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Interactions of the *Escherichia coli* Primosomal PriB Protein with the Single-Stranded DNA. Stoichiometries, Intrinsic Affinities, Cooperativities, and Base Specificities§

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Abstract

Quantitative analyses of the interactions of the *E. coli* primosomal PriB protein with a single-stranded DNA have been performed, using quantitative fluorescence titration, photo-crosslinking, and analytical ultracentrifugation techniques. Stoichiometry studies were performed using a series of etheno-derivatives of ssDNA oligomers. Interactions with the unmodified nucleic acids were addressed, using the Macromolecular Competition Titration (MCT) method. The total site-size of the PriB dimer - ssDNA complex, *i.e.*, the maximum number of nucleotides occluded by the PriB dimer in the complex is 12 ± 1 nucleotides. The protein has a single DNA-binding site, which is centrally located within the dimer and has a functionally homogeneous structure. The determined stoichiometry and photo-crosslinking data show that only a single monomer of the PriB dimer engages in interactions with the nucleic acid. The analysis of the PriB binding to long oligomers was performed using the statistical thermodynamic model that takes into account the overlap of potential binding sites and cooperative interactions. The PriB dimer binds the ssDNA with strong positive cooperativity. Both the intrinsic affinity and cooperative interactions are accompanied by a net ion release, with anions participating in the ion exchange process. The intrinsic binding process is an entropy-driven reaction, strongly suggesting that the DNA association induced a large conformational change in the protein. The PriB protein shows a dramatically strong preference for the homo-pyrimidine oligomers with an intrinsic affinity higher by ~3 orders of magnitude, as compared to the homo-purine oligomers. The significance of these results for the PriB protein activities is discussed.

Keywords

E. coli PriB Protein; Primosome; DNA Binding; Fluorescence Titrations

Priming of the DNA strand during the replication process is catalyzed by a multiple-protein complex called the primosome^{1–18}. The complex synthesizes short oligoribonucleotide primers, which are used to initiate synthesis of the complementary DNA strand. The

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assembly process of the primosome was first characterized for the specific primosome assembly site (PAS) in the synthesis of the complementary DNA strand of phage ϕ X174 DNA¹⁻⁴. Current data show that the assembly of the primosome is a fundamental step in the restart of the stalled replication fork at the damaged DNA sites¹⁰⁻¹⁶.

The PriB protein is an essential replication protein in *Escherichia coli* that plays a fundamental role in the ordered assembly of the primosome¹⁻¹⁸. The assembly process is initiated by recognition of the PAS sequence or damaged DNA site by the PriA protein. The next key step includes the association of the PriB protein with the PriA - DNA complex, followed by binding of the DnaT and the PriC protein. The formed entity constitutes a scaffold recognized by the DnaB helicase - DnaC protein complex and, subsequently, by the primase resulting in a functional primosome. The PriB protein was originally discovered to be an essential factor during synthesis of the complementary DNA strand of phage ϕ X174 DNA^{3,4}. The gene encoding the PriB protein has been cloned and its sequence determined^{7,8}. The molecular weight of the protein monomer is ~11.4 kDa^{5,7,8,9}. The native protein is a homo-dimer and the dimer is the predominant form of the protein in solution^{5,7,8,9}. The crystal structures of the PriB dimer and its complex with the ssDNA oligomer have been solved to a resolution of 2 – 2.7 Å¹⁹⁻²³. The structure of the PriB dimer alone is depicted in Figure 1a¹⁹⁻²². The structure of the PriB dimer - ssDNA complex with the proposed engagement of the nucleic acid in interactions with the protein, based on crystallographic analyses, is shown in Figure 1b²² (see below).

Studies *in vitro* indicate that the PriB protein displays multiple activities: 1) Specific interactions with the PriA protein and the PriA - ssDNA complex, which is considered the major biological function of the PriB protein in the assembly process of the primosome^{5,6,10,11,15,18,23}; 2) Specific interactions with the DnaT protein, which is considered a key step in the recruitment of the replicative helicase, DnaB protein, to the primosome^{10-12,23}; 3) Nonspecific binding to the ssDNA, which is proposed to be crucial for recognition of the PriA - ssDNA complex and recruiting the DnaT protein to the primosome^{10-12,18,23}; 4) Specific interactions with the SSB protein⁵; 5) Strong binding to the ssDNA coated by the SSB protein⁵. These multiple activities reflect complex interactions of PriB with different ingredients of the primosome, including protein - protein and protein - ssDNA interactions⁴⁻¹⁸.

Most of the *in vivo* functions of the PriB protein are related to the ability of the protein to interact with the ssDNA and ssDNA-protein complexes^{4-18,20,22,23}. In crystal, the structure of the PriB dimer and the ssDNA-binding pocket of each monomer resemble that of the *E. coli* single-stranded binding protein (SSB), suggesting a similar mode of engaging the DNA by both proteins¹⁹⁻²³. Although the importance of understanding the PriB protein - ssDNA interactions has been recognized, quantitative aspects of these interactions remain obscure. Such fundamental quantities as the stoichiometry (site-size) of the PriB - ssDNA complex, *i.e.*, the number of nucleotides occluded by the protein in the complex, the number of the ssDNA-binding sites on the PriB dimer, are unknown. Computer modeling of the crystal structure inferred that both monomers of the dimer can engage in interactions with the ssDNA, *i.e.*, the presence of two ssDNA-binding sites on the PriB dimer, as depicted in Figure 1b²². However, this has never been experimentally established in solution. Little is known about the energetics of the PriB protein - DNA complexes^{19,22}. Nothing is known about the intrinsic affinities and cooperativities of the PriB protein - ssDNA interactions, and the effects of solution conditions, salt and type of salt on the complex formation.

Knowledge of the energetics and mechanisms of the PriB - ssDNA complex formation is of fundamental importance for our understanding of the activities of this essential protein in DNA metabolism¹⁻²³. In this communication, we report the quantitative analyses of the

PriB interactions with the ssDNA. We establish that, in solution, the PriB dimer occludes 12 ± 1 nucleotides in the complex and the dimer has a single, functionally homogeneous ssDNA-binding site. Moreover, only one monomer of the dimer engages in interactions with the nucleic acid. The PriB dimer binds the ssDNA with strong positive cooperativity. The protein shows a very strong preference for the homo-pyrimidine oligomers, as compared to the homo-purine ssDNA with intrinsic affinities differing by ~ 3 orders of magnitude.

RESULTS

Determination of the Total Site-Size of the PriB Protein- ssDNA Complex

Although binding of the PriB protein to the ssDNA homopolymers is accompanied by a modest change in the protein fluorescence emission²⁰ (data not shown), we have found that formation of the complex with the etheno-derivative of the adenosine polymer and oligomers causes a strong nucleic acid fluorescence increase, providing an excellent signal to perform the high-resolution measurements of the stoichiometry and mechanism of the protein - ssDNA complex formation (Materials and Methods). To address the fundamental problem of the stoichiometry of the PriB - ssDNA complex, we performed a series of quantitative studies using several ssDNA oligomers with different numbers of nucleotides^{25–30}. The use of the oligomers is also dictated by the fact that the PriB - polymer ssDNA complex precipitates at higher protein concentrations (data not shown). Using the analytical ultracentrifugation technique, we determined that the molecular weight of the protein is 23 ± 2 kDa and remains constant, within experimental accuracy, over a large protein concentration range (see below). These results indicate that the PriB protein exists as a stable dimer in the protein concentration range studied in this work.

Fluorescence titrations of the 20-mer, dεA(pεA)₁₉, with the PriB protein at two different nucleic acid concentrations, in buffer C (pH 7.0, 10°C), are shown in Figure 2a. The shift of the titration curve at a higher nucleic acid concentration, results from the fact that more protein is required to obtain the same total average degree of binding, $\Sigma\Theta_i$. The selected nucleic acid concentrations provide separation of the binding isotherms up to the relative fluorescence increase of ~ 0.8 . To obtain thermodynamic binding parameters, independent of any assumption about the relationship between the observed signal and the total average degree of binding, $\Sigma\Theta_i$ the fluorescence titration curves, shown in Figure 2a, have been analyzed, using the quantitative approach outlined in Materials and Methods^{31–36}. Figure 2b shows the dependence of the relative fluorescence increase of the 20-mer, ΔF , as a function of the total average degree of binding, $\Sigma\Theta_i$ of the PriB protein. Extrapolation to the maximum fluorescence change, $\Delta F_{\max} = 1.18 \pm 0.03$, provides the stoichiometry of the complex 0.93 ± 0.15 . Thus, a single PriB dimer binds to the ssDNA 20-mer.

However, the maximum stoichiometry of the PriB - ssDNA oligomer changes for the 24-mer, dεA(pεA)₂₃, although this oligomer is only 4 nucleotides longer than the 20-mer. Fluorescence titrations of the dεA(pεA)₂₃ with the PriB protein at two different oligomer concentrations, in buffer C (pH 7.0, 10°C), are shown in Figure 2c. Separation of the titration curves allowed us to determine the total average degree of binding, $\Sigma\Theta_i$, up to ~ 1.4 . The dependence of the relative fluorescence increase of the 24-mer, as a function of $\Sigma\Theta_i$ of the PriB protein on the oligomer, is shown in Figure 2d. The plot is nonlinear, indicating the presence of two, high and low affinity, binding phases³⁴. Extrapolation of the weak affinity phase to the maximum fluorescence increase $\Delta F_{\max} = 1.32 \pm 0.03$ provides $\Sigma\Theta_i = 1.9 \pm 0.2$. Thus, the 24-mer provides enough interaction space for the binding of two PriB dimers.

On the other hand, further increase of the nucleic acid length by as much as 11 nucleotides does not affect the maximum stoichiometry of the two PriB dimers bound per ssDNA oligomer. Fluorescence titrations of the 35-mer, dεA(pεA)₃₄, with the PriB protein at two

different nucleic acid concentrations, in buffer C (pH 7.0, 10°C) are shown in Figure 3a. The maximum increase of the nucleic acid fluorescence is significantly higher than that observed for the 20- and 24-mer and the plot is, within experimental accuracy, linear. The dependence of the relative fluorescence increase of the 35-mer, as a function of the total average degree of binding of the PriB protein on the oligomer, is shown in Figure 3b. Extrapolation of the plot to the maximum fluorescence increase $\Delta F_{\max} = 2.0 \pm 0.05$ provides $\Sigma\Theta_i = 2.1 \pm 0.2$.

Maximum Stoichiometry of the PriB Dimer - ssDNA Complex. Sedimentation Equilibrium Studies

Further analysis of the PriB - ssDNA oligomers has been performed using the independent sedimentation equilibrium technique³⁶⁻⁴². In these studies, we utilize the 21- and 27-mer containing fluorescein at its 5' end, 5'Fl-dT(pT)₁₉ and 5'Flu-dT(pT)₂₅ (Materials and Methods). Thus, the sedimentation equilibrium profile of the ssDNA oligomer can be exclusively monitored at the fluorescein absorption band, without any interference of the protein absorbance. The experiments have been performed at the large excess of the PriB protein to assure complete saturation of the nucleic acid over the entire equilibrium profile³⁶⁻⁴¹. Because the molecular weights of the 21- or 27-mer are ~6700 and ~8600, respectively, the formation of the complex between the oligomer and the PriB protein dimer (M.W. ~23000) will be manifested by a large increase of the molecular weight of the nucleic acid.

Sedimentation equilibrium profiles of the ssDNA 21-mer, 5'Fl-dT(pT)₁₉, in the presence of the PriB dimer and recorded at the fluorescein absorption band (495 nm) is shown in Figure 4a. The protein and the nucleic acid concentrations are 1.86×10^{-5} M (dimer) and 1.56×10^{-6} M (oligomer), respectively. The solid line is the nonlinear least squares fit, using the single exponential function defined eq. 14 (Materials and Methods). Adding additional exponents does not improve the statistics of the fit (data not shown). The fit provides an excellent description of the experimental curve indicating the presence of a single species with the molecular weight of 27200 ± 2900 . Therefore, the data show that a single PriB dimer binds to the ssDNA 21-mer.

Corresponding sedimentation equilibrium profiles of the ssDNA 27-mer, 5'Fl-dT(pT)₂₅, in the presence of the PriB dimer, is shown in Figure 4b. The protein and the nucleic acid concentrations are 1×10^{-5} M (dimer) and 1.58×10^{-6} M (oligomer), respectively. The solid line is the nonlinear least squares fit, using the single exponential function defined by eq. 14 (Materials and Methods), which indicates the presence of a single species with the molecular weight of 51300 ± 5100 . Thus, the data show that two PriB dimers bind to the 27-mer, which is in excellent agreement with the fluorescence titration data (Figures 2 and 3) (see below).

Number of Nucleotides Directly Engaged in Interactions with the ssDNA-Binding Site of the PriB Dimer

Analogous quantitative analysis of the maximum stoichiometry of PriB - ssDNA complexes, as described above, has been performed for a series of ssDNA oligomers²⁵⁻³⁰. The dependence of the maximum number of bound PriB dimers per ssDNA oligomer upon the length of the oligomer is shown in Figure 5. The selected ssDNA oligomers range from 14 to 35 nucleotides in length. A single PriB dimer binds to the oligomers containing 14, 16, 18, and 20 nucleotides. Sharp transition from a single dimer bound per ssDNA oligomer to two dimers bound per oligomer occurs between 20- and 24-mers (Figure 5). However, a further increase in the length of the oligomer, up to 35 nucleotides, does not lead to the increase of the number of bound PriB dimers. These data indicate that a total site-size of the

PriB - ssDNA complex must contain at least 11 but less than 18 nucleotides per protein dimer (see below).

Intrinsic Affinities of PriB - ssDNA Interactions

Binding of a single PriB dimer to 14-, 16-, 18-, and 20-mer can be analyzed using a single-site-binding isotherm described by

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

where K_N is the macroscopic binding constant characterizing the affinity for a given ssDNA oligomer, containing N nucleotides, and $\Delta F_{\max}$ is the maximum relative fluorescence increase. The solid lines in Figures 2a are nonlinear least squares fits of the experimental titration curve for the PriB - 20-mer system using equation 1, and a single set of binding and spectroscopic parameters. The values of K_N for all studied ssDNA oligomers, which can accept only a single PriB dimer, are included in Table 1.

The value of K_N , within experimental error, increases as the length of the ssDNA oligomers increase (Table 1). This behavior indicates the presence of a statistical factor hidden in K_N and resulting from the fact that the number of nucleotides engaged in direct interactions with the ssDNA-binding site of the PriB dimer must be less than the length of the examined ssDNA oligomers^{34,43-45}. These direct intrinsic interactions are characterized by the intrinsic binding constant, K_i . Using K_i , the expression describing the binding of the PriB dimer to the ssDNA oligomers, which can accommodate only a single dimer molecule, is defined as

$$\Delta F = \Delta F_{\max} \left(\frac{(N - n + 1)K_i P_F}{1 + (N - n + 1)K_i P_F} \right) \quad (2)$$

where n is the number of nucleotides engaged in direct interactions with the DNA-binding site of the protein. Thus, expression 2 takes into account the overlap of the potential binding sites^{34,43-45}. The determined macroscopic binding constant, K_N , is then defined as

$$K_N = (N - n + 1)K_i \quad (3a)$$

and

$$K_N = NK_i - (n - 1)K_i \quad (3b)$$

Therefore, K_N should be a linear function of N with the slope $\partial K_N / \partial N = K_i$. Moreover, for $K_N = 0$, the plot of K_N , as a function of the nucleic acid length, will intercept the N axis at the value of $N = n - 1$. Figure 6 shows the dependence of the macroscopic equilibrium constant, K_N , for the PriB dimer binding to the ssDNA oligomers, which can accept only a single dimer, as functions of the ssDNA oligomer length. The plot is strictly linear. Such behavior of K_N as a function of the length of ssDNA provides very strong evidence for the existence of several potential binding sites on the ssDNA oligomers (eqs. 3a and 3b)^{34,43-45}.

Extrapolation of the plot to zero value of the macroscopic equilibrium constant intercepts the DNA length axis at $N = n - 1 = 11.3 \pm 1$ (Figure 6). Thus the obtained data show that the site-size of the PriB dimer - ssDNA complex is $n = 12 \pm 1$ nucleotides (see Discussion).

Statistical Thermodynamic Model of the PriB Dimer Binding to the ssDNA Oligomers, Which Can Accept Two Dimer Molecules. Intrinsic Affinities and Cooperativities

Quantitative analysis of the PriB dimer binding to the 24-, 26-, 30-, and 35-mer is much more complex. The partition function, Z_N , for these systems must account for the potential overlap of the binding sites and the possible cooperative interactions between the bound protein molecules. Such a binding system can be directly treated by the exact combinatorial theory for large ligand binding to a finite linear, homogeneous lattice^{34,44}. The partition function, Z_N , of the system is defined as

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

where g is the maximum number of ligand molecules which may bind to the finite nucleic acid lattice (for the nucleic acid lattice N residues long, $g = N/n$), ω is the cooperative interactions 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

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

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

$$\Delta F = \Delta F_1 \left[\frac{(N - n + 1) K_i P_F}{Z_N} \right] + \Delta F_{\max} \left[\frac{\sum_{j=0}^{k-1} P_N(k, j) (K_i P_F)^k \omega^j}{Z_N} \right] \quad (7)$$

where ΔF_1 and $\Delta F_{\max}$ are the relative molar fluorescence increases accompanying the binding of one and two PriB dimers. The values of the total site-size of the PriB - ssDNA

complex, $n = 12$, is known (see above). The value of ΔF_1 can be estimated for each particular ssDNA oligomer as $\Delta F_1 = \partial \Delta F / \partial \Sigma \Theta_i$, from the initial part of the plot of ΔF as a function of $\Sigma \Theta_i$, as shown in Figures 2b and 3b for the 24- and 35-mers. The value of $\Delta F_{\max}$ is the maximum observed relative fluorescence increase and can be estimated from the parental fluorescence titration curves, as shown in Figures 2c and 3a. Thus, two independent parameters, K_i , and ω must be determined. The solid lines in Figures 2c and 3a are nonlinear least squares fits of the titration curves using eqs. 4 – 7, which provide an excellent description of the experimental data. The obtained spectroscopic and binding parameters for all examined etheno-derivatives of the ssDNA oligomers, which can accommodate two PriB dimers, are included in Table 1.

The values of the determined intrinsic binding constants for the 24-, 26-, 30-, and 35-mer are very similar to each other and to the values of K_i , obtained for the oligomers, which can accommodate only a single dimer molecule. Such similarity indicates that the same intrinsic binding process is observed. The value of the cooperativity parameter, $\omega \approx 45 - 50$ is large, indicating the PriB dimer binds the ssDNA with strong positive cooperative interactions^{34,44}. Moreover, within experimental accuracy, ω is the same for the 24-, 26-, 30-, and 35-mer. As pointed out above for the intrinsic affinity, the same value of ω indicates the same nature of the cooperative interactions in complexes with different oligomers. The large value of the cooperativity parameter indicates that the PriB dimer is capable of forming long clusters on the nucleic acid lattice (see Discussion). The value of ΔF_1 , which characterizes the binding of the first PriB dimer, is lower than those observed for the ssDNA oligomers, which accept only a single PriB dimer (Table 1). Such a lower value of ΔF_1 reflects the fact that a single PriB dimer exerts less pronounced effect on the nucleic acid structure in the case of the longer oligomer, where large part of the nucleic acid is not engaged in interactions with the protein. Nevertheless, with the exception of the 35-mer, the value of $\Delta F_{\max}$ is similar to the analogous parameter obtained for shorter oligomers, indicating similar nucleic acid structure in the examined complexes (Table 1).

Salt Effect on the Intrinsic PriB - ssDNA Interactions

Fluorescence titrations of dεA(pεA)₁₉ with the PriB protein, in buffer C (pH 7.0, 10°C) containing different NaCl concentrations, are shown in Figure 7a. Analogous titrations have been performed in the presence of NaBr (data not shown). As the salt concentration increases, there is a modest decrease of the maximum fluorescence increase at saturation, $\Delta F_{\max}$, from ~1.2 at 100 mM to ~1.0 at 154 mM NaCl, indicating the similar structure of the nucleic acid in the complex. The solid lines in Figures 7a are nonlinear least squares fits to a single-site binding model with two fitting parameters, K_{20} , and the maximum relative fluorescence increase, $\Delta F_{\max}$ (eq. 1). Figure 7b shows the dependence of the logarithm of K_{20} upon the logarithm of NaCl and NaBr concentrations (log-log plots)^{46,47}. The plots are linear in the examined salt concentration range and are characterized by the slopes $\partial \log K_{20} / \partial \log [\text{NaCl}] = -5.7 \pm 0.5$ and $\partial \log K_{20} / \partial \log [\text{NaBr}] = -5.4 \pm 0.5$, respectively. The values of the slopes indicate that there is a net release of ~ 5 – 6 ions upon the complex formation. However, there is a clear anion effect on the interactions reflected in the value of the binding constant, which is lower in the presence of NaBr, as compared to K_{20} in the analogous NaCl concentrations (see Discussion)^{46,47}.

Magnesium Effect on the Intrinsic PriB - ssDNA Interactions

A series of fluorescence titrations of dεA(pεA)₁₉ with the PriB protein, in buffer C (pH 7.0, 10°C), containing different MgCl₂ concentrations, are shown in Figure 7c. Both macroscopic affinity and $\Delta F_{\max}$ decrease as a result of the magnesium concentration increase. The solid lines in Figure 7c are nonlinear least squares fits to a single-site-binding model, with two fitting parameters, intrinsic binding constant, K_{20} , and $\Delta F_{\max}$ (eq. 1).

Figure 7d shows the dependence of the logarithm of K_{20} upon the logarithm of $[\text{MgCl}_2]$ (log-log plot)^{46,47}. The plot is clearly nonlinear. At low magnesium concentrations, the slope, $\partial \log K_{20} / \partial \log [\text{MgCl}_2] = \sim 0 \pm 0.2$. Above $\sim 1 \text{ mM}$ MgCl_2 the affinity dramatically decreases, indicating that the intrinsic binding process is accompanied by a net ion release. At high magnesium concentrations, the linear part of the plot is characterized by the $\partial \log K_{20} / \partial \log [\text{MgCl}_2] = -1.8 \pm 0.4$, which is significantly lower than the -5.7 ± 0.6 obtained in the presence of NaCl alone (Figure 7a, see Discussion).

Salt Effect on Intrinsic Affinity and Cooperativity of the PriB Dimer Binding to the ssDNA 26-mer, dεA(pεA)₂₅

To address the salt effect on the intrinsic affinity and cooperativity of the PriB protein binding to the ssDNA, we selected the ssDNA 26-mer, which can accommodate two PriB dimer molecules (Table 1). Fluorescence titrations of dεA(pεA)₂₅, with the PriB protein in buffer C (pH 7.0, 10°C), containing different NaCl concentrations, are shown in Figure 8a. Analysis of the titration curves has been performed, as described above, and the solid lines in Figure 8a are nonlinear least squares fits using eqs. 4 – 7. The dependence of the logarithm of the intrinsic binding constant, K_i , upon the logarithm of $[\text{NaCl}]$ is shown in Figure 8b. The plot is linear in the studied salt concentration range and is characterized by the slope $\partial \log K_i / \partial \log [\text{NaCl}] = -5.3 \pm 0.6$, which is, within experimental accuracy, the same as observe for the 20-mer, which can accommodate only one PriB dimer (see above). Thus, the intrinsic interactions between the PriB dimer and the 26-mer are accompanied by the release of $\sim 5 - 6$ ions (see Discussion). The dependence of the logarithm of the cooperative interactions parameter, ω , upon the logarithm of $[\text{NaCl}]$, is shown in Figure 8c. The value of ω strongly decreases with increasing $[\text{NaCl}]$, indicating that positive cooperative interactions are weakened by high salt concentrations. The salt effect on the free energy of cooperative interactions between the two bound PriB molecules is characterized by the negative slope, $\partial \log \omega / \partial \log [\text{NaCl}] = -2.8 \pm 0.6$. Thus, cooperative interactions between the bound protein molecules are accompanied by the net release of ~ 3 ions (see Discussion).

Temperature Effect on the Intrinsic PriB - ssDNA Interactions

We further address the nature of the intrinsic interactions in the PriB - ssDNA complex, by examining the temperature effect on the protein binding to the ssDNA 20-mer, dεA(pεA)₁₉. Fluorescence titrations of dεA(pεA)₁₉ with the PriB protein performed at different temperatures, are shown in Figure 9. It is evident that in the range from 5°C to 20°C, neither the intrinsic affinity nor the values of ΔF_{max} is affected by temperature. The solid line in Figure 9 is the nonlinear least squares fit of the experimental titration curve, obtained at 10°C, using $K_i = 1.9 \times 10^5 \text{ M}^{-1}$ and $\Delta F_{\text{max}} = 1.18$ (Table 1). Within experimental accuracy, the fit provides an adequate description for titration curves obtained at all examined temperatures. Thus, the PriB - ssDNA interactions are characterized by the apparent enthalpy change $\Delta H^\circ \approx 0$. In other words, the intrinsic interactions are completely driven by the apparent entropy change, ΔS° . Using the standard thermodynamic formulas, $\Delta G^\circ = -RT \ln K_i$, and $\Delta S^\circ = (-\Delta G^\circ + \Delta H^\circ) / T$, one obtains the free energy of binding at 10°C, $\Delta G^\circ \approx -6.8 \text{ kcal/mole}$, which provides $\Delta S^\circ \approx 24 \text{ cal/mol deg}$ (see Discussion).

Base Specificity of PriB - ssDNA Interactions. Lattice Competition Titrations Using the MCT Method

Quantitative determination of affinities of the PriB protein for unmodified ssDNAs, differing by the type of base, has been performed, using the macromolecular competition titration (MCT) method^{34,48–51}. The approach is based on the same thermodynamic arguments as applied to quantitative titrations of fluorescent nucleic acids with the protein (Materials and Methods). In the presence of the competing unmodified ssDNA oligomer, the protein binds to two different nucleic acids that are present in the solution, but the observed

signal originates only from the fluorescent “reference” nucleic acid. In studies described in this work we use, as a reference lattice, the 20-mer, dεA(pεA)₁₉, and the base specificity of the PriB protein has been examined using different 20-mers, dN(pN)₁₉. At a given titration point, “i”, the total concentration of the bound protein, P_b, is defined as^{34,48–51}

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

where $(\sum \Theta_i)_R$ and $(\sum \Theta_i)_S$ are the total average degree of binding of the PriB protein on the reference dεA(pεA)₁₉ and the examined oligomer, dN(pN)₁₉, respectively, M_{TR} and M_{TS} are the total concentrations of the reference and the unmodified 20-mer, respectively. Using the corresponding macroscopic binding constant, K_{20R} and K_{20S}, the above expression is defined as

$$P_b = \left(\frac{K_{20R} P_F}{1 + K_{20R} P_F} \right) M_{TR} + \left(\frac{K_{20S} P_F}{1 + K_{20S} P_F} \right) M_{TS} \quad (9b)$$

The concentration of the free protein, P_F, is then

$$P_F = P_T - P_b \quad (10)$$

where P_T is the known total concentration of the PriB protein. The observed relative fluorescence increase, ΔF, is then defined by eq. 1^{34,48–51}.

Fluorescence titrations of dεA(pεA)₁₉ with the PriB protein in buffer C (pH 7.0, 10°C) in the presence of two different dT(pT)₁₉ concentrations, are shown in Figure 10a. For comparison, the titration curve of dεA(pεA)₁₉ in the absence of the competing dT(pT)₁₉ is also included. The titration curve dramatically shifts, with increasing dT(pT)₁₉ concentration, indicating a strong competition between dεA(pεA)₁₉ and dT(pT)₁₉ for the PriB protein. Because K_{20R} = 1.7 × 10⁶ M^{−1} and ΔF_{max} = 1.18 are known from independent titration experiments (Figure 2a), the solid lines in Figure 10a are nonlinear least squares fits of the experimental titration curves, with a single fitting parameter, K_{20S}, using eqs. 9 – 10. Analogous fluorescence titrations of dεA(pεA)₁₉ with the PriB protein, in the presence of dA(pA)₁₉ and dC(pC)₁₉, at the same concentration of both ssDNA oligomers, are shown in Figure 10b, together with the titration curve of dεA(pεA)₁₉ in the absence of the competing oligomers. As observed for dT(pT)₁₉, the titration curve significantly shifts in the presence of dC(pC)₁₉, while it is barely affected by the same concentration of dA(pA)₁₉. The solid lines in Figure 10a are nonlinear least squares fits of the experimental titration curves, with a single fitting parameter, K_{20S}, for dA(pA)₁₉ and dC(pC)₁₉, respectively, using eqs. 9 – 10. The binding constants for all examined 20-mers, differing by the type of the base, are included in Table 2. The obtained value of K_{20S} for pyrimidine oligomers dT(pT)₁₉ and dC(pC)₁₉ are ~3 and ~2 of magnitude higher than the analogous parameter determined for the homo-purine oligomer, dA(pA)₁₉ (see Discussion).

Photo-Cross-Linking of the PriB Dimer To the ssDNA

The involvement of the monomers of the PriB dimer in interactions with the ssDNA, has been addressed using the UV irradiation⁶². UV irradiation produces covalent linkage between nucleic acid bases and amino acid residues through free-radical mechanisms

between photo-excited nucleic acid bases and the amino acid residues in very close proximity, resulting in a “zero-length” cross-linking with minimal perturbation to the protein - nucleic acid complex. Among the nucleic acid bases, thymine is by far the most reactive in the photo-cross-linking reactions. Therefore, parallel to our thermodynamic binding studies, we performed photo-cross-linking experiments of the PriB dimer - dT(pT)₁₉ complex. Only a single PriB dimer can detectably associate with the ssDNA 20-mer (see above). The nucleic acid has been labeled at the 5' end with [³²P], using polynucleotide kinase^{63,64}. Figure 11a shows the autoradiogram of the SDS polyacrylamide gel of the PriB - dT(pT)₁₉ complex, after irradiation, at different protein concentrations. At the highest protein concentration applied, the nucleic acid is completely saturated with the protein. Only a single radioactive band appears on the gel, at the molecular weight of ~17,000, corresponding to the PriB monomer - 20-mer complex. The same gel but stained with Coomassie Brilliant Blue is shown in Figure 11b. A single protein band at ~12,000 indicates the location of the PriB monomer. The same location of the PriB monomer band is observed for the non-irradiated complex and the PriB protein alone (Figure 11b). It is evident that only one monomer of the PriB dimer is engaged in interactions with ssDNA in the complex, independent of the protein concentration (see Discussion).

DISCUSSION

The Total Site-Size of the PriB Dimer - ssDNA Complex Is 12 ± 1 Nucleotides

The total site-size of a large protein ligand - DNA complex corresponds to the DNA fragment, which includes nucleotides prevented from interacting with another protein molecule by the protruding protein matrix of the previously bound protein⁴³⁻⁴⁵. This fundamental quantity is of paramount importance in any quantitative analysis of the energetics and dynamics of the protein - DNA complex and, in turn, in any analyses of the physiological activities of the protein. The strategy applied in this work relies on examination of the maximum stoichiometry and macroscopic affinity of the PriB - ssDNA complexes with the ssDNA oligomers, which can accommodate one or two PriB dimers²⁵⁻³⁰. The strictly linear dependence of the macroscopic binding constant for the oligomers, which can accommodate only a single PriB dimer, upon the length of the ssDNA oligomer (Figure 6) indicates that the minimum length of the nucleic acid, which forms all necessary contacts with the binding site on the protein, is 12 ± 1 nucleotides.

The change of the maximum stoichiometry from one to two bound PriB dimers occurs between the oligomers containing 20 and 24 nucleotides, indicating that a stretch of 12 nucleotides is long enough for the second PriB dimer to associate with the oligomer (Figure 5). Moreover, only two PriB dimers associate with the 35-mer, which is only possible if the total site-size is 12 nucleotides. Furthermore, these data indicate that the ssDNA-binding site must be centrally located, with respect to the dimer molecule. As pointed out above, the very similar intrinsic affinities determined for the oligomers, which can accept one PriB dimer or two dimers, indicate that the same intrinsic binding process, *i.e.*, association with the same number of 12 nucleotides is observed (Table 1).

The PriB Dimer Has Only One DNA-Binding Site Located on a Single Monomer

Based on crystallographic analysis, the model of the PriB dimer - ssDNA interactions was proposed, where both protein monomers are able to engage the ssDNA 15-mer in interactions in the assumed DNA-binding pockets, although located on two different dimers (Figure 1b)²². In other words, the PriB dimer would have two possible DNA-binding sites, though not necessarily equivalent ones. Moreover, the proposed model also indicates that two PriB dimers can bind to the ssDNA 15-mer. However, this is not experimentally observed in solution. First, the determined total site-size of the protein DNA-binding site is

only 12 ± 1 nucleotides (see above). This is just enough to fill a single DNA-binding pocket of one monomer but not long enough to wrap the nucleic acid around the protein dimer²². Second, binding of the 14-, 16, 18, and 20-mer to the PriB protein shows that even an ~10 fold increase of the oligomer concentration does not change the 1:1 stoichiometry of the complex (Figures 2a and 2b). These results provide strong thermodynamic evidence that the dimer has a single ssDNA-binding site. If there is another binding site, its affinity must be ~2 orders of magnitude lower than the affinity of the strong site and it would be observed as a dramatic change of the stoichiometry. Third, only two PriB dimers bind to 26-, 30-, and 35-mers (Figures 2d and 3b). The length of these oligomers is large enough to fully access to the presumed second DNA-binding site on another monomer.

Notice, the local concentration of these longer oligomers in the complex with the PriB dimer is very high. If there was a second binding site with detectable affinity, it would be accessed by the long oligomers and manifested in the large change of the total site-size of the complex, and the intrinsic affinity. Yet, this is not experimentally observed. The total site-size of the PriB dimer with the longer oligomers is 12 ± 1 nucleotides and the intrinsic affinity is very similar to the corresponding affinity of the shorter oligomers (Table 1). Finally, the photo-cross-linking data clearly indicate that only a single monomer engages the ssDNA in the complex, independent of the protein concentration (Figures 11a and 11b). It seems that the model arrangement of the PriB dimers on the ssDNA, proposed on the basis of crystallographic studies, is a result of the crystal packing forces, rather than reflecting functional interactions of the PriB dimer with the ssDNA in solution²².

Our data indicate that the PriB dimer behaves like a protein with half-site reactivity, where only one monomer of the dimer can engage in interactions with the nucleic acid⁵². This is a well-known phenomenon in enzymology, where only half of protomers of the multi-subunit enzyme engages in its activity⁵². Nevertheless, the functional significance of such a behavior, in the case of the PriB dimer, is unknown. Also, it is unknown whether or not the PriB dimer exists in solution in a pre-equilibrium between two conformational states and only one state is selected by the nucleic acid, or the dimer has an asymmetric structure with only one monomer designated to engage the nucleic acid and the other to engage other protein components of the primosome. Our laboratory is currently examining these possibilities.

The ssDNA-Binding Site of the PriB Dimer Has Functionally Homogenous Structure

Although the total site-size of the protein - DNA complex includes nucleotides inaccessible for the binding of another protein molecule, it may have a heterogeneous structure and contain an area, which is directly involved in interactions with the nucleic acid, the strong DNA-binding subsite, or the DNA-binding site proper, as well as the nucleotides not engaged in direct interactions, but occluded by the protruding protein matrix. Such a pronounced heterogeneous structure of the total DNA-binding site has been found, *e.g.*, for the *E. coli* PriA and DnaB helicases, plasmid RSF1010 Rep helicase, rat and human pol β , as well as polymerase X of the African Swine Fever virus and plays a significant functional role in activities of these enzymes^{25–29,53–56}. However, the transition from a single PriB dimer bound per ssDNA oligomer to two bound dimers occurs between 20- and 24-mers, indicating that the DNA-binding site, which occludes the total site-size of ~12 nucleotides, is fully engaged in the protein - nucleic acid interactions. Binding of only two PriB dimers to 26-, 30-, and 35-mers requires that the protein occludes the same number of ~12 nucleotides in all of these complexes, *i.e.*, there is not protein matrix protruding beyond the total site-size. In other words, the DNA-binding site of the PriB dimer is, in its entirety, the functional DNA-binding site, or it has a functionally homogeneous structure (see below).

PriB Dimer Binds the ssDNA With Significant Positive Cooperativity

The cooperative binding of the PriB dimer to ssDNA has been previously inferred on the basis of the Hill coefficient²². However, the values of the Hill coefficient significantly higher than 1, have been determined for the ssDNA oligomers containing 15 nucleotides, which bind only a single PriB dimer, and the cooperative interactions cannot occur²². Apparent affinities of the PriB protein for the ssDNA oligomers have also been obtained assuming a noncooperative binding process²⁴. The statistical thermodynamic approach applied in this work allows us to quantitatively extract both the intrinsic affinities and the cooperative interactions parameter, ω , characterizing the interactions between the bound PriB dimers^{43–45}. The obtained value of $\omega \sim 45 - 50$ indicates that PriB dimer engages in strong positive cooperative interactions when associated with the ssDNA (Table 1). Thus, the protein can form long clusters on the nucleic acid lattice. The number of PriB dimers participating in the primosome assembly is still not completely clear. Nevertheless, some reports indicated the presence of at least two PriB dimers in the primosome¹⁰. The finding of strong positive cooperativity in the PriB protein binding to the ssDNA indicates that, indeed, multiple PriB dimers may participate in the initial stages of the primosome assembly¹⁰. However, the cooperative interactions strongly decrease with the increase of the salt concentration in solution (Figure 8c). On the other hand the weakening effect of the salt on cooperative interactions may be much less pronounced in the assembly process of the primosome, due to the presence of other protein components of the system associated with the DNA.

Salt Effect on the Intrinsic Affinity of the PriB - ssDNA Complex Indicates Engagement of the Entire Total DNA-Binding Site of the Protein in Interactions with the Nucleic Acid

The data discussed above indicate that the entire total DNA-binding site of the PriB dimer engages in direct interactions with the nucleic acid. The salt effect on the intrinsic affinity of the protein strongly supports that conclusion. The slopes, $\partial \log K_{20} / \partial \log [\text{NaCl}] = -5.7 \pm 0.5$ and $\partial \log K_{26} / \partial \log [\text{NaCl}] = -5.3 \pm 0.4$ indicate that a net release of 5 – 6 ions accompanied the engagement in intrinsic interactions (Figure 7b and 8b). A similar number of ions released is obtained from the slope $\partial \log K_{20} / \partial \log [\text{NaBr}] = -5.4 \pm 0.5$, although the protein affinity is lower than in the presence of the chloride ions. Bromide anions, Br^- , are known to have significantly higher affinity for protein amine groups than Cl^- ⁵⁷. The apparent independence of the slope of the log - log plot upon the type of anion most probably results from the fact that in the studied salt concentration range (>100 mM) all anion-binding sites are fully saturated with Br^- , or Cl^- .

The DNA-binding pocket, identified in crystallographic studies, contains Arg13, Lys18, Arg34, Lys84, Lys88, and Lys89 in close contact with the bound nucleic acid²². Thus, the number of possible ionic contacts in the DNA-binding site corroborates very well the net number of ions released at the protein - nucleic acid interface. Moreover, the arginine and lysine residues are spread over the entire DNA-binding site of the PriB protein²². In order to engage all these basic residues, as indicated from the log - log plots, the entire total DNA-binding site must participate in interactions with the nucleic acid. Contrary to monovalent salts, the log-log plot, in the presence MgCl_2 is clearly nonlinear and the linear part at the high MgCl_2 concentration range indicate that ~2 ions are released upon formation of the complex (Figure 7d). The lower absolute value of the slope of the linear part of the plot, as compared to NaCl or NaBr, is expected because of the lower value of the thermodynamic binding for magnesium cations to the nucleic acid^{46,47}. Because the thermodynamic degree of Mg^{+2} binding on the 20-mer changes in the studied salt concentration ranges, the nonlinear behavior of the log-log plot suggests that the released ions may originate predominantly from the nucleic acid. It should be mentioned that, due to the presence of multiple components in solution, the studied system is extremely complex. Nevertheless, a

strong decrease of the intrinsic binding constant of the PriB protein - 20-mer complex, occurs around 1 mM MgCl₂ and suggests that the ions are released from the binding sites characterized by the affinity constant of at least 500 M⁻¹.

The PriB Protein Shows a Very Strong Preference For the Homo-Pyrimidine ssDNAs

The difference in the intrinsic affinity of the PriB protein for the ssDNA, differing by the type of base is dramatic. This is particularly pronounced for dT(pT)₁₉ and dA(pA)₁₉ with the intrinsic binding constant is $\sim 3.3 \times 10^7 \text{ M}^{-1}$ and $\sim 3.9 \times 10^4 \text{ M}^{-1}$, respectively (Table 2). Thus, the protein shows very strong preference for the homo-pyrimidine nucleic acids. The ~ 3 orders of magnitude difference in the intrinsic affinity between dT(pT)₁₉ and dA(pA)₁₉ indicates an important contribution of the base in ssDNA binding, which is not obvious in the crystal structure of the complex²². What is obvious from the crystal structure is, that in spite of the low sequence homology, the structure of the DNA binding pocket of the PriB dimer is very similar to the structure of the DNA-binding site of the *E. coli* SSB protein, which shows exceptionally high affinity for thymine polymers²⁴. In this context, the lack of temperature effect on the PriB dimer interactions with the ssDNA is puzzling (Figure 9). Such a significant participation of nucleic acid bases in interactions with the PriB protein would suggest a rather large enthalpy contribution to the free energy of binding. The observed exclusively, apparent entropy-driven binding reaction strongly suggests that a large conformational change of the protein is induced in the association reaction, whose enthalpy change would compensate the enthalpy change of the intrinsic, DNA binding process.

Notice, the estimated concentration of the PriB dimer in the *E. coli* cell is $\sim 6 \times 10^{-8} \text{ M}$, which strongly suggests that, at this concentration, the protein predominantly associates with the stretches of thymine nucleotides on the DNA⁵. It should be pointed out that the *E. coli* PriA helicase also shows a significantly higher intrinsic affinity for homo-pyrimidine oligomers than for homo-purine oligomers, although the difference is only ~ 1 order of magnitude²⁶. The physiological importance of the strong preference of both proteins for the homo-pyrimidine ssDNA is still unknown. However, recognition of the stalled replication fork through the initial formation of the PriA - PriB complex, is one of two major pathways of the restart of the chromosomal DNA replication in *E. coli* at the damaged DNA site¹³⁻¹⁷. The mechanism of the selection of the restart pathway is still unclear. Nevertheless, it invokes the structure of the ssDNA gap of the damaged DNA. On the other hand, the finding of a very strong preference of PriB for homo-thymine ssDNAs and a significant preference of the PriA for the same pyrimidine stretches strongly suggests that the sequence of the nucleic acid around the damaged site may play an important role in such a pathway selection of the restart of the replication fork.

MATERIALS & METHODS

Reagents and Buffers

All solutions were made with distilled and deionized >18 MΩ (Milli-Q Plus) water. All chemicals were reagent grade. Buffer C is 10 mM sodium cacodylate adjusted to pH 7.0 with HCl, 100 mM NaCl, 1 mM DTT, and 25% glycerol. Temperatures and concentrations of salts in the buffer are indicated in the text.

PriB Protein

The gene of the *E. coli* PriB protein has been directly isolated from the *E. coli* K12 strain. The isolated gene of the PriB protein has been placed in pET30a plasmid (Novagen). The isolation and purification procedure were as described in the literature¹⁸⁻²³. The protein used in our studies was > 99% pure as judged by polyacrylamide electrophoresis with Coomassie Brilliant Blue staining. The concentration of the PriB protein was

spectrophotometrically determined, with the extinction coefficient $\epsilon_{280} = 1.0776 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$ (dimer) obtained using an approach based on Edeldoch's method^{58,59}.

Nucleic Acids

Oligomers, dA(pA)₁₃, dA(pA)₁₅, dA(pA)₁₇, dA(pA)₁₉, dA(pA)₂₃, dA(pA)₃₀, dA(pA)₃₄, dC(pC)₁₉, dT(pT)₁₉, and fluorescein-labeled 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' through phosphoramidate chemistry and are referred to as 21-mer, 5'Fl-dT(pT)₁₉ and 27-mer, 5'Fl-dT(pT)₂₅, respectively. The concentration of dC(pC)₁₉, dT(pT)₁₉, and dA(pA)₁₉ were determined using extinction coefficients (nucleotide)²⁶: $\epsilon_{270} = 7200 \text{ cm}^{-1} \text{ M}^{-1}$, $\epsilon_{260} = 8100 \text{ cm}^{-1} \text{ M}^{-1}$, and $\epsilon_{260} = 10,000 \text{ cm}^{-1} \text{ M}^{-1}$. Concentrations of the fluorescein-labeled ssDNA oligomers have been spectrophotometrically determined as previously described by us^{53–56}. The etheno-derivatives of adenosine oligomers were obtained by modification with chloroacetaldehyde^{60–62}. This modification goes to completion and provides a fluorescent derivative of the nucleic acid. The concentrations of the etheno-derivative of the nucleic acids were determined using the extinction coefficient, $\epsilon_{257} = 3700 \text{ cm}^{-1} \text{ M}^{-1}$ (nucleotide)^{60–62}.

Fluorescence Measurements

Steady-state fluorescence titrations were performed using Fluorolog F-11 spectrofluorometer (Jobin Yvon). In order to avoid possible artifacts, due to the fluorescence anisotropy of the sample, polarizers were placed in excitation and emission channels and set at 90° and 55° (magic angle), respectively. The PriB binding was followed by monitoring the fluorescence of the etheno-derivatives of the nucleic acids ($\lambda_{\text{ex}} = 325 \text{ nm}$, $\lambda_{\text{em}} = 410 \text{ nm}$). Computer fits were performed using KaleidaGraph software (Synergy Software, PA) and Mathematica (Wolfram Research, IL). The relative fluorescence increase of the nucleic acid, ΔF , upon binding the PriB protein is defined as, $\Delta F = (F_i - F_o)/F_o$, where F_i is the fluorescence of the nucleic acid solution at a given titration point "I" and F_o is the initial value of the fluorescence of the same solution.

Determination of Thermodynamically Quantitative Binding Isotherms of the PriB Protein - ssDNA Complexes

To obtain quantitative estimates of the total average degree of binding, $\Sigma\Theta_i$ (number of bound protein molecules per ssDNA 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^{31–36}. Briefly, each different possible "I" complex of the PriB protein with the ssDNA contribute to the experimentally observed relative fluorescence increase, ΔF_{obs} . Thus, ΔF_{obs} is functionally related to $\Sigma\Theta_i$ by

$$\Delta F_{\text{obs}} = \sum \Theta_i \Delta F_i \quad (11)$$

where ΔF_i is the molecular parameter characterizing the maximum fluorescence increase of the nucleic acid with the PriB protein bound in complex "I". The same value of ΔF_{obs} , obtained at two different total nucleic acid concentrations, M_{T1} and M_{T2} , indicates the same physical state of the nucleic acid, *i.e.*, the total average degree of binding, $\Sigma\Theta_i$, and the free PriB protein concentration, P_F , must be the same. The value of $\Sigma\Theta_i$ and P_F is then related to

the known, total protein concentrations, P_{T1} and P_{T2} , and the known total nucleic acid concentrations, M_{T1} and M_{T2} , at the same value of ΔF_{obs} , by

$$\sum \Theta_i = \frac{(P_{T1} - P_{T2})}{(M_{T1} - M_{T2})} \quad (12)$$

$$P_F = P_{Tx} - (\sum \Theta_i) M_{Tx} \quad (13)$$

where $x = 1$ or 2^{31-36} .

Photo-cross-linking Experiments

The ssDNA oligomer, dT(pT)₁₉ has been labeled at the 5' end with [³²P], using polynucleotide kinase^{63,64}. The labeled ssDNA were mixed with the PriB protein in buffer C (pH 7.0, 10°C). The samples (total volume 40 μ l) were placed on Parafilm, immersed in a 10°C, and irradiated for 20 minutes, 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. After irradiation, the samples were loaded on 15% 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, PA).

Analytical Ultracentrifugation Measurements

Analytical ultracentrifugation experiments were performed with an Optima XL-A analytical ultracentrifuge (Beckman Inc., Palo Alto, CA), as we previously described³⁶⁻⁴¹. Sedimentation equilibrium scans were collected at the absorption band of the fluorescein of the labeled ssDNA oligomers (495 nm) at a large excess of the protein to assure complete saturation of the DNA. The sedimentation was considered to be at equilibrium when consecutive scans, separated by time intervals of 8 hrs, did not indicate any changes. For the n-component system, the total concentration at radial position r , c_r , is defined by

$$c_r = \sum_{i=1}^n c_{bi} \exp \left[\frac{(1 - \bar{v}_i \rho) \omega^2 M_i (r^2 - r_b^2)}{2RT} \right] + b \quad (14)$$

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 base-line error term⁴². Equilibrium sedimentation profiles were fitted to eq. 14 with M_i and b as fitting parameters³⁶⁻⁴¹.

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Abbreviations

Tris	tris(hydroxymethyl)aminomethane
PAS	primosome assembly site
SSB	single-strand binding protein; dithiothreitol

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Figure 1.

a. Structure of the PriB protein dimer based on crystallographic data²². The structure has been generated using data from Brookhaven Protein Data Bank, under the code 1V1Q, using PyMOL (DeLano Scientific, San Carlos, CA). The two monomers in the PriB dimer are marked with different colors. **b.** Proposed structure of the PriB dimer complex with a single ssDNA 15-mer, dT₁₅ (blue), obtained in crystallographic studies²². Two PriB dimers make contact with the nucleic acid, although using different monomers, as marked with different colors. The structure has been generated using data from Brookhaven Protein Data Bank, under the code 2CCZ, using PyMOL (DeLano Scientific, San Carlos, CA).

Figure 2.

a. Fluorescence titrations of the ssDNA 20-mer, dεA(pεA)₁₉, with the PriB protein ($\lambda_{\text{ex}} = 325 \text{ nm}$, $\lambda_{\text{em}} = 410 \text{ nm}$) in buffer C (pH 7.0, 10°C), containing 100 mM NaCl, at two different nucleic acid concentrations: (■) $4.8 \times 10^{-7} \text{ M}$, and (□) $4.8 \times 10^{-6} \text{ M}$ (oligomer). The solid lines are nonlinear least squares fits of the titration curves, using the single-site binding isotherm, described by eq. 1 (Table 1). **b.** Dependence of the relative fluorescence increase ΔF , of dεA(pεA)₁₉, upon the total average degree of binding of the PriB dimer, $\Sigma\Theta_i$ (■). The solid line follows the experimental points and has no theoretical basis. The dashed line is the extrapolation of ΔF to its maximum value, $\Delta F_{\text{max}} = 1.18$. **c.** Fluorescence titrations of the ssDNA 24-mer, dεA(pεA)₂₃, with the PriB protein ($\lambda_{\text{ex}} = 325 \text{ nm}$, $\lambda_{\text{em}} = 410 \text{ nm}$) in buffer C (pH 7.0, 10°C), containing 100 mM NaCl, at two different nucleic acid concentrations: (■) $6.0 \times 10^{-7} \text{ M}$, and (□) $2.75 \times 10^{-6} \text{ M}$ (oligomer). The solid lines are nonlinear least squares fits of the titration curves, using the statistical thermodynamic model, described by eqs. 4 – 7. **d.** Dependence of the relative fluorescence increase ΔF , of dεA(pεA)₂₃, upon the total average degree of binding of the PriB dimer, $\Sigma\Theta_i$ (■). The solid lines indicate the slopes of the high and low affinity phases of the plot. The dashed line is the extrapolation of ΔF to its maximum value, $\Delta F_{\text{max}} = 1.14$. The solid line is the computer simulation of the dependence of ΔF upon $\Sigma\Theta_i$ using eqs. 4 – 7 and the obtained binding parameters (Table 1) (details in text).

Figure 3.

a. Fluorescence titrations of the ssDNA 35-mer, dεA(pεA)₃₄, with the PriB protein ($\lambda_{\text{ex}} = 325$ nm, $\lambda_{\text{em}} = 410$ nm) in buffer C (pH 7.0, 10°C), containing 100 mM NaCl, at two different nucleic acid concentrations: (■) 4.0×10^{-7} M, and (□) 2.0×10^{-6} M (oligomer). The solid 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 ΔF , of dεA(pεA)₃₄, upon the total average degree of binding of the PriB dimer, $\Sigma\Theta_i$ (■). The solid line follows the experimental points and has no theoretical basis. The dashed line is the extrapolation of ΔF to its maximum value, $\Delta F_{\text{max}} = 2.0$ (details in text).

Figure 4.

a. Sedimentation equilibrium concentration profile of the ssDNA 21-mer, 5'Fl-dT(pT)₁₉, labeled at the 5' end with fluorescein (Materials and Methods) in the presence of PriB protein in buffer C (pH 7.0, 10°C). The concentrations of the nucleic acid and the protein are 1.56×10^{-6} M (oligomer) and 1.86×10^{-5} M (dimer), respectively. The profile has been recorded at 495 nm and at 28000 rpm. The solid line is the nonlinear least squares fit to a single exponential function (eq. 14), with single species having a molecular weight of 27200 ± 2900 . **b.** Sedimentation equilibrium concentration profile of the ssDNA 27-mer, 5'Fl-dT(pT)₂₅, labeled at the 5' end with fluorescein (Materials and Methods), in the presence of the PriB protein in buffer C (pH 7.0, 10°C). The concentrations of the nucleic acid and the

PriB protein are 1.58×10^{-6} M and 1×10^{-5} M, respectively. The profile has been recorded at 495 nm and at 18000 rpm. The solid line is the nonlinear least squares fit to single exponential function (eq. 14), with a single species having a molecular weight of 51300 ± 5000 .

Figure 5.

The maximum number of the PriB protein dimers bound per ssDNA oligomer, as a function of the length of the nucleic acid, in buffer C (pH, 7.0, 10°C). The solid lines follow the experimental points and have no theoretical bases. The number of the bound PriB protein dimers has been determined using the quantitative approach outlined in the Materials and Methods section^{31–36}.

Figure 6.

The dependence of the macroscopic, equilibrium binding constant, K_N , characterizing the binding of the PriB protein dimer to different etheno-derivatives of the ssDNA oligomers, which accept only a single PriB dimer, upon the length of the ssDNA oligomer (nucleotides). The solid line is the linear least squares fit of the plot according to eqs. 3a and 3b. The dashed line is the extrapolation of the plot to the zero value of the equilibrium binding constant.

Figure 7.

a. Fluorescence titrations of the ssDNA 20-mer, dεA(pεA)₁₉, with the PriB protein ($\lambda_{\text{ex}} = 325 \text{ nm}$, $\lambda_{\text{em}} = 410 \text{ nm}$) in buffer C (pH 7.0, 10°C), containing different NaCl concentrations: 100 mM (■); 113.5 mM (□); 127 mM (●); 136 mM (○); 154 mM (◆). The concentration of the ssDNA 20-mer is $4.8 \times 10^{-7} \text{ M}$. The solid lines are nonlinear least squares fits of the titration curves, using the single-site binding isotherm described by eqs. 1, with ΔF_{max} and K_{20} : 1.18, $1.7 \times 10^6 \text{ M}^{-1}$ (■); 1.17, $7.5 \times 10^5 \text{ M}^{-1}$ (□); 1.15, $4.5 \times 10^5 \text{ M}^{-1}$ (●); 1.07, $2.7 \times 10^5 \text{ M}^{-1}$ (○); 0.99, $1.4 \times 10^5 \text{ M}^{-1}$ (◆). **b.** The dependence of the logarithm of K_{20} upon the logarithm of NaCl (■) and NaBr (□) concentration, respectively. The solid lines are linear least squares fits, which provide the slope $\partial \text{Log} K_{20} / \partial \text{Log} [\text{NaCl}] = -5.7 \pm 0.6$ and $\partial \text{Log} K_{20} / \partial \text{Log} [\text{NaBr}] = -5.4 \pm 0.6$, respectively. **c.** Fluorescence titrations of the ssDNA 20-mer, dεA(pεA)₁₉, with the PriB protein ($\lambda_{\text{ex}} = 325 \text{ nm}$, $\lambda_{\text{em}} = 410 \text{ nm}$), in buffer C (pH 7.0, 10°C), containing 0.1 mM EDTA and different MgCl_2 concentrations: 0 mM (■); 1.9 mM (□); 2.9 mM (●); 4.9 mM (○); 5.9 mM (◆). The solid lines are nonlinear least squares fits of the titration curves, using the single-site isotherm described by eqs. 1 with F_{max} , and K_{20} : 1.17, $1.45 \times 10^6 \text{ M}^{-1}$ (■); 1.16, $1.0 \times 10^6 \text{ M}^{-1}$ (□); 1.13, $7.0 \times 10^5 \text{ M}^{-1}$ (●); 1.09, $2.9 \times 10^5 \text{ M}^{-1}$ (○); 1.0, $2.0 \times 10^5 \text{ M}^{-1}$ (◆). **d.** The dependence of the logarithm of K_{20} upon the logarithm of $[\text{MgCl}_2]$ (■). The solid line is the linear least squares fit of the plot in the high $[\text{MgCl}_2]$ range, which provides the slope, $\partial \text{Log} K_{20} / \partial \text{Log} [\text{MgCl}_2] = -1.8 \pm 0.4$.

Figure 8.

a. Fluorescence titrations of the ssDNA 26-mer, dεA(pεA)₂₅, with the PriB protein ($\lambda_{\text{ex}} = 325 \text{ nm}$, $\lambda_{\text{em}} = 410 \text{ nm}$) in buffer C (pH 7.0, 10°C), containing different NaCl concentrations: 100 mM (■); 127 mM (□); 154 mM (●); 172 mM (○). The concentration of the ssDNA 26-mer is $6 \times 10^{-7} \text{ M}$. The solid lines are nonlinear least squares fits of the titration curves, using the statistical thermodynamic model described by eqs. 4 – 7, with ΔF_1 , ΔF_{max} , K_i and ω : 0.8, 1.08, $2.8 \times 10^5 \text{ M}^{-1}$, 45 (■); 0.8, 1.02, $8 \times 10^4 \text{ M}^{-1}$, 28 (□); 0.8, 0.91, $3.5 \times 10^4 \text{ M}^{-1}$, 15 (●); 0.77, 0.78, $1.4 \times 10^4 \text{ M}^{-1}$, 10 (○). **b.** The dependence of the logarithm of the intrinsic binding constant, K_i upon the logarithm of [NaCl]. The solid line is the linear least squares fit, which provides the slope, $\partial \text{Log} K_i / \partial \text{Log} [\text{NaCl}] = -5.3 \pm 0.6$. **c.**

The dependence of the logarithm of the cooperativity parameter, ω , upon the logarithm of [NaCl]. The solid line is the linear least squares fit, which provides the slope, $\partial \text{Log} \omega / \partial \text{Log} [\text{NaCl}] = -2.8 \pm 0.6$.

Figure 9.

a. Fluorescence titrations of the ssDNA 20-mer, dεA(pεA)₁₉, with the PriB protein ($\lambda_{\text{ex}} = 325 \text{ nm}$, $\lambda_{\text{em}} = 410 \text{ nm}$) in buffer C (pH 7.0, 10°C) at different temperatures: 5°C (■), 10°C (□), 15°C (●), 20°C (◆). The concentration of the ssDNA 20-mer is $4.8 \times 10^{-7} \text{ M}$. The solid line is the nonlinear least squares fit of the titration curve, obtained at 10°C, using the single-site binding isotherm defined by eq. 1, with $K_{20} = 1.7 \times 10^6 \text{ M}^{-1}$ and $\Delta F_{\text{max}} = 1.18$, respectively (details in text).

Figure 10.

a. Fluorescence titrations of the ssDNA 20-mer, dεA(pεA)₁₉, with the PriB protein ($\lambda_{\text{ex}} = 325 \text{ nm}$, $\lambda_{\text{em}} = 410 \text{ nm}$) in buffer C (pH 7.0, 10°C) in the absence (■) and presence of two different concentrations of dT(pT)₁₉. The concentration of dεA(pεA)₁₉, is $4.8 \times 10^{-7} \text{ M}$ (oligomer). The concentrations of dT(pT)₁₉ are: (□) $1.5 \times 10^{-7} \text{ M}$ and (◆) $5 \times 10^{-7} \text{ M}$, respectively. The solid lines are nonlinear least squares fits of the binding titration curves, using eqs. 9 – 10, with the binding constant for dεA(pεA)₁₉, $K_{20} = 1.7 \times 10^6 \text{ M}^{-1}$ and $\Delta F_{\text{max}} = 1.18$, respectively, and the intrinsic binding constant $K_{20S} = 3 \times 10^8 \text{ M}^{-1}$ for dT(pT)₁₉. **b.** Fluorescence titrations of the ssDNA 20-mer, dεA(pεA)₁₉, with the PriB protein ($\lambda_{\text{ex}} = 325 \text{ nm}$, $\lambda_{\text{em}} = 410 \text{ nm}$) in buffer C (pH 7.0, 10 °C) in the absence (■) and presence of dA(pA)₁₉

(□) and dC(pC)₁₉ (●). The concentration of dεA(pεA)₁₉, is 4.8×10^{-7} M (oligomer). The concentrations of dA(pA)₁₉ and dC(pC)₁₉ are 5×10^{-7} M (oligomer). The solid lines are nonlinear least squares fits of the binding titration curves using eqs. 9 – 10, with the binding $K_{20} = 1.7 \times 10^6 \text{ M}^{-1}$ and $\Delta F_{\text{max}} = 1.18$ for dεA(pεA)₁₉ and the binding constant $K_{20S} = 3.5 \times 10^5 \text{ M}^{-1}$ and $K_{20S} = 2.5 \times 10^7 \text{ M}^{-1}$ for dA(pA)₁₉ and dC(pC)₁₉, respectively.

Figure 11.

a. Autoradiogram of the 15% SDS polyacrylamide gel of the PriB - 5' [³²P]-dT₂₀ complex, after UV-mediated cross-linking of the protein to the DNA, formed at different concentration of the PriB dimer, buffer C (pH 7.0, 10°C) (Materials and Methods). The concentration of the 5' [³²P]-dT₂₀ is 5 × 10⁻⁶ M (oligomer). The 0 lane contains protein markers. Lane 1 contains the control sample, which is the PriB - dT₂₀ complex not subjected to the UV irradiation. Lanes 2 to 4 contain the constant concentration of the ssDNA 20-mer and the increasing concentration of the PriB protein (dimer): lane 2, 5 × 10⁻⁶ M; lane 3, 8 × 10⁻⁶ M; and lane 4, 1.3 × 10⁻⁵ M. Lane 5 contains only the PriB protein (5 × 10⁻⁶ M) (dimer) in the absence of the 5' [³²P]-dT₂₀. Lane 6 contains the 5' [³²P]-dT₂₀ alone. **b.** The same SDS polyacrylamide gel of the PriB - dT(pT)₁₉ complex, as shown in panel a, but stained with Coomassie Brilliant Blue.

Thermodynamic and spectroscopic parameters characterizing the binding of the *E. coli* PriB protein to ethenoderivatives of ssDNA oligomers in buffer C (pH 7.0, 10°C)*

Table 1

	14-mer	16-mer	18-mer	20-mer	24-mer	26-mer	30-mer	35-mer
	$d\epsilon\Delta(\text{p}\epsilon\Delta)_{13}$	$d\epsilon\Delta(\text{p}\epsilon\Delta)_{15}$	$d\epsilon\Delta(\text{p}\epsilon\Delta)_{17}$	$d\epsilon\Delta(\text{p}\epsilon\Delta)_{19}$	$d\epsilon\Delta(\text{p}\epsilon\Delta)_{23}$	$d\epsilon\Delta(\text{p}\epsilon\Delta)_{25}$	$d\epsilon\Delta(\text{p}\epsilon\Delta)_{29}$	$d\epsilon\Delta(\text{p}\epsilon\Delta)_{34}$
Stoichiometry	1 ± 0.1	1 ± 0.1	1 ± 0.1	1 ± 0.1	2 ± 0.2	2 ± 0.2	2 ± 0.2	2 ± 0.2
Site-size p	12	12	12	12	12	12	12	12
$K_N (\text{M}^{-1})$	$(5.2 \pm 0.7) \times 10^5$	$(9.0 \pm 1.3) \times 10^5$	$(1.3 \pm 0.2) \times 10^6$	$(1.7 \pm 0.2) \times 10^6$	-	-	-	-
$K_I (\text{M}^{-1})$	$(1.7 \pm 0.2) \times 10^5$	$(1.8 \pm 0.3) \times 10^5$	$(1.9 \pm 0.3) \times 10^5$	$(1.9 \pm 0.3) \times 10^5$	$(2.5 \pm 0.4) \times 10^5$	$(2.8 \pm 0.5) \times 10^5$	$(2.8 \pm 0.5) \times 10^5$	$(1.5 \pm 0.3) \times 10^5$
ω	-	-	-	-	50 ± 10	45 ± 10	45 ± 10	45 ± 10
ΔF_I	-	-	-	-	0.90 ± 0.03	0.80 ± 0.03	0.70 ± 0.03	0.9 ± 0.03
ΔF_{max}	0.77 ± 0.03	1.04 ± 0.03	1.19 ± 0.03	1.18 ± 0.03	1.32 ± 0.2	1.14 ± 0.03	1.22 ± 0.03	2.0 ± 0.05

* The errors are standard deviations determined using 3–4 independent titration experiments.

Macroscopic and intrinsic binding constants, K_{20} and K_{in} , and the site-size, n , characterizing the binding of the PriB protein to different ssDNA homo-oligomers, dN(pN)₁₉, in buffer C (pH 7.0, 10 °C). The values of K_{20} for the unmodified 20-mers have been determined using the MCT Method (details in text). *

Table 2

	dεA(pεA) ₁₉	dA(pA) ₁₉	dT(pT) ₁₉	dC(pC) ₁₉
K_{20} (M ⁻¹)	(1.7 ± 0.4) × 10 ⁶	(3.5 ± 0.8) × 10 ⁵	(3.0 ± 0.7) × 10 ⁸	(2.5 ± 0.4) × 10 ⁷
K_{in} (M ⁻¹)	(1.9 ± 0.5) × 10 ⁵	(3.9 ± 0.8) × 10 ⁴	(3.3 ± 0.7) × 10 ⁷	(2.8 ± 0.4) × 10 ⁶
n	12	12	12	12

* Errors are standard deviations determined using 3–4 independent titration experiments.