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A mutation in *E. coli* SSB protein (W54S) alters intra-tetramer negative cooperativity and inter-tetramer positive cooperativity for single-stranded DNA binding

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Abstract

E. coli SSB tetramer binds with high affinity and cooperatively to single-stranded (ss) DNA and functions in replication, recombination and repair. Curth et al. (Biochemistry, 32 (1993) 2585–2591) have shown that a mutant SSB protein, in which Trp-54 has been replaced by Ser (W54S) in each subunit, binds preferentially to ss-polynucleotides in the $(SSB)_{35}$ mode in which only 35 nucleotides are occluded per tetramer under conditions in which wild-type (wt) SSB binds in its $(SSB)_{65}$ mode. The W54S mutant also displays increased UV sensitivity and slow growth phenotypes, suggesting defects in vivo in both repair and replication (Carlini et al. (Molecular Microbiology, 10 (1993) 1067)). We have characterized the energetics of SSBW54S binding to poly(dT) as well as short oligodeoxyribonucleotides ($dA(pA)_{69}$, $dT(pT)_{34}$, $dC(pC)_{34}$) to determine the basis for this dramatic change in binding mode preference. We find that the W54S mutant remains a stable tetramer; however, its affinity for ss-DNA as well as both the intra-tetramer negative cooperativity and its inter-tetramer positive cooperativity in the $(SSB)_{35}$ mode (ω_{35}) are altered significantly compared to wtSSB. The increased intra-tetramer negative cooperativity makes it more difficult for ss-DNA to bind the third and fourth subunits of the W54S tetramer, explaining the increased stability of the $(SSB)_{35}$ mode in complexes with poly(dT). When bound to $dA(pA)_{69}$ in the $(SSB)_{35}$ mode, W54S tetramer also displays a dramatically lower inter-tetramer positive cooperativity ($\omega_{35} = 77(\pm 20)$) than wtSSB ($\omega_{35} \geq 10^5$) as well as a significantly lower affinity for ss-DNA. These results indicate that a single amino acid change can dramatically influence the ability of SSB tetramers to bind in the different SSB binding modes. The altered ss-DNA properties of the W54S SSB mutant are probably responsible for the observed defects in replication and repair and support the proposal that the different SSB binding modes may function selectively in replication, recombination and/or repair.

Keywords: Fluorescence; Thermodynamics; Energetics; Replication; Recombination; Repair

1. Introduction

The *E.coli* single-stranded DNA binding (SSB) protein, a member of the general class of helix destabilizing proteins, has essential roles in DNA

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replication and repair and facilitates RecA mediated recombination [1–6]. The protein forms a stable homotetramer [7–10] (subunit $M_r = 18{,}843$) [11,12] and the tetramer is the functional form of the protein [10,13–15]. The SSB tetramer not only binds with high affinity to ss nucleic acids, but can bind in several binding modes, referred to as $(SSB)_n$, where n represents the average number of nucleotides occluded per tetramer [16–18]. At 25°C (pH 8.1), three binding modes have been identified with $n = 35 \pm 2$, 56 ± 3, and 65 ± 3 nucleotides per tetramer, whereas an additional binding mode with $n = 40 \pm 2$ occurs at 37°C [16,18–20]. The relative stabilities of these binding modes are quite sensitive to solution conditions, especially the type and concentration of monovalent salt, divalent and multivalent salts such as polyamines, as well as pH, temperature and SSB binding density [17–19,21]. The $(SSB)_{35}$ mode is favored at high SSB binding density and low salt concentration ($\leq$ 10 mM NaCl) [16,19], whereas the higher site size modes are stabilized at higher salt concentrations and low SSB binding density. Only two of the subunits of the tetramer interact with ss nucleic acid in the $(SSB)_{35}$ mode, whereas all four subunits interact with DNA in both the $(SSB)_{56}$ and $(SSB)_{65}$ modes [15,22]. The transition from lower to higher site size modes results in a compaction of the protein–DNA complex [17,19,23], consistent with the greater number of subunit contacts in the higher site size modes. Two different SSB–M13 DNA complexes have been observed by electron microscopy [17,23]; a "beaded" complex, which correlates with the $(SSB)_{56}$ and/or $(SSB)_{65}$ binding modes, is observed at higher salt and low SSB binding density, whereas a "smooth-contoured" complex, which correlates with the $(SSB)_{35}$ mode, is observed at low salt and high SSB concentrations.

The relative stability of the $(SSB)_{35}$ mode at low salt concentrations is due at least in part to a high degree of negative cooperativity among the DNA binding sites on the SSB tetramer [14,15,22]. This negative cooperativity manifests itself as a decreased affinity of ss-oligodeoxynucleotides for the third and fourth subunits of the SSB tetramer and becomes more pronounced at lower salt concentration [14,15,24]. This negative cooperativity is a property of the SSB tetramer since it is observed for $dT(pT)_{15}$ binding to the SSB-1 tetramer, but not the SSB-1 monomer [25]. Although there is clearly an electrostatic component to the negative cooperativity, it is also dramatically influenced by the base composition of the ss-DNA [24]. The greatest degree of negative cooperativity is observed with the binding of $dA(pA)_{34}$, followed by $dT(pT)_{34}$, and then $dC(pC)_{34}$. This correlates well with the inability of the $(SSB)_{35}$ mode to form on poly(dC), even at [NaCl] $\leq$ 10 mM [24].

The SSB tetramer also displays positive cooperativity in its binding to ss-polynucleotides. However, the type and magnitude of the positive cooperative interactions between tetramers differs dramatically for the $(SSB)_{65}$ and $(SSB)_{35}$ modes [26–28]. SSB tetramers bind with an "unlimited" type of cooperativity in the $(SSB)_{35}$ mode, and thus can form long protein clusters which can saturate the DNA [3,17,26,29], in a manner similar to that observed for the phage T4 gene 32 protein [30–32]. The nearest-neighbor cooperativity parameter for this binding mode (ω_{35}) is estimated to be $\geq 10^5$ (25.0°C, 0.125 M NaCl, pH 8.1) [28]. In contrast, a "limited" type of positive inter-tetramer cooperativity is observed in the $(SSB)_{65}$ mode, such that protein clustering is limited to the formation of dimers of tetramers [23,27]. The "limited" cooperativity parameter, $\omega_{T/O}$, is 420 ± 80 (25°C, 0.2 M NaCl, pH 8.1) [9,27], but increases with temperature [2,33].

Since the different SSB polynucleotide binding modes display such very different properties, we have proposed that some of the binding modes may be used selectively in DNA replication, recombination, and repair [2–4]. For example, if the ability to form long contiguous clusters of protein along ss-DNA is an important feature for the function of helix destabilizing proteins in DNA replication, then the $(SSB)_{35}$ mode, with its high degree of "unlimited" cooperativity, is well suited for this purpose [3,4,28]. However, since the SSB tetramer binds with "limited" cooperativity in its $(SSB)_{65}$ mode, some regions of ss-DNA remain accessible, even at high SSB binding densities. Thus, the $(SSB)_{65}$ mode may be used selectively in repair and recombination, allowing access to the DNA by other proteins involved in these processes. In fact, there is some evidence to support the selective role of the $(SSB)_{56}$ and/or $(SSB)_{65}$ binding modes in RecA-mediated recombination events (for a review see [4]).

A determination of the roles of the different SSB binding modes in DNA metabolism would be greatly facilitated if SSB mutants could be found for which one of the SSB–ss-DNA binding modes is selectively stabilized. Along these lines, Curth et al. [34] have recently made a series of SSB mutants in which the four tryptophan (W) residues within each of the four SSB subunits (at positions 40, 54, 88, and 135) were systematically replaced. Surprisingly, the W54S (Trp-54 to Ser) mutation resulted in preferential stabilization of the $(SSB)_{35}$ polynucleotide binding mode. Carlini et al. [35] further showed that when the *ssbW54S* gene is expressed in *E. coli*, the cells exhibit slow growth and increased UV sensitivity, consistent with defects in DNA replication and repair. Although Curth et al. showed that the $(SSB)_{35}$ polynucleotide binding mode is preferentially formed in vitro at 0.3 M NaCl, whereas wtSSB forms the $(SSB)_{65}$ mode [34], the W54S mutant was not characterized further in terms of its DNA binding properties. In this report, we present a more detailed characterization of the various ss-DNA binding properties of the W54S mutant, including quantitative estimates of its negative inter-tetramer cooperativity and the two types of positive inter-tetramer cooperativity associated with the $(SSB)_{35}$ and $(SSB)_{65}$ polynucleotide binding modes. Our results support the suggestion that changes in the relative stabilities of the SSB binding modes can influence the role of SSB in vivo.

2. Materials and methods

2.1. Reagents and buffers

All chemicals were reagent grade. All solutions were prepared with distilled and deionized (Milli-Q) water. Buffer T is 10 mM Tris (tris(hydroxymethyl)aminomethane), pH 8.1, 0.1 mM Na_3EDTA (ethylenediamine-tetraacetic acid). The salt concentration contained in the buffer is indicated for each experiment.

2.2. SSB proteins and DNA

Wild-type *E. coli* SSB protein was purified as described [36] and its concentration determined spectrophotometrically in Buffer T + 0.20 M NaCl ($\epsilon_{280} = 1.13 \times 10^5$ M^{-1}(tetramer) cm^{-1}) [16]. The W54S SSB mutant protein was purified from *E. coli* strain K12 containing the plasmid pSF1-W54S (a generous gift of Drs. G. Maass and C. Urbanke (Medizinische Hochschule, Hannover, Germany)). W54S protein was purified using the procedure for wtSSB [36] with one modification. The NaCl concentration used to load the W54S protein onto the ss-DNA cellulose column was decreased to 0.1 M NaCl (50 mM Tris, pH 8.3, 1 mM EDTA, 10% glycerol). The W54S concentration was determined spectrophotometrically in Buffer T + 0.20 M NaCl ($\epsilon_{280} = 9.04 \times 10^4$ M^{-1}(tetramer) cm^{-1}). This extinction coefficient was calculated by accounting for the loss of one tryptophan residue per SSB monomer [37].

Poly(dT) was from Sigma (St. Louis, MO) and had $s_{20,w} = 10.1$ S, corresponding to an average length of > 1000 nucleotides [38]; it was dialyzed extensively before use. Poly(dT) concentration was determined spectrophotometrically in Buffer T (pH 8.1), 0.1 M NaCl ($\epsilon_{260} = 8100$ M^{-1}(nucleotide) cm^{-1}) [39]. The oligodeoxynucleotides, $dA(pA)_{69}$, $dC(pC)_{34}$, and $dT(pT)_N$ (where $N = 69$, 34, and 15), were synthesized and purified (≥ 98% pure) as described [28]. The DNA was resuspended in Buffer T containing the appropriate NaCl concentration, and dialyzed extensively before use. Oligodeoxynucleotide concentrations were determined spectrophotometrically in Buffer T (pH 8.1), 0.1 M NaCl, using the following extinction coefficients (per nucleotide): $dT(pT)_n$: $\epsilon_{260} = 8.1 \times 10^3$ M^{-1} cm^{-1}; $dA(pA)_n$: $\epsilon_{257} = 1.0 \times 10^4$ M^{-1} cm^{-1}; $dC(pC)_n$: $\epsilon_{270} = 7.2 \times 10^3$ M^{-1} cm^{-1} [39].

2.3. Fluorescence titrations

Equilibrium binding of SSB and SSBW54S tetramers to DNA was monitored by the quenching of protein tryptophan fluorescence ($\lambda_{ex} = 300$ nm, (2 nm band pass); $\lambda_{em} = 347$ nm, (8 nm band pass)), using an SLM 8000C spectrofluorometer. The temperature of the sample was maintained at 25.0 ± 0.1°C. "Reverse" titrations were performed by addition of DNA to a constant concentration of SSB in either Buffer T containing the appropriate NaCl concentration. The observed quenching of the fluorescence was calculated as $Q_{obs} = (F_0 - F_i)/F_0$; where

F_0 is the initial fluorescence of the free protein, and F_i is the fluorescence after addition of the ith aliquot of DNA. All measurements were corrected for dilution, background fluorescence, and inner filter effects as previously described [40]. Photobleaching was not observed under these conditions.

2.4. Determination of K_N for formation of a 1:1 W54S–dT(pT)$_{34}$ complex

Under conditions where a 1:1 W54S–dT(pT)$_{34}$ complex is formed, values of K_N, the equilibrium association constant, and Q_{max}, the maximum fluorescence quenching at saturating DNA concentrations, were determined by non-linear least squares analysis of the experimental isotherms using Kaleidagraph (Synergy Software, Reading, PA). For this model, $Q_{obs}/Q_{max} = [PD]/P_T = K_N D_f/(1 + K_N D_f)$, where $[PD]/P_T$ is the fraction of SSB tetramers bound to DNA and D_f is the free DNA concentration. An expression for Q_{obs} written in terms of total SSB tetramer concentration, P_T, and total DNA concentration, D_T, is given in Eq. (1), where $K_D = 1/K_N$

$$Q_{obs} = \frac{Q_{max}\left(D_T + K_D + P_T - \left((D_T + K_D + P_T)^2 - 4P_T D_T\right)^{1/2}\right)}{2P_T} \quad (1)$$

2.5. Analysis of SSB–dN(pN)$_{34}$ binding under conditions such that two dN(pN)$_{34}$ can bind per SSB tetramer

Equilibrium titrations of SSB with dT(pT)$_{34}$ in the presence of $MgCl_2$ were analyzed using the square model [27] (see Eq. (3)). This model is described by two equilibrium constants, K_{35}, the intrinsic equilibrium constant for binding dT(pT)$_{34}$ to two SSB subunits, and σ_{35}, the negative cooperativity parameter. The data were simultaneously analyzed using the nonlinear least squares algorithm, NONLIN [41] on a Hewlett-Packard Apollo 9000/730 computer as described [42]. Binding isotherms were simulated based on Eq. (2) using Kaleidagraph Software (Synergy Software, Reading, PA), where $Q_{1/35}$ and $Q_{2/35}$ are the extents of

$$Q_{obs} = \frac{Q_{1/35}(4\sigma_{35} + 2)K_{35}L_f + Q_{2/35}3\sigma_{35}^4(K_{35}L_f)^2}{1 + 4\sigma_{35}K_{35}L_f + 2K_{35}L_f + 3\sigma_{35}^4(K_{35}L_f)^2} \quad (2)$$

tryptophan fluorescence quenching associated with binding one and two dT(pT)$_{34}$ molecules, respectively.

2.6. Analysis of W54S– and SSB–dC(pC)$_{34}$ titrations in 0.2 M NaCl

Under these conditions, two dC(pC)$_{34}$ molecules bind to the W54S and wtSSB tetramers. However, the affinity for the first site is too high to accurately determine K_{35} and σ_{35} (see Eq. (2)), thus the equilibrium titrations were analyzed using Eq. (3), where $K_{1/35}$ and $K_{2/35}$ are the macroscopic binding constants for the first and second molecule of dC(pC)$_{34}$ [14]

$$Q_{obs} = \frac{Q_{1/35}K_{1/35}L_f + Q_{2/35}K_{1/35}K_{2/35}L_f^2}{1 + K_{1/35}L_f + K_{1/35}K_{2/35}L_f^2} \quad (3)$$

We emphasize that for wtSSB, the binding is sufficiently tight so that $K_{1/35}$ and $K_{2/35}$ represent minimum estimates.

2.7. Analysis of SSB W54S tetramer binding to poly(dT)

Multiple titrations were performed at different W54S concentrations (0.6 M NaCl, pH 8.1, 25°C) and model-independent equilibrium binding isotherms were constructed using the binding density function method [40,43,44]. Since the spectroscopic signal (fluorescence quenching) is from the protein, the average moles of SSB tetramer bound per mole nucleotide, $\Sigma\nu_i$, is related to the binding density function, $Q_{obs}(P_T/D_T)$, as in Eq. (4)

$$Q_{obs}(P_T/D_T) = \Sigma\nu_i Q_i \quad (4)$$

where Q_{obs} is the observed protein fluorescence quenching at total poly(dT) concentration (in nucleotides) D_T and total tetramer concentration P_T, and Q_i is the extent of fluorescence quenching when

a W54S tetramer is bound in mode "i". A series of titrations of W54S protein with poly(dT) were performed at several total protein concentrations, P_{Tx}, and the binding density function, $Q_{obs}(P_T/D_T)$, plotted as a function of log [poly(dT)] for each titration. At each constant value of $Q_{obs}(P_T/D_T)$, we obtain a set of values of P_{Tx} and D_{Tx} ($x = 1$, 2...5). A plot of P_T as a function of D_T is analyzed to obtain $\Sigma\nu_i$, the average number of SSB tetramers bound per nucleotide, and P_f, the corresponding free W54S tetramer concentration, according to

$$P_T = P_f + (\Sigma\nu_i) D_T \quad (5)$$

From these data one can construct a model-independent equilibrium binding isotherm without knowledge of the relationship between the extent of binding and fluorescence quenching.

Using the binding density function analysis, we established that a linear relationship exists between Q_{obs} and the fraction of bound W54S tetramer, P_b/P_T, allowing us to use the following three equations

$$Q_{obs}/Q_{max} = P_b/P_T \quad (6)$$

$$\nu_Q = (Q_{obs}/Q_{max})(P_T/D_T) \quad (7)$$

$$P_f = 1 - (Q_{obs}/Q_{max}) P_T \quad (8)$$

to analyze the data. A closed form expression for Q_{obs} as a function of DNA concentration can be obtained by substituting Eq. (7) and Eq. (8) into Eq. (9). The root of Eq. (9) gives the theoretical value of Q_{obs} based on the McGhee–von Hippel model [45,46] for the "unlimited" cooperative binding of large ligands to a homogeneous lattice

$$\frac{V_Q}{P_f} - K t_1 t_2^{n-1} t_3^2 = 0 \quad (9)$$

where

$$t_1 = 1 - n\nu_Q$$

$$R = \sqrt{(1 - (n+1)\nu_Q)^2 + 4\omega_{unlim}\nu_Q t_1}$$

$$t_2 = \frac{2\omega_{unlim} t_1}{(2\omega_{unlim} - 1)t_1 + \nu_Q + R}$$

$$t_3 = \frac{1 - (n+1)\nu_Q + R}{2t_1}$$

Best fits of the data to Eq. (9) were obtained by iteratively adjusting the values of K and ω_{unlim} using the non-linear least squares algorithm NONLIN [41] on a Hewlett-Packard Apollo 9000/730 computer. The site size, n, was fixed at 40 nucleotides based on an estimate of the site size from titrations performed at 11.2 μM W54S. Constraining n to a value of 35 nucleotides did not significantly alter the quality of the fit. Q_{max} was fixed at 0.75, the observed plateau value in the titration curves.

2.8. Determination of intertetramer SSB cooperativity (ω_{35}) in the $(SSB)_{35}$ mode

Multiple titrations of SSB W54S with $dA(pA)_{69}$ were performed at several W54S concentrations in 30 mM NaCl (25°C, pH 8.1), and model-independent equilibrium binding isotherms were constructed using the binding density function method [43,44]. These isotherms were analyzed to estimate the intertetramer positive cooperativity in the $(SSB)_{35}$ mode using a model described previously for wtSSB [28]. However, due to the extreme negative cooperativity for ss DNA binding by the W54S tetramer, 1:1 complexes in which all 4 SSB subunits interact with $dA(pA)_{69}$ are not populated. Therefore, the model used considered only complexes in which W54S tetramers bind to DNA using 2 subunits and occluding 35 nucleotides. A 1:1 W54S tetramer–$dA(pA)_{69}$ complex forms with binding constant K^*_{35}, in which only two of the W54S subunits interact with the DNA occluding 35 nucleotides (Complex I, Fig. 1). The other complex is one in which two W54S tetramers bind to $dA(pA)_{69}$, each with binding constant K^*_{35}, so that only two protomers of each tetramer interact with the ss-DNA, occluding 35 nucleotides each (Complex II, Fig. 1). The partition function for the SSB–$dA(pA)_{69}$ system, with free $dA(pA)_{69}$ as the reference state, is given in Eq. (10), where $S_1 = 70 - 35 + 1 = 36$ is the statistical factor for the number of binding sites available to one SSB tetramer bound using only 2 subunits

$$Z = 1 + S_1 K^*_{35} P_f + \omega_{35}(K^*_{35})^2 P_f^2 \quad (10)$$

The degree of binding, ν (W54S tetramers bound per $dA(pA)_{69}$), is then given by

$$\nu = \frac{S_1 K^*_{35} P_f + 2\omega_{35}(K^*_{35} P_f)^2}{1 + S_1 K^*_{35} P_f + \omega_{35}(K^*_{35} P_f)^2} \quad (11)$$

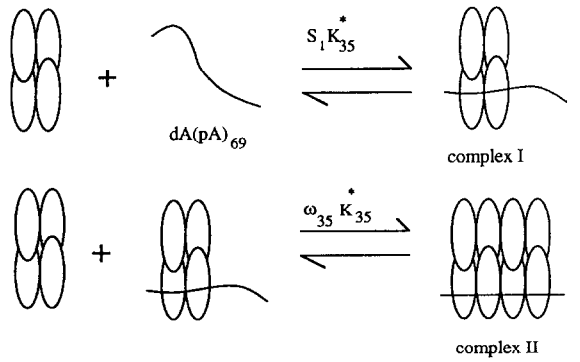

Fig. 1. Cartoon depicting the different possible complexes that can form upon interaction of SSB W54S tetramers with $dA(pA)_{69}$. Complex I: $(SSB)_{35}$ complex in which only two SSB subunits interact with the ss-DNA; Complex II: two SSB tetramers bound to $dA(pA)_{69}$, each in the $(SSB)_{35}$ mode.

The observed SSB fluorescence quenching, Q_{obs}, is related to ν as in the equation

$$Q_{obs} = \frac{D_f\left(Q_{35}S_1K_{35}^* + 2Q_{35}\omega_{35}\left(K_{35}^*\right)^2P_f\right)}{1 + D_f\left(S_1K_{35}^* + 2\omega_{35}\left(K_{35}^*\right)^2P_f\right)} \quad (12)$$

Estimates of K_{35}^* and ω_{35} were made by simultaneous analysis of the multiple titrations performed at six W54S concentrations at 30 mM NaCl according to Eq. (12) using the nonlinear least squares algorithm NONLIN [41] as described previously [28]. The simulated isotherms shown in Fig. 10 were calculated using Eq. (12) and the interaction parameters obtained from the non-linear least squares fitting, using Kaleidagraph Software. The value of $Q_{35} = 0.45 \pm 0.02$ was determined from the plateau value (Q_{max}) of titrations performed at 30 mM NaCl.

2.9. Sedimentation studies

Sedimentation velocity experiments with SSBW54S were performed at several W54S concentrations (0.16 to 0.54 μM (tetramer)) in 10 mM Tris pH 8.1, 0.2 M NaCl at 20°C using an Optima XL-A analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA). The absorbance profile was obtained by scanning each cell at 230 nm. The sedimentation coefficient was determined from the position of the second moment of the boundary vs. $\omega^2 t$ using the XLAGAMMA software provided by the manufacturer. The sedimentation coefficients are reported as $s_{20,w}$. Sedimentation velocity experiments with poly(dT) were performed in 10 mM Tris pH 8.1, 0.5 M NaCl, 20°C.

3. Results

Curth et al. [34] characterized a series of SSB mutants in which each of the four tryptophan (W) residues (Trp-40, 54, 88, and 135) were systematically mutated. Replacement of Trp-54 with Ser (W54S) yielded a protein which showed preferential stabilization of the $(SSB)_{35}$ polynucleotide binding mode relative to the $(SSB)_{56}$ and $(SSB)_{65}$ modes. For example, whereas wtSSB binds to poly(dT) in the $(SSB)_{65}$ polynucleotide mode at 0.30 M NaCl, occluding 65 nucleotides per tetramer [16,18], the W54S mutant binds in the $(SSB)_{35}$ mode occluding ~ 35 nucleotides per tetramer. However, the W54S mutant was not characterized further in terms of its DNA binding properties. We therefore performed more extensive DNA binding studies to determine the basis for the increased stability of the $(SSB)_{35}$ mode, and to investigate the cooperative interaction between W54S tetramers on DNA.

3.1. SSBW54S protein is tetrameric

We examined the assembly state of the W54S protein by performing sedimentation velocity experiments as a function of protein concentration from 0.64 to 2.2 μM total monomer (10 mM Tris, pH 8.1, 0.2 M NaCl, 20°C). At each protein concentration, we measured $s_{20,w} = 4.23 \pm 0.09$ S, in good agreement with the value of 4.3 ± 0.3 S obtained for wtSSB under the same conditions [9,33]. Therefore, the W54S protein is tetrameric at concentrations at least as low as 0.16 μM tetramer.

3.2. The W54S mutation preferentially stabilizes the $(SSB)_{35}$ polynucleotide binding mode

Curth et al. [34] reported that the W54S mutant binds to poly(dT) with an apparent site size of ~ 35 nucleotides per tetramer in 0.30 M NaCl (20 mM potassium phosphate, pH 7.4). However, at this same

Table 1
Apparent Site Size of W54S–poly(dT) as a Function of [NaCl] and [$MgCl_2$]

[NaCl]/mM	[SSBw54s]/μM	Site size, *n*
5	0.709	§
100	0.709	§
200	0.709	§
300	0.161	39
	0.709	34
	1.29	32
600	0.17	Weak binding
	0.709	Weak binding
	1.42	Weak binding
	2.75	Weak binding
	5.5	Weak binding
	11.2	≈ 42
[$MgCl_2$]/mM		
1	0.12	
5	0.12	33
10	0.12	33
	0.24	33
20	0.12	40
30	0.12	45
	0.24	45
40	0.12	49
	0.24	49
	0.24	47
50	0.24	52
60	0.12	53
	0.24	53
70	0.24	58
80	0.12	63
	0.252	63
	0.5	63
	0.63	63
100	0.12	64
	0.24	57
	0.125	64

§: presence of two binding modes.All measurements performed in Buffer T, pH 8.1, 25°C.

[NaCl], the wild-type SSB tetramer binds to poly(dT) in the $(SSB)_{65}$, covering ~ 65 nucleotides per tetramer [16,18,19]. We have extended these studies by measuring the apparent site size of the W54S tetramer bound to poly(dT) at different [NaCl] as well as [$MgCl_2$] as summarized in Table 1. At low [NaCl] or [$MgCl_2$] (see Fig. 2A), the change in protein Trp fluorescence upon binding poly(dT) is complex, showing an initial quenching followed by a partial recovery of fluorescence upon further addition of poly(dT). This behavior was first observed by Curth et al. [34] in titrations performed at [NaCl] ≤ 0.2 M NaCl, and was interpreted to reflect the coexistence of two binding modes with $n = 27$ and $n = 33$. However, at 0.30 M NaCl, this complex behavior is no longer observed (see Fig. 2B), and we

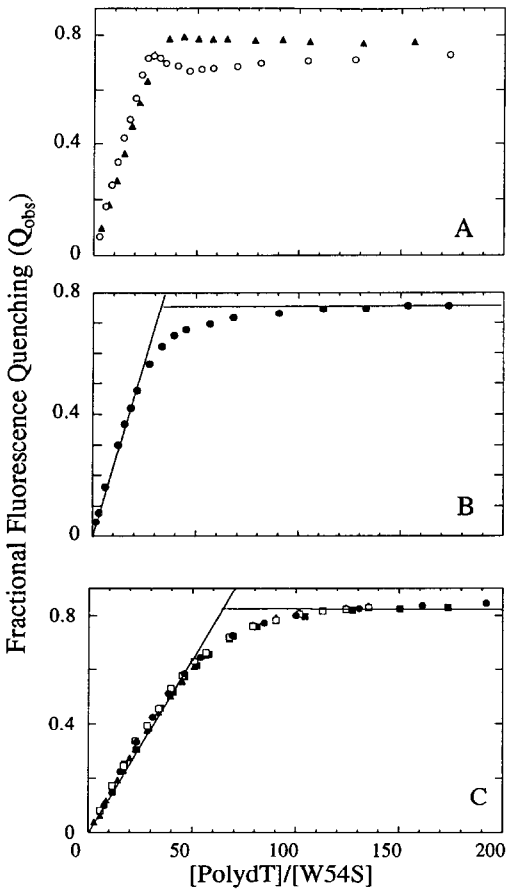

Fig. 2. Titrations of SSB W54S with poly(dT) performed under different solution conditions to determine the occluded site size. The binding was monitored by the quenching of the protein tryptophan fluorescence upon titration with poly(dT). All titrations were performed in Buffer T, pH 8.1 at 25.0°C. (A) (▲) 5 mM NaCl, (0.71 μM W54S tetramer); (○) 1 mM $MgCl_2$, (0.12 μM W54S tetramer). Titrations performed at low salt concentrations showed biphasic behavior, consistent with the presence of two binding modes. (B) 0.3 M NaCl, (1.2 μM W54S tetramer); the apparent site size, $n = 33 \pm 2$ nucleotides per SSB W54S tetramer. (C) 80 mM $MgCl_2$, (■) 0.12, (▲) 0.25, (□) 0.5, (●) 0.63 μM W54S tetramer. The binding is stoichiometric as shown by the overlay of titrations performed at different SSB W54S concentrations (apparent size, $n = 63 \pm 3$ nucleotides per W54S tetramer).

estimate $n = 33 \pm 2$ nucleotides per tetramer, consistent with the results of Curth et al. [34]. Several titrations were performed at different W54S concentrations to ensure that binding is stoichiometric under these conditions. Although the binding of W54S to poly(dT) is sufficiently weak at 0.6 M NaCl to make accurate determination of the site size difficult, we have determined an upper limit of ~ 40 nucleotides per W54S tetramer from experiments performed at 11.2 μM W54S (tetramer). Therefore, the $(SSB)_{35}$ polynucleotide binding mode appears to be favored up to at least 0.60 M NaCl. This is in stark contrast to the wt SSB tetramer which when bound to poly(dT) undergoes a [NaCl]-dependent transition from the $(SSB)_{35}$ to the $(SSB)_{56}$ binding mode which is complete by 50 mM NaCl, and which is then followed by a transition to the $(SSB)_{65}$ binding mode which is complete by ~ 0.2 M NaCl (pH 8.1, 25°C) [18].

In order to compare directly the ability of the W54S mutant, relative to wt SSB, to form the higher site size polynucleotide binding modes we had to perform experiments in buffers containing $MgCl_2$. This was due to the weak binding of W54S to poly(dT) at [NaCl] > 0.3 M. For wtSSB, the transition from the $(SSB)_{35}$ to the $(SSB)_{65}$ mode occurs at lower concentrations of di- and multivalent cations compared to NaCl [16,18,19,21]. We measured a site size of 63 ± 3 nucleotides per W54S tetramer at 80 mM $MgCl_2$, with ~ 77% quenching of the Trp fluorescence at saturation in titrations at three W54S concentrations (0.12–0.63 μM (tetramer)). We note that the large increase in fluorescence quenching accompanying the $(SSB)_{35}$ to $(SSB)_{65}$ binding mode transition on poly(dT) with wt SSB protein is not observed with the W54S mutant (compare Fig. 2A and Fig. 2C). This indicates that it can be very misleading to use the extent of fluorescence quenching as the sole indication of a change in polynucleotide binding modes.

Apparent site sizes for W54S binding to poly(dT) were measured as a function of $[MgCl_2]$ and are compared in Fig. 3 with those measured previously for wt SSB on poly(dT) [18] (see also Table 1). The W54S protein undergoes a transition from the $(SSB)_{35}$ to the $(SSB)_{65}$ mode over a $[MgCl_2]$ range from 0.01 to 0.1 M with a midpoint near ~ 40 mM $MgCl_2$. No intermediate plateau corresponding to the

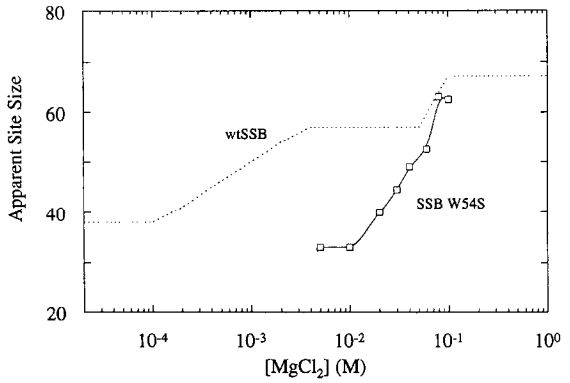

Fig. 3. Apparent site size (nucleotides per tetramer) determined for the SSB W54S mutant binding to poly(dT) (□) as a function of $MgCl_2$ concentration. Experiments were performed in Buffer T, pH 8.1 at 25°C. The dashed line shows the apparent site size measurements of wtSSB tetramer binding to poly(dT) as reproduced from Bujalowski and Lohman [18].

$(SSB)_{56}$ mode is apparent for W54S. However, the $(SSB)_{35}/(SSB)_{65}$ transition for the W54S protein occurs at a ~ 60-fold higher $[MgCl_2]$ than for wt SSB protein. At 10 mM $MgCl_2$, a site size of 33 ± 2 nucleotides per tetramer is measured for the W54S mutant, while wtSSB still binds to poly(dT) in the $(SSB)_{56}$ mode. Therefore, although the W54S tetramer can form the $(SSB)_{65}$ mode on poly(dT), a much higher $[MgCl_2]$ is required than for wtSSB.

3.3. The W54S tetramer displays greater negative cooperativity for ss-DNA binding than does wtSSB

Studies of oligodeoxynucleotide binding to the wt SSB tetramer [14,15,22] indicate that ss-DNA binds with extreme negative cooperativity within individual wtSSB tetramers. We therefore performed equilibrium titrations with $dT(pT)_{34}$ to determine if the extent of this negative cooperativity is affected by the W54S mutation. Fig. 4A shows equilibrium titrations of W54S protein (0.161 μM tetramer) with $dT(pT)_{34}$ performed at 0.1, 0.3, and 0.5 M NaCl (pH 8.1, 25°C). The solid lines in Fig. 4A represent 1:1 binding isotherms simulated using values of K_{35} and Q_{max} determined from nonlinear least squares fitting of the isotherms to Eq. (1) (see Table 2). At 0.1 M NaCl, binding is stoichiometric and too tight to obtain an accurate estimate of K_{35}, but the binding affinity decreases at higher salt concentrations so

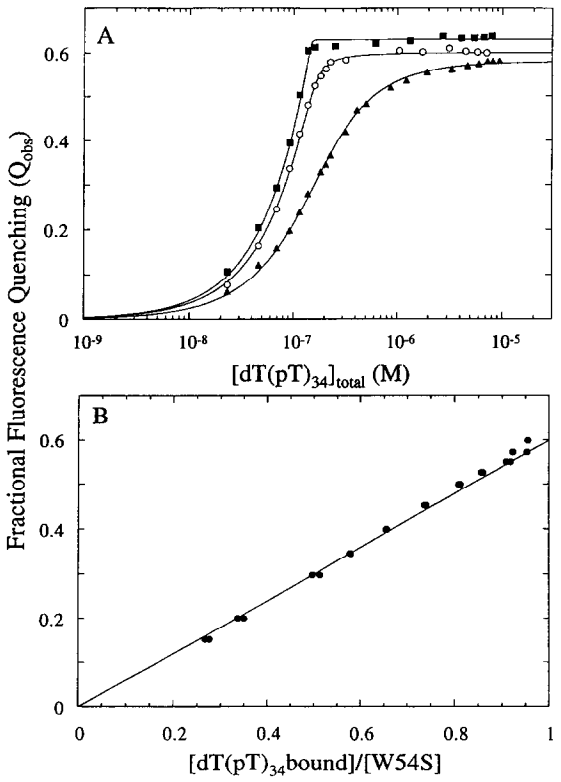

Fig. 4. Equilibrium titrations of SSB W54S with $dT(pT)_{34}$, monitored by the quenching of the SSB tryptophan fluorescence. (A) Titrations were performed in Buffer T, pH 8.1, 25°C under the following conditions: (■) 0.1 M NaCl, 0.148 μM W54S tetramer; (○) 0.3 M NaCl, 0.161 μM W54S tetramer; (▲) 0.5 M NaCl, 0.161 μM W54S tetramer. The binding at 0.1 M NaCl is too tight to measure a binding constant, but yields a stoichiometry of 1 $dT(pT)_{34}$ per tetramer. The solid curves for titrations performed at 0.3 and 0.5 M NaCl are non-linear least squares fits of the data to a 1:1 binding model (Eq. (1)), with the binding parameters given in Table 2. (B) The dependence of the quenching of the W54S protein fluorescence upon the average number of $dT(pT)_{34}$ bound per W54S tetramer determined from the analysis of 3 titrations performed at 0.12, 0.3 and 0.71 μM W54S tetramer in 0.1 M NaCl (data not shown). The binding stoichiometry is one $dT(pT)_{34}$ per W54S tetramer, and $Q_{max} = 0.6 \pm 0.02$. Under these same conditions the maximum binding stoichiometry is two $dT(pT)_{34}$ per wtSSB tetramer.

that K_{35} can be determined. These results are consistent with the site size measurements on poly(dT) which show that the $(SSB)_{65}$ mode is not formed appreciably at [NaCl] ≤ 0.6 M.

Fig. 4B shows the results of a model-independent binding density function analysis [43,44] for titrations of W54S with $dT(pT)_{34}$ performed at three different W54S concentrations (0.12,0.3, 0.71 μM tetramer) (data not shown). The results indicate that the plateau values reached in the titrations in Fig. 4A correspond to one $dT(pT)_{34}$ bound per W54S tetramer. However, under these same conditions, the wtSSB tetramer is able to bind two $dT(pT)_{34}$ molecules per tetramer [15]. Even upon increasing the [NaCl] to 0.5 M, the W54S tetramer still binds only a single molecule of $dT(pT)_{34}$ with high affinity ($K_{app} = 1.4 \pm 0.04 \times 10^7$ M^{-1} (Fig. 4A), whereas the wtSSB tetramer readily forms a 2:1 $dT(pT)_{34}$–protein complex [15]. These results indicate that the W54S tetramer has a much higher degree of negative cooperativity for ss-DNA binding than does the wtSSB tetramer.

The same general result is observed for the interaction of W54S with $dT(pT)_{15}$ (data not shown). Two titrations were performed in 50 mM NaCl (pH 8.1, 25°C) at two different W54S concentrations (1.3×10^{-7} and 5.2×10^{-7} M). Fig. 5 shows a model-independent binding density function analysis performed on these data which shows that W54S only binds two molecules of $dT(pT)_{15}$ under these conditions. The wtSSB tetramer can bind in excess of three molecules of $dT(pT)_{15}$ under these same conditions, although saturation of the fourth site with $dT(pT)_{15}$ is also difficult due to the high degree of negative cooperativity ($\sigma_{35} = 0.034$) [14,15].

3.4. Quantitative estimate of the negative cooperativity parameter for the W54S tetramer

The degree of negative cooperativity for ss-DNA binding to the wtSSB tetramer also depends on base composition, with oligodeoxycytidines displaying the least negative cooperativity [24]. We therefore per-

Table 2
Equilibrium binding and quenching constants for the W54S–dT_{35} interaction [a,b]

[NaCl]/M	$K_{obs} \times 10^7/(M)^{-1}$	Q_{max}
0.3	36(±3)	0.60
0.5	1.4 (±0.04)	0.58

[a] K_{obs} and Q_{obs} obtained from fitting to a 1:1 binding model. Error in Q_{max} is ±2%. [b] Measurements performed in Buffer T, pH 8.1, 25°C, [W54S] = 0. 161 μM.

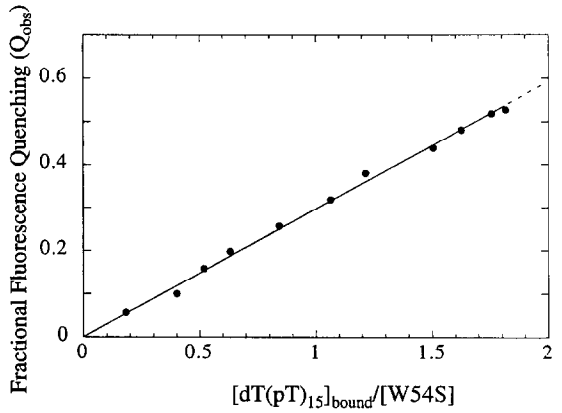

Fig. 5. The dependence of the quenching of the W54S protein fluorescence upon the average number of $dT(pT)_{15}$ bound per W54S tetramer determined from 2 titrations performed at 0.13 and 0.52 μM W54S tetramer in Buffer T+50 mM NaCl (pH 8.1), 25°C. The broken line represents a linear extrapolation from the initial portion of the curve. The maximum binding stoichiometry is 2 molecules of $dT(pT)_{15}$ per W54S tetramer with $Q_{max} = 0.59 \pm 0.02$.

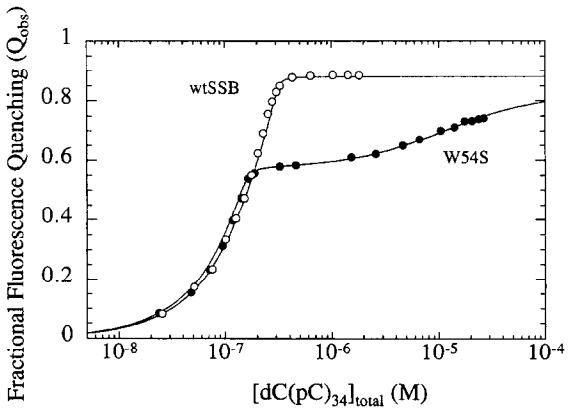

Fig. 6. Titrations of (●) W54S (0.161 μM tetramer) and (○) wtSSB (0.168 μM tetramer) monitoring tryptophan fluorescence quenching upon addition of $dC(pC)_{34}$ (0.2 M NaCl, pH 8.1, 25°C). $dC(pC)_{34}$ binds with high affinity to two sites on the wtSSB tetramer. However, negative cooperativity is observed for $dC(pC)_{34}$ binding to the W54S tetramer, with high affinity binding to the first site and weak binding to the second site. The solid lines are simulations based on a two-site binding model (Eq. (3)) with the equilibrium parameters determined from the analysis given in Table 3. The macroscopic binding constants for binding the second molecule of $dC(pC)_{34}$ ($K_{2/35}$) are 7×10^8 and 9.0×10^4 M^{-1} for wtSSB and W54S, respectively.

formed equilibrium titrations of W54S with $dC(pC)_{34}$ in an attempt to find conditions that might allow a quantitative estimate of the degree of negative cooperativity to compare with that measured for the wtSSB tetramer. Fig. 6 compares equilibrium titrations for $dC(pC)_{34}$ binding to both wtSSB and W54S tetramers ([W54S] = 0.161 μM tetramer, [wtSSB] = 0.168 μM tetramer) (0.2 M NaCl, pH 8.1, 25°C). Under these conditions we observe binding of two molecules of $dC(pC)_{34}$ per W54S tetramer as indicated by the biphasic character of the titration curves, although significantly higher concentrations of $dC(pC)_{34}$ are needed than for binding to the wtSSB tetramer. Binding of the first molecule of $dC(pC)_{34}$ occurs with a relatively large Trp fluorescence quenching; the second molecule binds with much lower affinity and a smaller fluorescence quenching. Since the binding of $dC(pC)_{34}$ is very tight to the first site on both the wt and the W54S tetramers under these conditions, we cannot accurately determine the binding constant, K_{35}, or the negative cooperativity parameter, σ_{35}, from these data. We instead fit the data to Eq. (3), represented by the solid lines in Fig. 6; the best fit parameters $K_{1/35}$, $K_{2/35}$, $Q_{1/35}$, and $Q_{2/35}$ are given in Table 3.

Since the binding of $dC(pC)_{34}$ to the first site is very tight, the values of $K_{1/35}$ only represent minimum estimates. However, the macroscopic binding constants for the second molecule of $dC(pC)_{34}$, $K_{2/35}$, can be determined accurately. These results show that W54S can bind a second molecule of $dC(pC)_{34}$, but with ~ 8000-fold lower affinity compared to wtSSB. Increasing the salt concentration to 0.50 M NaCl weakens the binding of $dC(pC)_{34}$ to the first site on the tetramer sufficiently to allow an

Table 3
Equilibrium interaction constants for the W54S– and wtSSB–$dC(pC)_{34}$ interaction [a,b]

	W54S	wtSSB
$K_{1/35}$ (M^{-1})	1.0×10^9	1.0×10^{10}
$K_{2/35}$ (M^{-1})	9.0×10^4	7.0×10^8
$Q_{1/35}$	0.58 (±0.02)	0.52 (±0.05)
$Q_{2/35}$	0.82 (±0.02)	0.88 (±0.02)

[a] Measurements performed in Buffer T, pH 8.1, 25°C, 0.2 M NaCl. Errors in $K_{2/35}$ are ±20%. Values of $K_{1/35}$ are minimum estimates due to tight binding. [b] [W54S] = 0.161 μM, [SSB] = 0.168 μM.

accurate measure of affinity, but binding to the second site is also weakened sufficiently so that its affinity could no longer be measured accurately. This precluded use of these data to estimate σ_{35}; however, these results suggest that the W54S tetramer displays a greater degree of negative cooperativity than the wtSSB tetramer.

To obtain a quantitative comparison of σ_{35} for wt and W54S tetramers, we performed titrations of W54S with $dT(pT)_{34}$ in the presence of $MgCl_2$. Fig. 7A shows titrations performed at 0.177 and 0.7 μM W54S in the presence of 0.125 M $MgCl_2$ (pH 8.1, 25°C). The titrations are clearly biphasic, indicating a significant negative cooperativity. The experimental data at both protein concentrations are well described by the solid curves simulated according to

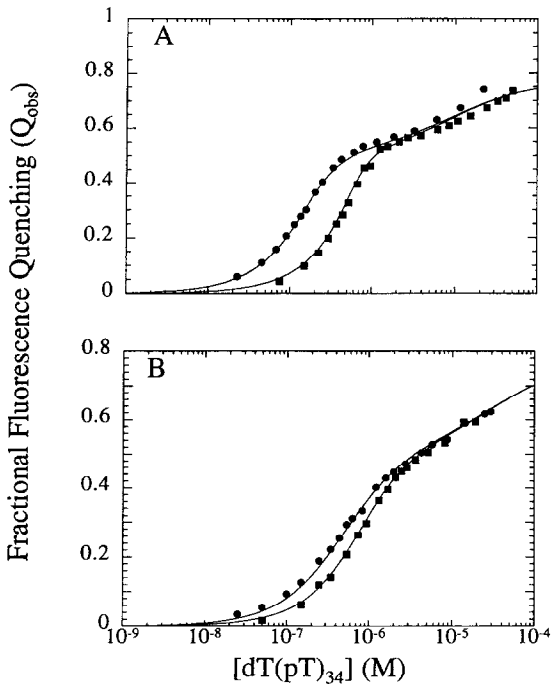

Fig. 7. Tryptophan fluorescence quenching of the W54S tetramer upon titrating with $dT(pT)_{34}$ in the presence of $MgCl_2$ to determine the negative cooperativity parameter, σ_{35}. All titrations were performed in Buffer T, pH 8.1, 25°C. (A) 0.125 M $MgCl_2$ at: (●) 0.177 μM; (■) 0.70 μM W54S tetramer. (B) 0.2 M $MgCl_2$ at: (●) 0.145 μM; (■) 0.65 μM W54S tetramer. The solid curves are simulations based on the equilibrium binding parameters obtained from non-linear least squares analysis of the data according to Eq. (2) and given in Table 4.

the square model [14] (see Eq. (2)), using the equilibrium parameters determined from non-linear least squares analysis (Table 4). However, we could not obtain an accurate estimate of σ_{35} for wtSSB at this $[MgCl_2]$ since $dT(pT)_{34}$ binds too tightly to the wtSSB tetramer.

We therefore performed titrations of W54S with $dT(pT)_{34}$ in 0.2 M $MgCl_2$ as shown in Fig. 7B. The isotherms are well described by the square model and the equilibrium binding parameters determined from non-linear least squares analysis given in Table 4. W54S binds $dT(pT)_{34}$ more weakly than does wtSSB; $K_{35} = 5.72\ (\pm 0.3) \times 10^5\ M^{-1}$ for W54S compared with $2.7\ (\pm 0.4) \times 10^8\ M^{-1}$ for wtSSB. For the W54S mutant, $\sigma_{35} = 0.49 \pm 0.04$, significantly lower than the value of 0.80 (±0.12) observed for the wtSSB under these conditions [15]. These results show that the mutant not only binds with lower affinity to ss-DNA, but also displays a greater degree of negative cooperativity compared to wtSSB. This greater degree of negative cooperativity is consistent with the higher $MgCl_2$ concentration required to stabilize the $(SSB)_{65}$ mode in W54S relative to wtSSB. Therefore, the greater relative stability of the $(SSB)_{35}$ polynucleotide binding mode on poly(dT) is due at least partly to a greater negative cooperativity for ss-DNA binding to the W54S tetramer, making it more difficult to bind ss-DNA to the third and fourth subunits of the tetramer.

3.5. Cooperative interactions of W54S tetramers in the $(SSB)_{35}$ mode

We have previously shown that SSB tetramers bind in the $(SSB)_{35}$ mode with a large positive nearest-neighbor cooperativity ($\omega_{35} \geq 10^5$), sufficiently large to cause formation of long protein clusters capable of saturating the DNA [28]. We therefore determined if this cooperativity was affected by the W54S mutation, which in turn might explain the defect in replication observed by Carlini et al. [35].

The affinity of the W54S tetramer for poly(dT) is sufficiently low to allow determination of the equilibrium binding parameters in 0.6 M NaCl (pH 8.1, 25°C). Fig. 8 shows the results of six titrations performed at [W54S] ranging from 0.17 to 11.2 μM

Table 4
Equilibrium interaction constants for the W54S–dT_{35} interaction in $MgCl_2$

$[MgCl_2]$/M	K_{35}/M^{-1}	σ_{35}	Q_1	Q_2
0.125	$7.03 (\pm 1.7) \times 10^5$	0.35 (±0.05)	0.54	0.77 (±0.03)
0.2	$5.72 (\pm 0.3) \times 10^5$	0.49 (±0.04)	0.54	0.77 (±0.03)

W54S tetramer, which were analyzed using a nearest-neighbor unlimited cooperativity model for uniform, infinitely long lattices [45–47]. The value of Q_{max} was fixed at 0.75, based on the plateau in the titration curve and n was fixed at 40 based on titrations performed at 11.2 μM W54S tetramer (see Table 1). Values of $K_{unlim} = 4.2 (\pm 0.95) \times 10^5$ M^{-1} and $\omega_{unlim} = 4 (\pm 3)$ were estimated from simultaneous non-linear least squares analysis of all six curves in Fig. 8. The solid lines in Fig. 8 are simulations based on Eq. (9) and the equilibrium parameters in Table 5. Therefore, the nearest-neighbor positive cooperativity between adjacent W54S tetramers in the $(SSB)_{35}$ mode cooperativity is very low. However, since it is possible that the "unlimited" cooperativity model is not an appropriate model to describe the cooperative binding used by W54S, we also used a second approach to estimate ω_{35}.

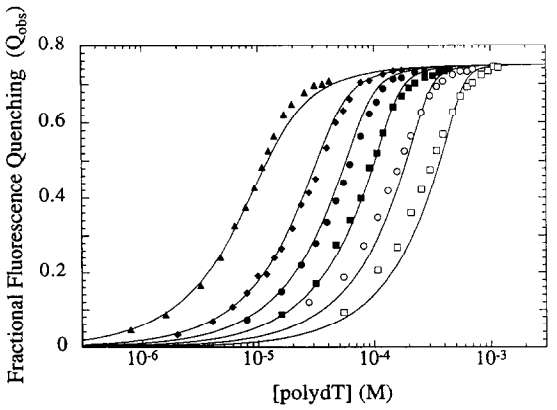

Fig. 8. Equilibrium titrations of W54S tetramer with poly(dT), plotted as the quenching of the SSB tryptophan fluorescence. Titrations were performed in Buffer T (pH 8.1), 0.6 M NaCl, 25°C at the following W54S tetramer concentrations: (▲) 0.17 μM, (◆) 0.71, (●) 1.4 μM, (■) 2.8 μM, (○) 5.5 μM, (□) 11.2 μM. The solid curves are simulations based on the "unlimited" cooperativity model (Eq. (9)) using the interaction constants in Table 5, which are based on simultaneous non-linear least squares analysis of the data from all six titration curves.

In previous studies, we estimated ω_{35} for wtSSB by examining the equilibrium binding of wtSSB tetramers to $dA(pA)_{69}$, under low salt conditions which permit the binding of two tetramers to $dA(pA)_{69}$ [28]. We used this approach, since the binding affinity of wtSSB is too high to allow accurate determination of the binding parameters under conditions which form exclusively the $(SSB)_{35}$ mode. $dA(pA)_{69}$ was chosen for these studies since the greatest degree of negative cooperativity is observed for $dA(pA)_N$, relative to $dT(pT)_N$ and $dC(pC)_N$ [24], thus increasing the relative population of the SSB binding mode in which only two subunits of the tetramer interact with $dA(pA)_{69}$. At low salt concentration (1 mM NaCl, pH 8.1, 25°C), wtSSB forms a 2:1 SSB–$dA(pA)_{69}$ complex [24,28]. At higher salt concentrations (> 0.15 M NaCl) a 1:1 SSB–$dA(pA)_{69}$ complex is formed in which DNA interacts with all four subunits of the tetramer. However, at intermediate salt concentrations (0.125 M NaCl, pH 8.1, 25°C), a mixture of these complexes exists at equilibrium. From analysis of titrations performed at intermediate salt concentration (0.125 M NaCl, pH 8.1, 25°C), a minimum estimate of $\omega_{35} \geq 10^5$ was obtained for wtSSB [28].

We first performed experiments in 1 mM NaCl (Buffer T, pH 8.1, 25°C) to determine the binding stoichiometry of the W54S–$dA(pA)_{69}$. Fig. 9a shows the results of a titration of 0.19 μM W54S (tetramer)

Table 5
Equilibrium binding parameters for the W54S tetramer–polydT interaction [a]

n	K_{unlim}/M^{-1}	ω
35	$4.0 (\pm 0.72) \times 10^5$	−0.7 (−2,8)
40	$4.2 (\pm 0.95) \times 10^5$	4 (±3)

[a] Q_{max} fixed at 0.75. Titrations performed at six different W54S tetramer concentrations ranging from 1.71×10^{-7} to 1.12×10^{-5} M. Titrations performed in Buffer T, pH 8.1, 25°C, containing 0.6 M NaCl.

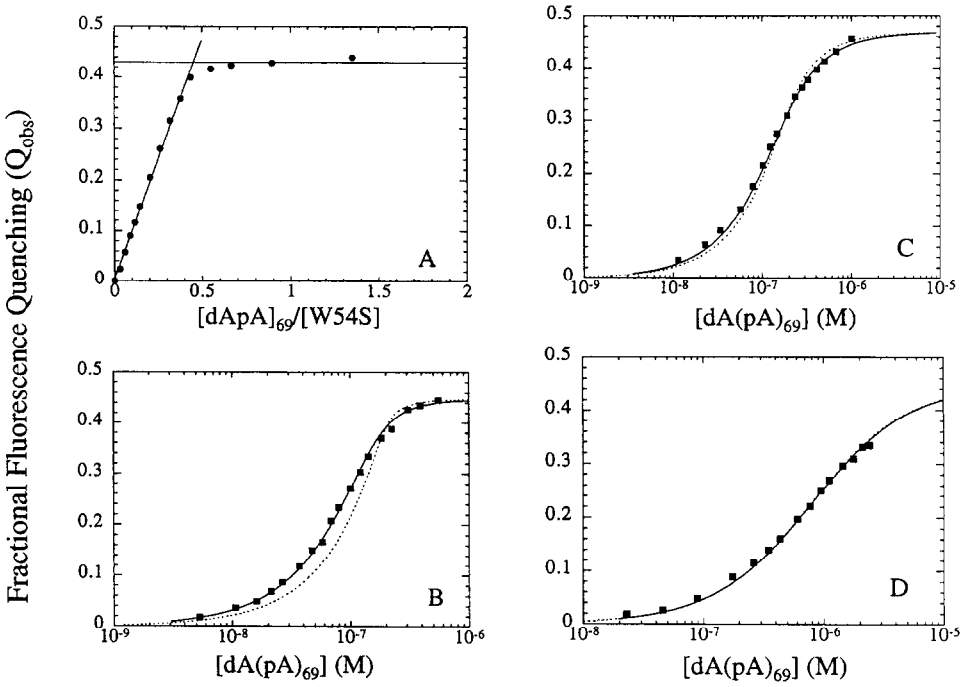

Fig. 9. The stoichiometry of the W54S–dA(pA)$_{69}$ interaction is salt-dependent. (A) 1 mM NaCl (Buffer T, pH 8.1, 25°C). Quenching of the W54S protein (0.19 μM tetramer) fluorescence plotted as a function of the molar ratio [SSB(tetramer)]/[dA(pA)$_{69}$]. At 1 mM NaCl, binding is stoichiometric with saturation occurring at 2 tetramers per dA(pA)$_{69}$, with a maximum quenching of 0.45 ± 0.02. (B) 30 mM NaCl (Buffer T, pH 8.1, 25°C) (0.2 μM W54S tetramer). Binding is well represented by a mixture of 2:1 and 1:1 complexes (Fig. 1) at equilibrium where each tetramer covers only 35 nucleotides. The solid lines represent a fit of the data to Eq. (12) with the binding parameters given in Table 6. The dashed line represents a non-linear least squares fit to a 1:1 binding model. (C) 45 mM NaCl (Buffer T, pH 8.1, 25°C) (0.19 μM W54S tetramer). (D) 85 mM NaCl (Buffer T, pH 8.1, 25°C) (0.19 μM W54S tetramer). The solid lines represent a fit of the data to Eq. (12) with the binding parameters given in Table 6.

with dA(pA)$_{69}$. Binding is stoichiometric under these conditions and the fluorescence quenching increases linearly with increasing dA(pA)$_{69}$ until saturation is reached at a stoichiometry of 2 tetramers per dA(pA)$_{69}$ ($Q_{max} = 0.45$). Therefore, the W54S mutant and wild-type proteins show the same stoichiometry under these conditions. However, as with wtSSB, the stoichiometry of the W54S–dA(pA)$_{69}$ complex depends on [NaCl] as shown in Fig. 9B–D. The isotherms at 30 mM (Fig. 9B) and 45 mM NaCl (Fig. 9C) are not well-described by a simple 1:1 binding model, although the deviations are less pronounced at 45 mM NaCl. However, the isotherm obtained at 85 mM NaCl is well-described by a 1:1 binding model with $K = 1.49 \times 10^6$ M^{-1} and $Q_{max} = 0.45$ (Fig. 9D). The deviations from a 1:1 binding model at 45 mM NaCl are due to the presence of an equilibrium mixture of 1:1 and 2:1 W54S–dA(pA)$_{69}$ complexes [28]. The solid lines are the best fits of the data to Eq. (12) (see below).

Based on these results, we performed titrations with dA(pA)$_{69}$ at different W54S concentrations ranging from 0.1 to 1.7 μM (30 mM NaCl, Buffer T, pH 8.1) (see Fig. 10) to obtain a quantitative estimate of ω_{35}. Under these conditions, the greatest deviation from 1:1 binding is observed, and about 40% of the total protein is predicted to exist in complexes with two tetramers bound per dA(pA)$_{69}$ (P_2D complex). The titrations at each protein concentration are well described by the solid lines, which are simulations based on Eq. (12) with the parameters determined by simultaneous non-linear

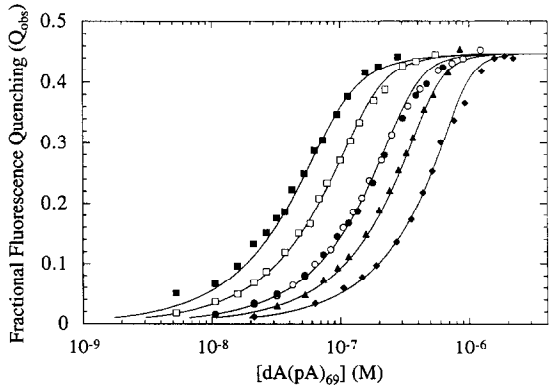

Fig. 10. Equilibrium titrations of W54S tetramer with dA(pA)$_{69}$, plotted as the quenching of the W54S tryptophan fluorescence. Experiments were performed in Buffer T (pH 8.1, 25°C) containing 30 mM NaCl at the following W54S tetramer concentrations; (■) 0.1 μM, (□) 0.22 μM, (●) 0.5 μM, (▲) 0.9 μM, (♦) 1.7 μM. The solid curves are simulations based on Eq. (12) using the interaction constants determined from non-linear least squares analysis of the data from all six titration curves: $K_{35}^{*} = 3.5\ (\pm 0.8) \times 10^{6}\ M^{-1}$, $\omega_{35} = 77(\pm 20)$, $Q_{max} = 0.45$.

Table 6
Equilibrium binding parameters for SSBW54S binding to dA(pA)$_{69}$ [a]

[NaCl]/mM	K_{35}^{*}/M^{-1} [b]	Q_{max}
30	$3.5\ (\pm 0.7) \times 10^{6}$	$0.46\ (\pm 0.01)$
45	$6.0\ (\pm 1.2) \times 10^{5}$	$0.47\ (\pm 0.01)$
85	$4.0\ (\pm 0.8) \times 10^{4}$	$0.45\ (\pm 0.01)$

[a] Buffer T, pH 8.1, 25°C. [W54S] = 0.2 μM (tetramer).
[b] Determined by fitting of the data to Eq. (12) with $\omega_{35} = 77$.

least squares analysis of all six titration curves. This analysis yields values of $K_{35}^{*} = 3.5\ (\pm 0.8) \times 10^{6}\ M^{-1}$, and $\omega_{35} = 77(\pm 20)$. This estimate of ω_{35} for W54S is very much lower than the minimum estimate of $\omega_{35} \geq 10^{5}$ obtained for wtSSB [28] and qualitatively consistent with the low value of ω_{35} obtained from the poly(dT) experiments performed in 0.6 M NaCl (see above).

Based on these results, we used Eq. (12) to fit the isotherms in Fig. 9 obtained at both 30 and 45 mM NaCl. However, since we do not know how K_{35}^{*} and ω_{35} vary with [NaCl], we made some simplifying assumptions. Since we know that the binding constant for the W54S–DNA interaction is salt-dependent, we varied K_{35}^{*} while keeping ω_{35} constant at the value determined at 30 mM NaCl (pH 8.1, 25°C). This assumption is consistent with the observation that the cooperativity parameter for wtSSB bound in the (SSB)$_{65}$ mode (ω_{65}) does not depend on NaCl concentration [9]. The solid lines in Fig. 9 represent simulations based on Eq. (12) and the values of the equilibrium parameters given in Table 6. At both 30 and 45 mM NaCl, the solid line provides a better fit to the experimental data than the dashed lines. However, both a simple 1:1 binding isotherm and the model described by Eq. (12) provide equally good fits to the data obtained at 85 mM NaCl. This can be understood from the predicted population distributions of the complexes in Fig. 11, which demonstrates that when K_{35}^{*} is decreased to $4 \times 10^{4}\ M^{-1}$, while maintaining $\omega_{35} = 80$, then the P_2D complex is not populated significantly at these protein and DNA concentrations.

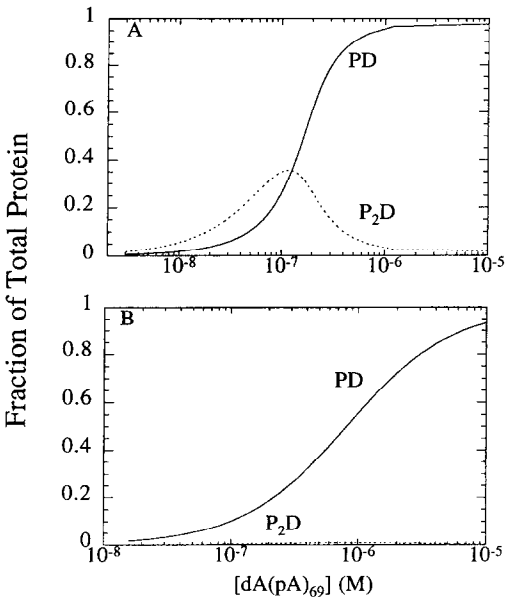

Fig. 11. The predicted population distributions of the two equilibrium W54S–dA(pA)$_{69}$ complexes (see Fig. 1) showing the effect of decreasing K_{35}^{*} while maintaining a constant value of $\omega_{35} = 80$. The simulations are based on the interaction constants in Table 6.

4. Discussion

Curth et al. [34] have shown that a mutant of the *E. coli* SSB protein in which Trp-54 has been substituted with Ser displays an increase in the relative stability of the $(SSB)_{35}$ polynucleotide binding mode. Furthermore, Carlini et al. [35] showed that expression of the *ssbW54S* gene in vivo causes slow growth and high UV sensitivity, suggesting defects in both repair and recombination. Based on these observations, we undertook further characterizations of the SSBW54S mutant in vitro to determine the extent to which the DNA binding properties of the W54S mutant are changed from those of wtSSB protein. We find that, although the W54S mutant remains a stable tetramer, the W54S mutation results in at least four major changes in the ss-DNA binding properties, compared to the wtSSB: (1) the stability of the $(SSB)_{35}$ mode is increased significantly relative to the $(SSB)_{65}$ mode, such that higher [NaCl] or $[MgCl_2]$ are required to form the $(SSB)_{65}$ mode; (2) the W54S mutant tetramer binds with significantly lower affinity to ss-DNA; (3) the W54S tetramer displays a greater degree of negative cooperativity (smaller value of σ_{35}) for ss-DNA binding; and (4) W54S displays a decreased positive inter-tetramer cooperativity in its $(SSB)_{35}$ polynucleotide binding mode (smaller ω_{35}).

Our results also show that in the low site size binding complex, the W54S tetramer interacts with the ss-DNA using only two of its subunits. This conclusion is based on the observation that at low [NaCl], only one molecule of $dC(pC)_{34}$ binds per tetramer, whereas upon raising the [NaCl], two molecules of $dC(pC)_{34}$ can bind per tetramer (see Fig. 6). This differs from the suggestion made by Curth et al. [34] that more than two subunits of the tetramer interact with ss-DNA in the low site size binding mode. Rather it supports the model that in the $(SSB)_{35}$ polynucleotide binding mode, only two SSB subunits interact with the ss-DNA, leaving two subunits unligated [16].

4.1. The W54S tetramer can form the $(SSB)_{65}$ mode on poly(dT) at $[MgCl_2] \geq 80$ mM

Our measurements of the W54S site size on poly(dT) agree with those of Curth et al. [34] and show that the W54S mutant is not able to form the $(SSB)_{65}$ mode at $[NaCl] \leq 0.6$ M NaCl. However, the $(SSB)_{65}$ mode can be populated by raising the $[MgCl_2]$ to ≥ 80 mM. This is consistent with our previous results indicating that an uptake of cations is required to progress from the lower to the higher site size binding modes [16,18,19]. Furthermore, the transition from the $(SSB)_{35}$ to the $(SSB)_{56}$ binding mode occurs at a much lower $[MgCl_2]$ for wtSSB (transition midpoint ~ 0.5 mM $MgCl_2$) (pH 8.1, 25.0°C) [18] than for the W54S tetramer. However, the $(SSB)_{56}$ mode does not seem to be populated significantly for the W54S mutant. Although it is possible that the W54S mutant can form the $(SSB)_{65}$ mode at $[NaCl] > 0.6$ M NaCl, the low affinity of the mutant for poly(dT) precludes accurate site size measurements at these higher [NaCl].

4.2. W54S shows a significantly greater negative cooperativity compared to wtSSB

Our studies with $dT(pT)_{34}$ and $dC(pC)_{34}$ indicate that the W54S mutant possesses a greater degree of negative cooperativity than wtSSB. In fact, at NaCl concentrations between 0.1 and 0.5 M, we can only detect the binding of one molecule of $dT(pT)_{34}$ per W54S tetramer due to the high negative cooperativity. This is consistent with a site size of ≈ 35 nucleotides per W54S tetramer at NaCl concentrations as high as 0.6 M. In the range of [NaCl] from 0.1 to 0.5 M NaCl, wtSSB tetramers can bind 2 molecules of $dT(pT)_{34}$ [15]. Our binding studies of W54S with $dC(pC)_{34}$ give further evidence that W54S has a greater degree of negative cooperativity between DNA binding sites. At 0.2 M NaCl, the W54S tetramer is able to bind a second molecule of $dC(pC)_{34}$, but with a binding constant ≈ 8000-fold lower than wtSSB. Our previous results show that with wtSSB [24], $dC(pC)_{34}$ displays the lowest degree of negative cooperativity (relative to $dA(pA)_{34}$ and $dT(pT)_{34}$), and therefore, the apparent affinity for binding a second $dN(pN)_{34}$ molecule is highest for $dC(pC)_{34}$. The observation that W54S can bind two molecules of $dC(pC)_{34}$, but only one $dT(pT)_{34}$ in the same range of [NaCl], supports the conclusion that the weaker binding to the second site of the W54S tetramer is a consequence of a greater degree of negative cooperativity, rather than some inability

of ss-DNA to interact with all four subunits of the W54S tetramer.

We have found one set of conditions (0.2 M $MgCl_2$, pH 8.1, 25°C) that allowed a direct measure of the negative cooperativity parameter, σ_{35}, for $dT(pT)_{34}$ binding to both the W54S and wtSSB tetramers, thus allowing a quantitative comparison of the change in negative cooperativity and binding affinity. Under these conditions, the W54S tetramer binds with lower affinity to $dT(pT)_{34}$ compared to wtSSB; the intrinsic binding constant, K^*_{35}, is 400 times weaker for W54S. The value of σ_{35} for W54S is 0.49 ± 0.04, whereas it is significantly greater (0.80 ± 0.12) for wtSSB. However, these differences in affinities will certainly change with solution and salt conditions and it is also likely that the relative values of σ_{35} will also change with solution conditions. In fact, we have previously demonstrated for wtSSB that the negative cooperativity for $dT(pT)_{15}$ binding decreases significantly with decreasing salt concentration below ~ 0.3 M NaCl [14,15].

4.3. W54S tetramers display a lower nearest-neighbor positive cooperativity than wtSSB in the $(SSB)_{35}$ mode

The strategy used for obtaining a quantitative estimate of ω_{35} for W54S binding to $dA(pA)_{69}$ is similar to that used previously for wtSSB [28]. Both the wt and W54S tetramers form a 2:1 tetramer–$dA(pA)_{69}$ complex at 1 mM NaCl, pH 8.1, 25°C. However, differences are observed upon increasing the salt concentration. At intermediate salt concentrations, wtSSB forms a mixture of 2:1 and 1:1 complexes, each covering 35 nucleotides per tetramer, as well as a fully wrapped 1:1 complex where all four subunits of the tetramer interact with $dA(pA)_{69}$. At [NaCl] > 0.15 M, the fully wrapped complex is formed exclusively. Analysis of titrations performed at 0.125 M NaCl where a mixture of complexes forms at equilibrium yielded an estimate of $\omega_{35} \geq 10^5$ for the wtSSB [28]. However, our results with the W54S tetramer are consistent with an equilibrium mixture of 2:1 and 1:1 complexes, each covering 35 nucleotides per W54S tetramer at NaCl concentrations ranging from 30 to 85 mM. The fully wrapped complex in which all four subunits of the tetramer interact with $dA(pA)_{69}$ does not appear to form, probably due to the higher degree of negative cooperativity for the W54S mutant. At 30 mM NaCl, we estimate $\omega_{35} = 77$ (± 20) for W54S, which is dramatically lower than the value of $\omega_{35} \geq 10^5$ obtained for wtSSB. Although we emphasize that different [NaCl] were used in these two measurements, this difference is not likely to have a large effect on the relative values of ω_{35}. In fact, we have shown that W54S also displays very low positive cooperativity in its binding to poly(dT) in the low site size mode at 0.6 M NaCl ($\omega_{35} \approx 4 \pm 3$).

In light of the major effects that the W54S mutation exerts on ss-DNA binding, it is not surprising that this mutation has a major impact on DNA metabolism in *E. coli*. Unfortunately, since the W54S mutation causes multiple changes in DNA binding properties, it is not possible to determine which of these might be responsible for the phenotypes observed in vivo. However, the effects of the W54S mutation on its ss-DNA binding properties in vitro are consistent with a selective role of the different SSB ss-DNA binding modes in various DNA metabolic processes in vivo as we have previously suggested [2–4].

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