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Negative Cooperativity within Individual Tetramers of *Escherichia coli* Single Strand Binding Protein Is Responsible for the Transition between the $(SSB)_{35}$ and $(SSB)_{56}$ DNA Binding Modes†

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ABSTRACT: We have examined the binding of the oligonucleotide $dT(pT)_{34}$ to the *Escherichia coli* SSB protein as a function of NaCl and $MgCl_2$ concentration (25 °C, pH 8.1) by monitoring the quenching of the intrinsic protein fluorescence. We find two binding sites for $dT(pT)_{34}$ per single strand binding (SSB) protein tetramer, with each site possessing widely different affinities depending on the salt concentration. At 200 mM NaCl, we observe nearly stoichiometric binding of $dT(pT)_{34}$ to both binding sites within the SSB tetramer, although a difference in the affinities is still apparent. However, when the NaCl concentration is lowered, the overall affinity of $dT(pT)_{34}$ for the second site on the SSB tetramer decreases dramatically. At 1.5 mM NaCl, only a single molecule of $dT(pT)_{34}$ can bind per SSB tetramer, even with a 10-fold molar excess of $dT(pT)_{34}$. $MgCl_2$ is effective at 100-fold lower concentrations than NaCl in promoting the binding of the second molecule of $dT(pT)_{34}$. This binding behavior reflects an intrinsic property of the SSB tetramer, since it is also observed upon binding of smaller oligonucleotides, and the simplest explanation is that a salt-dependent negative cooperativity exists between DNA binding sites within the SSB tetramer. This phenomenon is also responsible for the transition between the two SSB–single strand (ss) polynucleotide binding modes that cover 35 and 56 nucleotides per tetramer [Bujalowski, W., & Lohman, T. M. (1986) *Biochemistry 25*, 7799–7802]. Extreme negative cooperativity stabilizes the $(SSB)_{35}$ binding mode, in which the SSB tetramer binds tightly to ss DNA with only two of its subunits while the other two subunits remain unligated. At higher salt concentrations, negative cooperativity is reduced with the result that all four SSB subunits can interact with ss DNA, as in the $(SSB)_{56}$ and $(SSB)_{65}$ binding modes. The possible biological significance of this negative cooperativity is discussed.

The *Escherichia coli* single strand binding (SSB) protein is a helix-destabilizing protein that is required for DNA replication and a variety of repair processes in that organism (Sigal et al., 1972; Chase & Williams, 1986). It also stimulates the DNA strand exchange activity of the recA protein and hence is important in homologous recombination (Cox & Lehman, 1987). In these respects it is similar to the bacteriophage T4 gene 32 protein, although the *E. coli* SSB protein differs greatly in structural detail, as well as in its interactions with single-stranded (ss) nucleic acids. The SSB protein is a stable tetramer possessing D_2 symmetry (Ollis et al., 1983), and its tetrameric structure is maintained upon binding oligonucleotides (Bandyopadhyay & Wu, 1978; Krauss et al., 1981). The protein binds selectively and cooperatively to ss polynucleotides (Sigal et al., 1972; Lohman et al., 1986a; Bujalowski & Lohman, 1987a), although the interactions are complex since multiple binding modes can form on ss polynucleotides, depending on the solution conditions, particularly the salt concentration and type (Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Griffith et al., 1984; Chrysogelos & Griffith, 1982).

In studies with poly(dT), several binding modes have been identified for the *E. coli* SSB protein, and the transitions among the binding modes are dependent on cation and anion type and concentration, pH, temperature, and protein binding density (Lohman & Overman, 1985; Bujalowski & Lohman, 1986; Bujalowski et al., 1988). Different binding density dependent morphologies of SSB protein–ss M13 DNA complexes have also been observed by electron microscopy (Griffith et al., 1984). At 25 °C, pH 8.1, three major binding modes that differ in the number of nucleotides occluded by the SSB tetramer (i.e., the site size n) have been observed with $n = 35$, 56, and 65 nucleotides per SSB tetramer (Lohman & Overman, 1985; Bujalowski & Lohman, 1986). In the absence of di- or polyvalent cations, the $(SSB)_{35}$ mode is stable below 10 mM NaCl at high protein binding densities; the $(SSB)_{56}$ mode is stable in the region from 50 mM $\leq$ [NaCl] $\leq$ 0.1 M, whereas above 0.2 M NaCl, the $(SSB)_{65}$ mode is stable (Bujalowski & Lohman, 1986). $MgCl_2$ is much more effective than NaCl in promoting the transition from the $(SSB)_{35}$ to the $(SSB)_{56}$ binding mode, such that the $(SSB)_{56}$ mode is stable in the range from 4 to 50 mM $MgCl_2$. It has been proposed that, in the $(SSB)_{35}$ complex, the ss DNA interacts with only

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two subunits within each SSB tetramer, whereas the DNA interacts with all four subunits in the $(SSB)_{56}$ and $(SSB)_{65}$ complexes (Lohman & Overman, 1985; Lohman et al., 1986a). At equilibrium (25 °C, pH 8.1), in the $(SSB)_{65}$ mode [and possibly the $(SSB)_{56}$ mode] the SSB tetramer binds to ss polynucleotides with moderate "limited" positive cooperativity between nearest-neighbor-bound tetramers such that an equilibrium mixture of tetramers and octamers (dimers of tetramers) exists on the DNA (Lohman et al., 1986a; Bujalowski & Lohman, 1987a; Chrysogelos & Griffith, 1982) rather than protein clusters of unlimited size. However, a *metastable*, high-cooperativity complex that can transiently form long protein clusters can be observed under certain low salt conditions in vitro (Lohman et al., 1986a). It is currently not known which binding modes are involved in DNA replication or recombination or repair in *E. coli*.

In this paper, we have investigated the interaction of a short ss DNA, 35 nucleotides in length, which should only be long enough to occupy approximately half of the ss DNA binding sites on the SSB tetramer. We present direct evidence that there are two sites for binding of $dT(pT)_{34}$ per SSB tetramer; however, the affinities for these two sites differ dramatically, and this difference is highly salt dependent. We suggest that this reflects a strong, salt-dependent negative cooperativity that exists between the two sites within an individual SSB tetramer. This phenomenon is responsible for the salt-dependent transition between two of the SSB protein–ss polynucleotide binding modes that have recently been identified (Lohman & Overman, 1985; Bujalowski & Lohman, 1986).

Materials and Methods

Reagents and Buffers. All chemicals were of reagent grade; all solutions were made with distilled and deionized (Milli-Q) water. The standard buffer was buffer T, which is 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.1, and 0.1 mM trisodium ethylenediaminetetraacetate (Na_3EDTA). The NaCl and $MgCl_2$ concentrations in the buffers are indicated in the text. Na_3EDTA was not included in the buffers containing $MgCl_2$. The $MgCl_2$ stock solution was prepared as previously described (Bujalowski & Lohman, 1986), and the concentration was determined by measurement of the refractive index of the stock solution.

E. coli SSB Protein and Oligonucleotides. The *E. coli* SSB protein was purified as previously described (Lohman et al., 1986b), and the concentration was determined spectrophotometrically by using the extinction coefficient $\epsilon_{280} = 1.5$ mL mg^{-1} cm^{-1} [1.13×10^5 M^{-1} (tetramer) cm^{-1}] in buffer T + 0.20 M NaCl (Lohman & Overman, 1985). The oligodeoxynucleotide $dT(pT)_{34}$ was synthesized with an Applied Biosystems 380B instrument and purified by HPLC (Waters 600) on a Nucleogen DEAE 60-7 column (Rainin), with a linear NaCl gradient from 0.4 to 0.6 M. The $dT(pT)_{34}$ eluted at ~0.48 M NaCl and was ≥98% pure as judged by polyacrylamide gel electrophoresis and autoradiography of a sample that was labeled with ^{32}P at the 5′ end by using polynucleotide kinase. The concentration of $dT(pT)_{34}$ was determined spectrophotometrically by using an extinction coefficient $\epsilon_{260} = 8.1 \times 10^3$ M^{-1} (nucleotide) cm^{-1} [231 M^{-1} ($dT(pT)_{34}$) cm^{-1}].

Fluorescence Measurements. Titrations of SSB protein with the oligonucleotide $dT(pT)_{34}$ were performed while the quenching of the intrinsic tryptophan fluorescence of the SSB protein was being monitored ($\lambda_{ex} = 300$ nm; $\lambda_{em} = 347$ nm) in an SLM 8000 spectrofluorometer. The sample temperature was controlled at 25.0 (±0.1) °C, and all measurements were corrected for dilution, photobleaching, and inner filter effects as previously described (Bujalowski & Lohman, 1986).

Determination of Binding Stoichiometry. In the studies presented here, the signal change that is used to monitor $dT(pT)_{34}$ binding is from the SSB protein. Without knowledge of the relationship between the observed SSB protein fluorescence quenching Q_{obsd} and the average degree of binding of $dT(pT)_{34}$ per SSB tetramer $\sum\nu_i$, the following general procedure can be used to obtain an absolute estimate of $\sum\nu_i$ and the free $dT(pT)_{34}$ concentration L_F, thus rigorously establishing the relationship between Q_{obsd} and $\sum\nu_i$ (Bujalowski & Lohman, 1987b; Halfman & Nishida, 1972). In general, there will be i sites for binding of $dT(pT)_{34}$ per SSB tetramer, and ν_i is defined as the average number of moles of $dT(pT)_{34}$ bound to site i per SSB tetramer. In this case, Q_{obsd} is related to the average number of moles of $dT(pT)_{34}$ bound to all i sites per SSB tetramer, $\sum\nu_i$, as described in eq 1, where Q_{obsd} is

$$Q_{obsd} = \sum\nu_i(Q_{max})_i \quad (1)$$

the experimentally observed quenching of the SSB protein fluorescence at a total concentration of $dT(pT)_{34}$, L_T, and a total SSB tetramer concentration, M_T. $(Q_{max})_i$ is the maximum extent of SSB protein fluorescence quenching with $dT(pT)_{34}$ bound to site i. Since each $(Q_{max})_i$ is constant for a given site i, then at equilibrium, Q_{obsd} is dependent only upon the distribution of bound $dT(pT)_{34}$, i.e., on $\sum\nu_i$. Therefore, at any constant value of Q_{obsd}, L_F and $\sum\nu_i$ are also constant at equilibrium. This forms the basis by which one can calculate $\sum\nu_i$ as a function of L_F from a minimum of two titrations. From titrations [Q_{obsd} vs log L_T] performed at two (or more) different total SSB protein concentrations, M_{T1} and M_{T2} (e.g., see Figure 1), one determines the set of values of the total ligand [$dT(pT)_{34}$] concentration, L_{T1} and L_{T2}, for which Q_{obsd} is constant. This is done by drawing a horizontal line, defining a constant Q_{obsd} that intersects both titration curves (SSB concentrations M_{T1} and M_{T2}), and determining the values of L_{T1} and L_{T2} at the points of intersection. One can then calculate $\sum\nu_i$ and L_F from eq 2 and 3, respectively, which make

$$\sum\nu_i = (L_{T1} - L_{T2})/(M_{T1} - M_{T2}) \quad (2)$$

$$L_F = (M_{T1}L_{T2} - M_{T2}L_{T1})/(M_{T1} - M_{T2}) \quad (3)$$

use of the mass conservation equations, $L_{Tx} = L_F + (\sum\nu_i)M_{Tx}$, where $x = 1$ or 2. This procedure is repeated over the range of attainable values of Q_{obsd} that span the titration curves, thus yielding a full range of values of $\sum\nu_i$ and L_F. One can then obtain a binding isotherm or correlate $\sum\nu_i$ [$dT(pT)_{34}$ stoichiometry] vs Q_{obsd}, as shown in Figure 1B.

Results

There Are Two Sites for Binding of the Oligonucleotide $dT(pT)_{34}$ on Each SSB Protein Tetramer. In an effort to probe the different SSB protein–ss DNA binding modes, we have examined the binding of an oligodeoxythymidylate, $dT(pT)_{34}$, to SSB protein by monitoring the quenching of the intrinsic tryptophan fluorescence of the protein upon binding. The length of this oligonucleotide equals the number of nucleotides occluded in the low site size, $(SSB)_{35}$ complex formed between SSB protein and ss polynucleotides and is approximately half the site size of the $(SSB)_{56}$ and $(SSB)_{65}$ complexes (Lohman & Overman, 1985; Bujalowski & Lohman, 1986). Figure 1A shows two experiments in which different concentrations of SSB protein were titrated with $dT(pT)_{34}$ in 50 mM NaCl (buffer T, pH 8.1, 25.0 °C). The SSB protein fluorescence is quenched by ~90% upon saturation with $dT(pT)_{34}$. From the two titrations in Figure 1A, the absolute stoichiometry of $dT(pT)_{34}$ binding per SSB tetramer can be calculated, independent of any assumptions, as described under

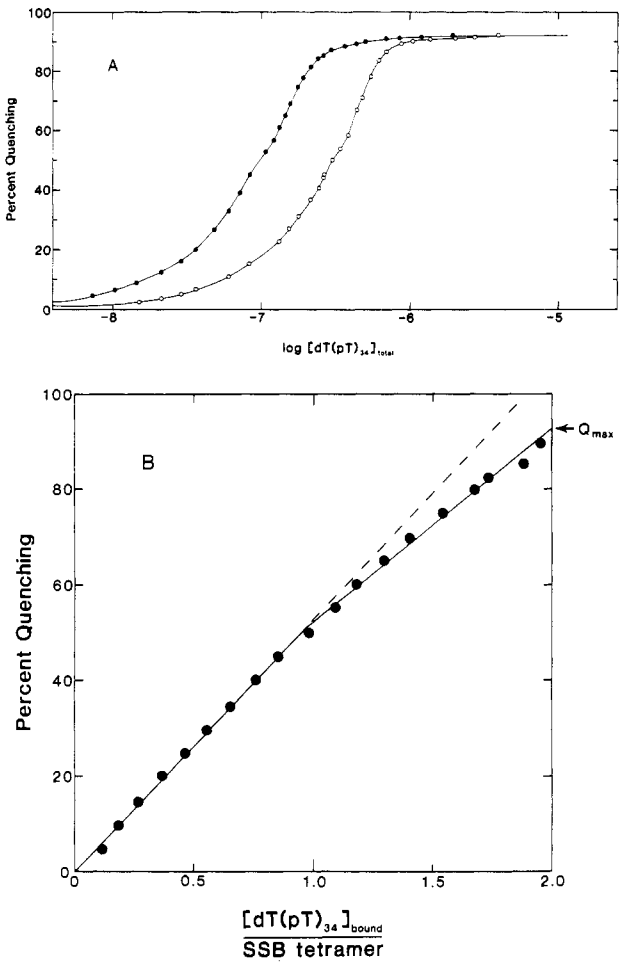

FIGURE 1: Fluorescence titrations to determine the stoichiometry for binding of $dT(pT)_{34}$ to the SSB protein tetramer. (A) Two concentrations of *E. coli* SSB protein were titrated with the oligodeoxynucleotide $dT(pT)_{34}$ in 10 mM Tris-HCl (pH 8.1)–50 mM NaCl at 25.0 °C, and the quenching of the SSB tryptophan fluorescence was monitored (λ_{ex} = 300 nm; λ_{em} = 347 nm). The total concentration of SSB protein in each titration was (●) 1.03×10^{-7} M (tetramer) and (○) 3.22×10^{-7} M (tetramer). (B) The relationship between the observed fluorescence quenching and the stoichiometry of $dT(pT)_{34}$ binding was determined from analysis of the data in (A) as described under Materials and Methods. The dashed line, which is a linear extrapolation of the quenching data for the binding of $dT(pT)_{34}$ to the first site on the SSB tetramer, emphasizes the different extents of fluorescence quenching observed for the binding of $dT(pT)_{34}$ to the first and second sites (50% vs 40%).

Materials and Methods. The correlation between the binding stoichiometry and the observed fluorescence quenching, Q_{obsd}, is shown in Figure 1B, and the analysis shows that 2 mol of $dT(pT)_{34}$ can bind per mole of SSB tetramer at saturation. The first mole of bound $dT(pT)_{34}$ results in 50% quenching of the SSB protein fluorescence; however, the second mole of bound $dT(pT)_{34}$ yields only an additional ~40% quenching, resulting in a maximum quenching of ~90%. Clearly, the binding of each successive $dT(pT)_{34}$ molecule does not exhibit an equivalent fluorescence change. Similar data obtained at different NaCl concentrations yield the identical correlation between binding stoichiometry and extent of fluorescence quenching.

Dramatic Salt-Dependent Differences in Apparent Affinities Exist for $dT(pT)_{34}$ Binding to the Two Sites within the SSB Tetramer. In Figure 2A, we show titrations of SSB protein with $dT(pT)_{34}$ performed at seven NaCl concentrations from 1.5 to 200 mM (pH 8.1, 25.0 °C). At all NaCl concentrations, the binding of the first molecule of $dT(pT)_{34}$ is stoichiometric; however, the binding of the second $dT(pT)_{34}$ molecule is clearly

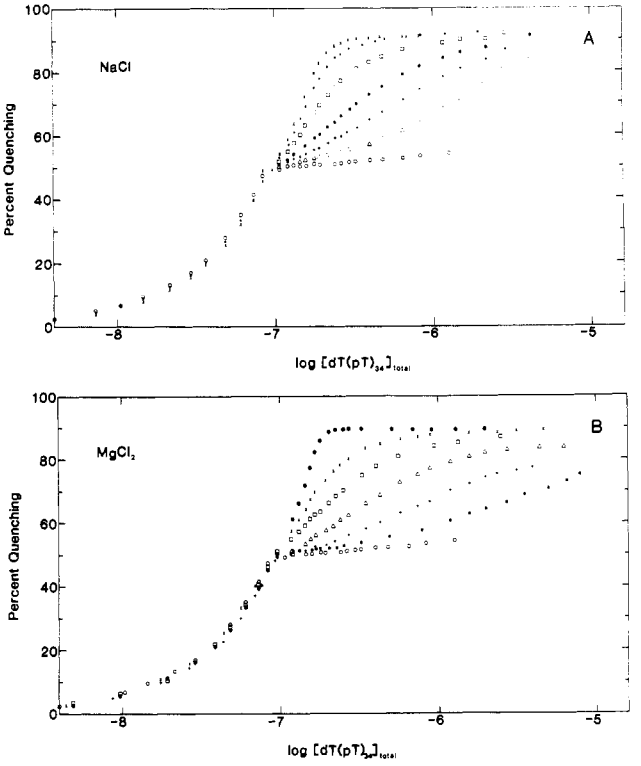

FIGURE 2: Fluorescence titrations of SSB protein with $dT(pT)_{34}$ at several salt concentrations showing the onset of apparent negative cooperativity in the binding of the second molecule of $dT(pT)_{34}$ to the SSB tetramer. The first 50% of the fluorescence quenching represents the filling of the first SSB binding site with $dT(pT)_{34}$, whereas the next 40% of the quenching corresponds to the filling of the second binding site with $dT(pT)_{34}$. (A) The titrations were performed in buffer T (pH 8.1) at 25 °C, with [SSB] = 1.03×10^{-7} M, containing the following NaCl concentrations: (○) 1.5 mM; (△) 10 mM; (+) 15 mM; (*) 20 mM; (□) 30 mM; (●) 50 mM; (×) 200 mM. (B) The titrations were performed in buffer T (pH 8.1) plus 1.5 mM NaCl, without EDTA, at 25 °C, with [SSB] = 1.04×10^{-7} M, containing the following $MgCl_2$ concentrations: (○) no $MgCl_2$, data taken from (A); (*) 49 μM; (+) 97 μM; (△) 146 μM; (□) 194 μM; (×) 291 μM; (●) 2.9 mM.

dependent upon the NaCl concentration. At 1.5 mM NaCl, a plateau at 50% quenching is observed, indicating that only one $dT(pT)_{34}$ molecule binds to each SSB tetramer at this low NaCl concentration, even with a 10-fold excess of $dT(pT)_{34}$ over the second SSB binding site. However, when the NaCl concentration is increased, it becomes easier to fill the second binding site on the SSB tetramer. The "apparent" affinity of $dT(pT)_{34}$ for the second SSB site *increases* with increasing NaCl concentration, in the range from 1.5 to 200 mM NaCl, although at each NaCl concentration, $dT(pT)_{34}$ has a much higher affinity for the first SSB binding site. There are two possible interpretations for the data in Figure 2. The simplest possibility is that the SSB tetramer exhibits a salt-dependent *negative* cooperativity for the binding of the second $dT(pT)_{34}$ molecule, and this negative cooperativity becomes weaker as the NaCl concentration is increased. The other possibility is that two classes of DNA binding sites, possessing different affinities for $dT(pT)_{34}$, exist on the SSB tetramer, and the affinities for these two sites exhibit different salt dependences. We favor the former explanation of negative cooperativity between DNA binding sites for the reasons given under Discussion.

In order to further probe the effects of salt on the apparent negative cooperativity, we compared the effect of $MgCl_2$ vs NaCl on the binding of $dT(pT)_{34}$ to SSB protein in Figure 2B. Six titrations were performed in buffer T (pH 8.1, 25.0 °C)

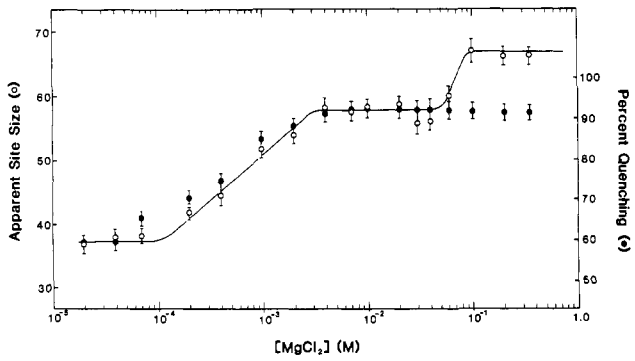

FIGURE 3: Quenching of the SSB protein fluorescence by poly(dT) as a function of the logarithm of the $MgCl_2$ concentration (●) compared directly with the apparent site size of the SSB protein on poly(dT) (○) [site size data are from Bujalowski and Lohman (1986)]. The increase in fluorescence quenching exactly corresponds to the transition from the $(SSB)_{35}$ mode to the $(SSB)_{56}$ mode.

+ 1.5 mM NaCl (without EDTA) in the presence of $MgCl_2$ concentrations in the range from 49 μM to 3 mM. We see the same general phenomenon as with NaCl; i.e., increasing the $MgCl_2$ concentration diminishes the differences in apparent affinity of $dT(pT)_{34}$ for the two binding sites. However, by comparison of the two titrations at 0.2 mM $MgCl_2$ and 20 mM NaCl in Figure 2, it is clear that $MgCl_2$ is effective at an approximately 100-fold lower concentration than NaCl. The data in Figure 2 indicate that a *net* binding of ions accompanies the binding of the second molecule of $dT(pT)_{34}$ to the SSB protein. The titrations in the presence of $MgCl_2$ display the same extent of fluorescence quenching upon binding the first $dT(pT)_{34}$ molecule (~50%) as well as the same total fluorescence quenching at saturation (~90%), although the titrations at the lower $MgCl_2$ concentrations seem to show a slightly lower extent of total fluorescence quenching. For the lower salt concentrations, the maximum quenching is estimated from an extrapolation of Q_{obsd} to saturation, as shown in Figure 1B.

Correlation between the Salt Dependence of the Apparent Negative Cooperativity and the SSB Protein–ss Polynucleotide Binding Mode Transitions. The data for $dT(pT)_{34}$ binding to the SSB tetramer can be compared with the measurements of SSB protein site sizes (number of nucleotides occluded per bound SSB tetramer), determined from titrations of SSB protein with poly(dT). We have previously observed multiple SSB protein–ss polynucleotide binding modes that differ in their site sizes, and the transitions from the lower site size to the higher site size modes are induced by NaCl and $MgCl_2$ in the same concentration range as is required to promote the binding of the second molecule of $dT(pT)_{34}$ (Lohman & Overman, 1985; Bujalowski & Lohman, 1986). Figure 3 shows a composite plot of the apparent site size per SSB tetramer on poly(dT) as a function of $MgCl_2$ concentration that we have previously determined (Bujalowski & Lohman, 1986), compared directly to the maximal fluorescence quenching observed for each titration with poly(dT) (25.0 °C, pH 8.1). Below 0.1 mM $MgCl_2$, we observe a plateau in fluorescence quenching at 56 ± 3%, corresponding to the site size plateau of 35 nucleotides per tetramer [$(SSB)_{35}$ mode]. Increasing the $MgCl_2$ concentration from 0.1 to 3 mM induces the $(SSB)_{35}$ to $(SSB)_{56}$ transition, with an accompanying increase in the extent of fluorescence quenching; the intermediate site sizes and fluorescence quenchings presumably reflect a mixed population of the two modes. The site size plateau at 56 ± 3 nucleotides per tetramer [$(SSB)_{56}$ binding mode] coincides with a plateau in the fluorescence quenching at 90 ± 2%, and no further change in the extent of quenching is observed upon formation of the $(SSB)_{65}$ mode. Therefore, the increased fluorescence quenching from 56 ± 3% to 90 ± 2% reflects the transition from the $(SSB)_{35}$ to the $(SSB)_{56}$ binding mode. In addition, the $MgCl_2$ concentration range over which the $(SSB)_{35}$ to $(SSB)_{56}$ transition occurs exactly corresponds to the range of $MgCl_2$ concentrations for which we observe the reduction in negative cooperativity, which allows the second molecule of $dT(pT)_{34}$ to bind to the SSB tetramer (see Figure 2B). These same correlations are observed for the $dT(pT)_{34}$ and poly(dT) data in NaCl.

The experiments in Figures 2 and 3 confirm our previous interpretation that poly(dT) interacts with only two subunits of the SSB tetramer in the $(SSB)_{35}$ mode, whereas poly(dT) interacts with all four SSB subunits in the $(SSB)_{65}$ binding mode (Lohman & Overman, 1985). However, Figure 3 shows that the $(SSB)_{56}$ mode also exhibits 90 ± 2% protein fluorescence quenching; hence, the ss DNA also interacts with all four SSB subunits in this mode. The differences between the $(SSB)_{56}$ and $(SSB)_{65}$ binding modes are clearly not at the level of the number of SSB subunits that interact with the ss DNA. The apparent negative cooperativity, whatever its molecular basis, is responsible for the difficulty in forming the $(SSB)_{56}$ binding mode in low salt concentrations. The $dT(pT)_{34}$ data in Figure 2 also suggest that the lower site size binding mode, with 35 nucleotides per tetramer, does not result from an intrinsic difficulty to wrap a long ss polynucleotide around the SSB tetramer. Rather, the stability of the lower site size binding mode results from an intrinsic property of an individual SSB tetramer, i.e., the extremely low affinity for binding of ss DNA to the second site on the SSB tetramer at low salt concentrations. Even the binding of a smaller oligonucleotide, $d(pT)_{16}$, which can bind to four sites on the SSB tetramer, displays pronounced negative cooperativity for filling the last two sites on the tetramer (Bujalowski and Lohman, unpublished data); hence, this is not simply a steric effect.

DISCUSSION

We report the observation of a salt-dependent apparent negative cooperativity between the two sites on the *E. coli* SSB protein tetramer for binding of the oligonucleotide $dT(pT)_{34}$. Under the conditions that we have examined, the binding of the first $dT(pT)_{34}$ is extremely tight such that there is essentially no free $dT(pT)_{34}$ up to a 1:1 molar ratio of $dT(pT)_{34}$ per SSB tetramer; hence, we have not been able to measure the affinity of $dT(pT)_{34}$ for the first binding site at [NaCl] ≤200 mM. The apparent affinity of $dT(pT)_{34}$ for binding to the second site on the SSB tetramer is lower than that for the first site at all salt concentrations; however, the apparent affinity for the second site decreases dramatically with decreasing NaCl concentration below 100 mM NaCl or 3 mM $MgCl_2$ (pH 8.1, 25.0 °C). These data could also be interpreted to indicate that the SSB tetramer possesses two nonequivalent, noninteracting classes of binding sites for $dT(pT)_{34}$, with each site possessing a different salt dependence. We cannot rule out this possibility solely on the basis of the binding experiments reported here. However, we favor the interpretation that this phenomenon reflects negative cooperativity between binding sites within an SSB tetramer for the following reasons. The SSB tetramer is composed of identical subunits (Sancar et al., 1981), and preliminary structural data indicate that the tetramer possesses D_2 symmetry (Ollis et al., 1983). In addition, previous studies show no evidence for a salt-induced structural transition of unliganded SSB protein (Lohman & Overman, 1985). Therefore, it is most likely that the four subunits in the free protein are initially equivalent and that negative cooperativity is induced upon binding of ss DNA to

the first one or two subunits. Moreover, the apparent binding constant of $dT(pT)_{34}$ for the second site, K_2, shows an optimum at ~0.1 M NaCl as a function of salt concentration (Bujalowski and Lohman, unpublished data). This behavior indicates that K_2 does not reflect a single independent binding process, since intrinsic SSB–ss polynucleotide interactions decrease with increasing salt concentration (Lohman et al., 1986a; Overman et al., 1988). At all NaCl concentrations, at pH 8.1 and 25 °C, the extent of SSB protein fluorescence quenching is ~50% upon binding a single molecule of $dT(pT)_{34}$ per tetramer; however, when the second site on the SSB tetramer is filled, an additional ~36–40% quenching is observed. This nonequivalent quenching also suggests the existence of interactions between the two ss DNA binding sites within the tetramer. Certainly, if the two sites were equivalent and noninteracting, one would expect equal degrees of fluorescence quenching upon filling each site.

The increase in the apparent binding constant for the second molecule of $dT(pT)_{34}$ upon increasing the NaCl concentration (in the range from 1.5 to 200 mM NaCl) may seem at odds with the typical observation of a decrease in protein–nucleic acid affinity at higher NaCl concentrations (Record et al., 1976; Lohman et al., 1980). However, due to the interactions between the two sites on the SSB tetramer, the apparent binding constant for $dT(pT)_{34}$ binding to the second site is composed of two factors: the intrinsic binding constant for the $dT(pT)_{34}$–protein site interaction and a negative cooperativity parameter. Therefore, the salt dependence of the apparent affinity is a composite of the salt dependences of these two factors. Since the intrinsic binding constant must decrease with increasing NaCl concentration over this range of salt concentrations,[1] the salt dependence of the negative cooperativity must be of opposite sign and greater than the salt dependence of the intrinsic binding constant (i.e., the cooperativity parameter is <1 and increases with increasing NaCl concentration). In fact, upon increasing the NaCl concentration above 200 mM, we do observe a decrease in apparent affinity for the second $dT(pT)_{34}$ molecule (unpublished data), indicating that the intrinsic binding constant does decrease with increasing salt concentration and that this feature dominates above 200 mM NaCl. These same effects are also observed at a 50–100-fold lower concentration of Mg^{2+}, in the range below 3 mM $MgCl_2$ (25 °C, pH 8.1), indicating that these salt effects are the result of the direct binding of ions to the SSB protein–ss oligonucleotide complex and are not ionic strength effects. The observed salt dependence of the apparent negative cooperativity reflects the requirement for some ion uptake to accompany the binding of the second molecule of $dT(pT)_{34}$ per SSB tetramer. One possible explanation for this ion uptake is that the two DNA binding sites on the tetramer may be in close proximity. If this is the case, then the increased negative charge due to the first bound $dT(pT)_{34}$ may make it difficult for the SSB protein to bind the second $dT(pT)_{34}$ without also binding cations in order to partially neutralize the negative charge from the first bound $dT(pT)_{34}$.

Our observation that each SSB protein tetramer is capable of binding two molecules of $dT(pT)_{34}$ or four molecules of $d(pT)_{16}$ at saturation supports the conclusion that a ss nucleic acid of sufficient length is able to occupy all of the nucleic acid binding sites and wrap around the SSB tetramer (Krauss et al., 1981). However, we find twice as many binding sites on the SSB tetramer for each of these oligonucleotides than reported by Krauss et al. (1981), who also did not report any differences in binding affinity for the multiple binding sites on the SSB tetramer. Although Krauss et al. (1981) determined their binding isotherms at 8 °C, their stoichiometries for oligonucleotide binding were determined at 20 °C, using sedimentation velocity; hence, these may have been underestimated due to the difficulty of saturating the SSB protein at this higher temperature.[2] The higher stoichiometries that we have measured are fully consistent with the SSB protein–ss polynucleotide site sizes that we have previously reported (Lohman & Overman, 1985; Bujalowski & Lohman, 1986).

The tetrameric *E. coli* SSB protein seems unique among helix-destabilizing proteins in its DNA binding properties, since it possesses both positive cooperativity between adjacent DNA-bound tetramers and an apparent negative cooperativity between DNA binding sites *within* a tetramer. Even the positive cooperativity that exists between ss DNA bound SSB tetramers is qualitatively different than that observed for T4 gene 32 protein and fd gene 5 protein. At equilibrium, in the $(SSB)_{65}$ binding mode, *E. coli* SSB protein binds to ss polynucleotides with *positive* cooperativity between adjacent tetramers, forming an equilibrium mixture of tetramers and octamers (Bujalowski & Lohman, 1987a). Nonequilibrium, *metastable* complexes, exhibiting high positive cooperativity of the "unlimited" type that results in long clusters of SSB tetramers on ss DNA have also been observed (Lohman et al., 1986a; Ruyechan & Wetmur, 1975; Sigal et al., 1972). These multiple cooperativities, both positive and negative, as well as the tetrameric structure of the protein, contribute to the fact that the *E. coli* SSB protein can bind to ss polynucleotides in a number of different modes.

The ranges of NaCl and $MgCl_2$ concentrations over which we observe the reduction in apparent negative cooperativity, which results in the ability to bind the second molecule of $dT(pT)_{34}$, are the same as those that induce the $(SSB)_{35}$ to $(SSB)_{56}$ transition (Bujalowski & Lohman, 1986). Therefore, it seems that this SSB protein–ss polynucleotide binding mode transition is directly linked to the reduction in apparent negative cooperativity within individual SSB tetramers. The transition between the $(SSB)_{56}$ and the $(SSB)_{65}$ binding modes occurs at significantly higher NaCl and $MgCl_2$ concentrations and is not accompanied by any further SSB protein fluorescence quenching, which suggests that sufficiently long ss DNA interacts with all four SSB subunits in both the $(SSB)_{56}$ and $(SSB)_{65}$ binding modes. The $(SSB)_{35}$ to $(SSB)_{56}$ binding mode transition also displays a very different dependence on pH, anions, and cations than does the $(SSB)_{56}$ to $(SSB)_{65}$ transition (Bujalowski & Lohman, 1986; Bujalowski et al., 1988); hence, they are clearly separate processes.

Negative cooperativity has been observed for a number of enzyme–substrate interactions (Levitzki & Koshland, 1976). What may be the biological significance of the apparent negative cooperativity exerted among the ss DNA binding sites within an individual SSB tetramer? The SSB protein is in-

[1] The binding of the first molecule of $dT(pT)_{34}$ to the SSB tetramer is stoichiometric in NaCl concentrations below 200 mM at the lowest SSB protein concentration that we have examined [10^{-7} M (tetramer)] at pH 8.1, 25.0 °C. We emphasize that this does not mean that the intrinsic binding constant, K_1, for this interaction is independent of NaCl concentration. Rather, this simply indicates that even though K_1 decreases with increasing NaCl concentration, it remains greater than a critical value ($\sim 1 \times 10^9$ M^{-1}) even up to 200 mM NaCl. As long as K_1 is greater than 10^9 M^{-1}, all titrations for binding of $dT(pT)_{34}$ to the first site will appear identical and stoichiometric, independent of the actual value of K_1.

[2] The stoichiometries measured by Krauss et al. (1981) may also have been underestimated since an extinction coefficient for SSB protein was used that is lower by a factor of 1.5 than the value of 1.5 mL mg^{-1} cm^{-1} that we have used (Lohman & Overman, 1985).

volved in a number of different processes in vivo, some of which may require different modes of binding of SSB protein to ss DNA. In addition to its ability to bind selectively to ss DNA, the *E. coli* SSB protein has been shown to interact specifically with a number of other proteins involved in DNA metabolism (Sigal et al., 1972; Molineux et al., 1974; Molineux & Gefter, 1975; Low et al., 1982). Therefore, in some of its functions, the SSB protein may be required to interact simultaneously with ss DNA and some of these proteins. Strong negative cooperativity would enable the SSB protein to bind tightly to ss DNA with two of its subunits, as in the $(SSB)_{35}$ mode, while the remaining two subunits could potentially interact with other proteins. In the absence of strong negative cooperativity, all four of the SSB protein subunits would bind ss DNA as in the $(SSB)_{56}$ or $(SSB)_{65}$ binding modes.

The ability to form long clusters on ss DNA is thought to be a necessary feature for the function of helix-destabilizing proteins during DNA replication, although there is no proof for this proposal. The solution conditions that favor the *metastable* long clusters of SSB protein are the same conditions that favor the formation of the $(SSB)_{35}$ binding mode and the onset of the apparent negative cooperativity between DNA binding sites within an SSB tetramer. The $(SSB)_{35}$ binding mode, in which two subunits within each SSB tetramer are free of ss DNA, may enable adjacent tetramers to interact more strongly and with "unlimited" nearest-neighbor positive cooperativity so that continuous clusters of SSB tetramers can form on ss polynucleotides. The apparent negative cooperativity that exists within an SSB tetramer may be important in regulating the switch between the low, "limited" cooperativity SSB binding mode that may be used for repair and recombination processes (Griffith et al., 1984) and the high, "unlimited" cooperativity SSB binding mode that may be used transiently during DNA replication.

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