strands with fluorescein (Materials and Methods), with the PriA protein ($\lambda_{\text{ex}} = 485$ nm, $\lambda_{\text{em}} = 520$ nm) in buffer C (pH 7.0, 10 °C), containing 50 mM NaCl, in the absence of nucleotide cofactors, at two different nucleic acid concentrations: ■, 1×10^{-8} M; □, 5×10^{-8} M (oligomer). The continuous lines are nonlinear least-squares fits of the titration curves, using the statistical thermodynamic model described by Eqs. (1)–(3) (Table 1). (b) Dependence of the relative fluorescence increase of the dsDNA 10-mer, ΔF , upon the total average degree of binding, $\Sigma \Theta_i$ (■). The continuous lines indicate the limiting slopes of the plot at the low and high enzyme concentration range, respectively, and have no theoretical basis. The broken line is the extrapolation of ΔF to the maximum value of $\Delta F_{\max} = 0.41$.

Fig. 3.

(a) Fluorescence titrations of the dsDNA 15-mer, labeled at the 5' end of one of the strands with fluorescein (Materials and Methods), with the PriA protein ($\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$) in buffer C (pH 7.0, 10 °C), containing 50 mM NaCl, in the absence of nucleotide cofactors, at two different nucleic acid concentrations: ■, $1 \times 10^{-8} \text{ M}$; □, $5 \times 10^{-8} \text{ M}$ (oligomer). The continuous lines are nonlinear least-squares fits of the titration curves, using the statistical thermodynamic model described by Eqs. (4)–(7). (b) Dependence of the relative fluorescence increase of the dsDNA 15-mer, ΔF , upon the total average degree of binding, $\Sigma \Theta_i$ (■). The continuous line is the computer simulation of the relative fluorescence increase of the dsDNA 15-mer upon the total average degree of binding of the PriA helicase on the nucleic acid, obtained using the determined intrinsic binding constant $K_{15} = (1 \pm 0.4) \times 10^7 \text{ M}^{-1}$, the cooperativity factor $\omega = 8$, and spectroscopic parameters $\Delta F_1 = 0.09$, $\Delta F_2 = 0.43$, and $\Delta F_3 = 0.19$.

Fig. 4. Sedimentation equilibrium concentration profile of the dsDNA 10-mer, labeled at the 5' end with fluorescein (Materials and Methods) in the presence of PriA helicase in buffer C (pH 7.0, 10 °C). The concentrations of the nucleic acid and the helicase are 2.6×10^{-7} M and 8×10^{-6} M, respectively. The profile has been recorded at 495 nm and at 8000 rpm. The continuous line is the nonlinear least-squares fit to single exponential function [Eq. (22)], with a single species having a molecular weight of $156,000 \pm 15,000$.

Fig. 5.

Fluorescence titrations of the fluorescent reference ssDNA 18-mer, dεA(pεA)₁₇, with the PriA protein ($\lambda_{\text{ex}} = 325$ nm, $\lambda_{\text{em}} = 410$ nm) in buffer C (pH 7.0, 10 °C) in the absence (■) and presence of two different concentrations of the unmodified dsDNA 10-mer (Materials and Methods). The concentration of the reference ssDNA 18-mer is 1×10^{-6} M. The concentrations of the unmodified dsDNA 10-mer are 1.5×10^{-7} M (□) and 5×10^{-7} M (●). The continuous lines are nonlinear least-squares fits of the binding titration curves using Eqs. (8)–(11) (lattice competition titrations) with the binding constant for dεA (pεA)₁₇, $K_{18} = 1.6 \times 10^6 \text{ M}^{-1}$ and $\Delta F_{\text{max}} = 1.67$, and the intrinsic binding constant, $K_{10S} = 8 \times 10^6 \text{ M}^{-1}$ and $\omega_S = 6$, for the unmodified dsDNA 10-mer.

Fig. 6.

(a) Fluorescence titrations of the dsDNA 10-mer, labeled at the 5' end of one of the strands with fluorescein (Materials and Methods), with the PriA protein ($\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$) in buffer C (pH 7.0, 10 °C), containing different NaCl concentrations: 50 mM (■), 69 mM (□), 88 mM (●), 107 mM (○), 126 mM (◆). The continuous lines are nonlinear least-squares fits of the titration curves, using the statistical thermodynamic model described by Eqs. (1)–(3), with $\Delta F_1 = 0.124$ and $\Delta F_{\text{max}} = 0.4$, and K_{10} and ω , respectively, as follows: $1.6 \times 10^7 \text{ M}^{-1}$ and 10 (■), $7.0 \times 10^6 \text{ M}^{-1}$ and 10 (□), $4.0 \times 10^6 \text{ M}^{-1}$ and 8 (●), $2.0 \times 10^6 \text{ M}^{-1}$ and 6 (○), $8.0 \times 10^5 \text{ M}^{-1}$ and 5 (◆). (b) The dependence of the logarithm of the intrinsic binding constant, K_{10} , upon the logarithm of NaCl concentration. The continuous line is a linear least-squares fit, which provides the slope $\partial \log K_{10} / \partial \log [\text{NaCl}] = -3.1 \pm 0.6$. (c) The dependence of the logarithm of the cooperativity parameter, ω , upon the logarithm of NaCl concentration. The continuous line is a linear least-squares fit, which provides the slope $\partial \log \omega / \partial \log [\text{NaCl}] = -1 \pm 0.3$.

Fig. 7.

(a) Fluorescence titrations of the dsDNA 10-mer, labeled with fluorescein at the 5' end (Materials and Methods), with the PriA helicase in buffer C (pH 7.0), at different temperatures ($^{\circ}\text{C}$): 5 (Δ), 7.5 ($\circ$), 10 ($\blacksquare$), 15 ($\square$), and 20 ($\bullet$). The continuous lines are nonlinear least-squares fits of the titration curves, using the statistical thermodynamic model described by Eqs. (1)–(3). The concentration of the DNA substrate is 1.5×10^{-8} M. (b) The dependence of the natural logarithm of the intrinsic binding constant, K_{10} , upon the reciprocal of the temperature (Kelvin) (van't Hoff plot). The continuous line is the nonlinear least-squares fit of the experimental plot to the model of binding of two PriA molecules in two different conformations, defined by Eqs. (15)–(18) (see the text for details). The dependence of the natural logarithm of the cooperativity parameter, ω , upon the reciprocal of temperature (Kelvin). The continuous line is a linear least-squares fit, which provides the slope $\Delta H_{\omega} = -28.9$ kcal/mol.

Fig. 8.

(a) Fluorescence titrations of the dsDNA 10-mer, labeled at the 5' end with fluorescein (Materials and Methods) with the PriA helicase in buffer C (pH 7.0, 10 °C), containing 1×10^{-5} M ADP, at two different nucleic acid concentrations: ■, 1×10^{-8} M; □, 2.5×10^{-8} M (oligomer). The continuous lines are nonlinear least-squares fits of the titration curves, using the statistical thermodynamic model described by Eqs. (1)–(3) (Table 1). (b) Dependence of the relative fluorescence increase of the dsDNA 10-mer, ΔF , upon the total average degree of binding of PriA helicase on the nucleic acid (■). The continuous line follows the experimental points and has no theoretical basis. The broken line is the extrapolation of ΔF to $\Delta F_{\max} = 0.29$.

Fig. 9.

(a) Fluorescence titrations of the dsDNA 10-mer, labeled at the 5' end with fluorescein (Materials and Methods) with the PriA helicase in buffer C (pH 7.0, 10 °C), containing 3×10^{-3} M ADP, at two different nucleic acid concentrations: ■, 1×10^{-8} M; □, 2.5×10^{-8} M (oligomer). The continuous lines are nonlinear least-squares fits of the titration curves, using the statistical thermodynamic model described by Eqs. (1)–(3) (Table 1). (b) Dependence of the relative fluorescence increase of the dsDNA 10-mer, ΔF , upon the average number of bound PriA molecules (■). The continuous lines indicate the limiting slopes of the plot at low and high enzyme concentration ranges, respectively, and have no theoretical basis. The broken line is the extrapolation of ΔF to the maximum value of $\Delta F_{\max} = 0.93$.

Fig. 10.

Fluorescence titrations of the dsDNA 10-mer, labeled at the 5' end with fluorescein (Materials and Methods) with the PriA helicase in buffer C (pH 7.0, 10 °C), containing 1×10^{-5} M ($\circ$) and 3×10^{-3} M ($\bullet$) ATP γ S, respectively. For comparison, the titrations of the same nucleic acid, in the presence of 1×10^{-5} M ($\square$) and 3×10^{-3} M ($\blacksquare$) ADP, are also included in the figure. The concentration of the nucleic acid is 2.5×10^{-8} M (oligomer). The continuous lines are nonlinear least-squares fits of the titration curves, using the statistical thermodynamic model described by Eqs. (1)–(3) (Table 1).

Fig. 11.

(a) The SDS polyacrylamide gel (10%) of the PriA protein- ^{32}P -dT₂₀ complex subjected to the time-dependent trypsin digestion and stained with Coomassie Brilliant Blue (Materials and Methods). Lane 0 contains the molecular markers; lane 1 contains 5' ^{32}P -dT₂₀ alone without irradiation; lane 2 contains 5' ^{32}P -dT₂₀ alone after irradiation; lane 3 contains the PriA protein- ^{32}P -dT₂₀ complex in the absence of the protease prior to irradiation; lane 4 contains the PriA protein- ^{32}P -dT₂₀ complex, in the absence of the protease after irradiation; lanes 5 to 11 contain the PriA protein- ^{32}P -dT₂₀ complex in the presence of the constant trypsin concentration, collected at different time intervals of the digestion reaction: lane 5, 1 min; lane 6, 3 min; lane 7, 5 min; lane 8, 10 min; lane 9, 20 min; lane 10, 30 min. (b) Autoradiogram of the same 10% SDS polyacrylamide gel of the PriA protein-dT(pT)₁₉ complex, as shown in (a) (see the text for details).

Fig. 12.

Schematic functional models of the PAS-like structures and the ssDNA gap recognition processes by the PriA helicase–nucleotide cofactor complexes. In the case of the PAS-like structure recognition (a), binding of ADP to the strong nucleotide-binding site, or hydrolysis of the bound ATP and the release of ADP from the weak nucleotide-binding site, induces a significant positive cooperativity, which reinforces the enzyme affinity for the dsDNA of the PAS structure. As a result, more than one PriA molecule may participate in the initial PAS recognition process. With both nucleotide-binding sites saturated with ADP, the positive cooperativity is strongly diminished, but the intrinsic affinity of the enzyme is strongly increased, preserving the specificity of the recognition of the PAS structure. In the case of the ssDNA gap at the stalled replication fork (b), mostly the complex, with both nucleotide-binding sites of the PriA protein saturated with ADP, seems to be involved, although the enzyme molecule without nucleotide cofactors may also participate in the recognition process.²¹ Low cooperativity of the PriA protein binding to the DNA, with two nucleotide-binding sites saturated with ADP, indicates that a single PriA molecule participates in the ssDNA gap recognition (see the text for details).²¹

Thermodynamic and spectroscopic parameters of the binding of the *E. coli* PriA helicase to the dsDNA 10-mer, in buffer C (pH 7.0, 10°C), containing 5 mM MgCl₂, and different concentrations of ADP and ATP γ S (see the text for details)

Table 1

Nucleotides	Stoichiometry	K	σ	ΔF_1	$\Delta F_{\max}$
No nucleotides	2	$(1.6\pm0.6)\times10^7$	10 $\pm$ 3	0.12 $\pm$ 0.03	0.41 $\pm$ 0.03
ADP, 1 $\times$ 10 ⁻⁵ M	2	$(8.0\pm2.5)\times10^6$	45 $\pm$ 14	0.14 $\pm$ 0.03	0.29 $\pm$ 0.03
ADP, 3 $\times$ 10 ⁻³ M	2	$(1.3\pm0.4)\times10^8$	2 $\pm$ 0.6	0.21 $\pm$ 0.03	0.93 $\pm$ 0.03
ATP γ S, 1 $\times$ 10 ⁻⁴ M	2	$(5.8\pm1.4)\times10^6$	12 $\pm$ 4	0.20 $\pm$ 0.03	0.35 $\pm$ 0.03
ATP γ S, 3 $\times$ 10 ⁻³ M	2	$(2.8\pm0.6)\times10^6$	16 $\pm$ 6	0.15 $\pm$ 0.03	0.40 $\pm$ 0.03

Errors are standard deviations determined using three to four independent experiments.